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Jun N-Terminal Kinase/Stress-Activated Protein Kinase (JNK/SAPK) Is Required for Lipopolysaccharide Stimulation of Tumor Necrosis Factor Alpha (TNF-α) Translation: Glucocorticoids Inhibit TNF-α Translation by Blocking JNK/SAPK

JENNIFER L. SWANTEK,[1,2] MELANIE H. COBB,[1] AND THOMAS D. GEPPERT[2]*

Departments of Pharmacology[1] and Internal Medicine,[2] The University of Texas Southwestern Medical Center, Dallas, Texas 75235-9041

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The adverse effects of lipopolysaccharide (LPS) are mediated primarily by tumor necrosis factor alpha (TNF-α). TNF-α production by LPS-stimulated macrophages is regulated at the levels of both transcription and translation. It has previously been shown that several mitogen-activated protein kinases (MAPKs) are activated in response to LPS. We set out to determine which MAPK signaling pathways are activated in our system and which MAPK pathways are required for TNF-α gene transcription or TNF-α mRNA translation. We confirm activation of the MAPK family members extracellular-signal-regulated kinases 1 and 2 (ERK1 and ERK2), p38, and Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), as well as activation of the immediate upstream MAPK activators MAPK/ERK kinases 1 and 4 (MEK1 and MEK4). We demonstrate that LPS also activates MEK2, MEK3, and MEK6. Furthermore, we demonstrate that dexamethasone, which inhibits the production of cytokines, including TNF-α, significantly inhibits LPS induction of JNK/SAPK activity but not that of p38, ERK1 and ERK2, or MEK3, MEK4, or MEK6. Dexamethasone also blocks the sorbitol but not anisomycin stimulation of JNK/SAPK activity. A kinase-defective mutant of SAPKβ, SAPKβ K-A, blocked translation of TNF-α, as determined by using a TNF-α translational reporting system. Finally, overexpression of wild-type SAPKβ was able to overcome the dexamethasone-induced block of TNF-α translation. These data confirm that three MAPK family members and their upstream activators are stimulated by LPS and demonstrate that JNK/SAPK is required for LPS-induced translation of TNF-α mRNA. A novel mechanism by which dexamethasone inhibits translation of TNF-α is also revealed.

Lipopolysaccharide (LPS), a surface component of gram-negative bacteria, is released upon host infection and causes tissue injury and shock (34). The adverse effects of LPS are mediated by toxic cytokines that are produced by LPS-activated macrophages. One of the most important cytokine mediators is tumor necrosis factor alpha (TNF-α) (3). Isolated macrophages exposed to LPS produce large quantities of TNF-α, constituting between 1 and 2% of the secreted proteins (3). Large amounts of TNF-α are also produced upon LPS treatment of animals (1). Passive immunization against TNF-α protects animals from the lethal effect of LPS (3), while purified TNF-α induces many of the deleterious effects of LPS in vivo (48).

LPS in the bloodstream binds to LPS-binding protein (LBP) (47). Interaction of LPS-LBP complexes with macrophages induces the synthesis of TNF-α by the macrophages (41). The receptor on macrophages for LPS-LBP complexes is CD14 (51), which is a glycosylphosphatidylinositol (GPI)-linked membrane-associated protein whose expression is limited to monocytes and macrophages. Because CD14 lacks transmembrane and intracellular domains, it has no obvious means of generating cytoplasmic signals; thus, the triggering of LPS signaling remains obscure. However, since a number of GPI-linked molecules, such as the receptor for ciliary neurotrophic factor, transduce signals (45), CD14 may initiate signals in a similar manner.

The signaling pathways utilized by LPS to induce TNF-α biosynthesis are unknown but are believed to be dependent on the activation of tyrosine kinases (14, 36, 49). LPS induced tyrosine phosphorylation in both monocytes and macrophages, and this response was blocked by tyrosine kinase inhibitors (14, 49). These inhibitors were also shown to inhibit LPS-induced TNF-α production as well as LPS-induced lethal toxicity in mice (36).

The LPS signaling cascade leading to TNF-α production bifurcates to control both transcription of the TNF-α gene and translation of TNF-α mRNA (4). Transcriptional control of the TNF-α gene is mediated primarily by NF-κB binding sites present within the TNF-α gene promoter (24, 44). Translational activation of TNF-α is dependent on a conserved element found within the 3′ untranslated regions (3′-UTRs) of various cytokines and proto-oncogenes (28). This element (TT ATTTAT) confers a repression of translation which must be derepressed in order for translation to proceed (5, 17–20).

Tyrosine kinase-initiated signaling often involves the subsequent activation of distinct mitogen-activated protein kinase (MAPK) signaling pathways. A MAPK pathway generally consists of a MAPK/extracellular-signal-regulated kinase (ERK) kinase kinase (MEKK) which activates a MAPK/ERK kinase (MEK) which in turn activates a MAPK/ERK. (MEKs are also called MKKs. For uniformity of nomenclature, MEK will be used to describe all members of this family throughout this paper.) The best-characterized MAPK pathway, the ERK pathway, consists of Raf, MEK1 and MEK2, and ERK1 and

* Corresponding author. Mailing address: Department of Internal Medicine, The University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235-9041. Phone: (214) 648-9112. Fax: (214) 648-7995.

ERK2 (9) and is activated in response to growth factors, including nerve growth factor (NGF), epidermal growth factor, and insulin (7). Upstream regulation of this pathway involves the activation of the small G-protein Ras (38). The ERK pathway is implicated in various cellular processes based in part on the diversity of ERK substrates. Recently, it has been shown that members of the ERK pathway are involved in the regulation of TNF-α production (15).

Another MAPK pathway involves the MAPK family member p38. p38 was cloned because its activation in monocytes was specifically blocked by a drug which inhibited TNF-α translation (30) and was cloned independently as a kinase activated by LPS (21). The putative three-kinase cascade consists of unknown MEKKs, MEK3 and MEK6, and p38 (12, 21, 22, 30, 43).

A third MAPK pathway, the Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathway, becomes activated in response to a wide variety of physiological and stress-related stimuli (11, 29). This three-kinase cascade is believed to consist of MEKK-1, MEK4 (also called SEK-1, JNKK, and MKK4), and JNK/SAPK (11, 23, 29, 31, 39, 53). However, there are at least two separate pathways leading to JNK/SAPK activation which are distinguishable by various stimuli (2, 33, 35). For example, sorbitol activates JNK/SAPK in a MEK4/SEK-1-independent manner, while anisomycin induction of JNK/SAPK is MEK4/SEK-1 dependent in cells derived from SEK-1 (−/−) mice (35). Thus, as-yet-undefined JNK/SAPK activators distinct from MEK4/SEK-1 exist.

The three MAPKs, JNK/SAPK, ERK, and p38, differ in their responses to extracellular stimuli. For example, ERKs are most responsive to growth factors and phorbol esters (9), while JNK/SAPK and p38 are only modestly stimulated by or are unresponsive to growth factors (37). JNK/SAPK and p38 are best activated following stress signals, including exposure of cells to UV or TNF-α (29, 37).

Here, we establish that LPS activates two tiers of the MAPK modules for ERK1 and ERK2, p38, and JNK/SAPK. We demonstrate that JNK/SAPK is required for LPS-induced translation of TNF-α mRNA and that dexamethasone inhibits LPS-induced JNK/SAPK activity. We also demonstrate that overexpression of wild-type SAPKβ overcomes dexamethasone-induced suppression of TNF-α translation, thus providing a molecular mechanism by which glucocorticoids may act to inhibit the inflammatory response.

MATERIALS AND METHODS

Reagents and antibodies. *Escherichia coli* LPS from strain O127:B8 was obtained from Difco and was dissolved in sterile saline and added to cells as indicated below. Dexamethasone, β-estradiol, and progesterone, purchased from Sigma, were dissolved in ethanol or dimethyl sulfoxide and were added to cells as indicated. Pentoxifylline (Sigma) was dissolved in water and was added to cells as indicated. Anisomycin (Sigma) was dissolved in ethanol and was added to cells as indicated. Sorbitol (Sigma) was dissolved in water and was added to cells as indicated. A monoclonal antibody to the hemagglutinin (HA) epitope was purchased from Babco (Richmond, Calif.). An antiserum to all forms of ERK1 and ERK2 (Y691) was previously described (6). An antiserum to active ERK1 and ERK2 was obtained from Promega. Antisera to MEK3 (Q804) and MEK6 (Q806) were produced in rabbits with peptides from MEK3 (LDSRT FITIGDRNFE) and MEK6 (LDSKACISIGNQNFE), by methods described earlier (6). Antiserum to MEK4 (0974) was produced by using the MEK4-derived peptide KRKALKLNFANPPVKSTART and was described elsewhere (26). Antisera to MEK1 (A2227) and MEK2 (A2228) were previously described (52). Antisera to JNK/SAPK (0977) and p38 (P287), against purified recombinant SAPKβ and p38 proteins, respectively, were raised in rabbits and are described elsewhere (27). The antibodies recognized their respective kinases in Western blots, and cross-reactivity with other MEK or MAPK family members was not observed.

DNA constructs. Constructs in which a TNF-α promoter drives the expression of chloramphenicol acetyltransferase (CAT) (TNF-α transcriptional reporter), in which a cytomegalovirus (CMV) promoter drives the expression of CAT (control), and in which a CMV promoter drives the expression of CAT with the TNF-α 3′-UTR following the CAT sequence (TNF-α translational reporter) were provided by Bruce Beutler and were described previously (15, 18, 19). Activity from the TNF-α translational reporter was previously shown to correlate with TNF-α translation (18). An expression vector encoding an HA-tagged MEK6 (3× HA-tag-MEK6-SRα3) was provided by B. Stein (46). Kinase mutant SAPKβ (SAPKβ K-A) and wild-type SAPKβ were provided in pGEX-KG by J. Kyriakis (29, 53) and were subcloned into pCMV5.

Cell culture. RAW 264.7 cells were maintained in Dulbecco modified Eagle medium containing 10% fetal bovine serum, 50 U of penicillin per ml, 50 μg of streptomycin per ml, and 2 mM L-glutamine at 37°C in 5% CO_2. For time courses of LPS-induced kinase activity or sorbitol- or anisomycin-induced kinase activity, cells were plated and grown until approximately 70% confluent. The cells were then starved in medium containing 0.5% fetal calf serum for 18 h and were stimulated with either LPS (1 μg/ml), sorbitol (0.4 M), or anisomycin (10 μg/ml) for the indicated times or were treated with diluent (control). Where indicated, dexamethasone, β-estradiol, or progesterone (or diluent) was added to the cells for the specified times prior to stimulation. The cells were washed with cold phosphate-buffered saline and were lysed in lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 100 mM NaF, 1% phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml) on ice for 15 min. Lysates were scraped and collected into Eppendorf tubes and were centrifuged at 16,000 × *g* for 10 min at 4°C. Protein amounts were determined with the Bio-Rad protein assay.

Western blotting of ERKs. A 20-μg amount of total protein was added to Laemmli buffer, and samples were boiled for 5 min and loaded on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels for electrophoresis in Tris-glycine-SDS buffer (25 mM Tris, 250 mM glycine, 0.1% SDS). Proteins were blotted onto 0.45-μm-pore-size nitrocellulose filters for 1.5 h at 125 mA on ice. The filters were blocked overnight in TBS-T (20 mM Tris HCl, 150 mM NaCl, 0.1% Tween 20)–5% milk at 4°C. All subsequent steps were done at room temperature. The filters were incubated with the Promega antibody (diluted 1:2,000) in TBS-T–0.5% milk for 1 h and were washed. Horseradish peroxidase-linked donkey anti-rabbit immunoglobulin (Amersham) was diluted 1:15,000 in TBS-T–1% milk and was added to the filters, which were then incubated for 1 h. The filters were washed with TBS-T. Bands were detected with ECL reagents (Amersham). The filters were then exposed to Kodak X-Omat AR film. The filters were stripped of antibodies by incubation in 2% SDS–62.5 mM Tris HCl (pH 6.7)–100 mM β-mercaptoethanol at 50°C for 30 min. The filters were washed with TBS-T and were then incubated with ECL detection reagents and exposed to X-ray film to ensure removal of antibodies. The filters were reprobed with Y691 (diluted 1:5,000 in TBS-T–0.5% milk) as described above.

In vitro kinase assays. JNK/SAPK, p38, and MEK1 to MEK4 were immunoprecipitated from equal amounts of protein (150 μg for JNK/SAPK and p38 or 400 μg for MEK1 to MEK4) from lysates of RAW 264.7 cells that had been stimulated with LPS (1 μg/ml), sorbitol (0.4 M), or anisomycin (10 μg/ml) (or diluent) for the indicated times, using 10 μl of the respective rabbit polyclonal antiserum, lysis buffer, and protein A-Sepharose (Pharmacia) overnight at 4°C on a rotating wheel. Where indicated, dexamethasone, β-estradiol, or progesterone was added to the cells for the indicated amounts of time prior to stimulation. Immunoprecipitates were washed three times with lysis buffer and once with kinase buffer (50 mM Tris [pH 7.4], 10 mM $MgCl_2$, 1 mM dithiothreitol). The pellets were resuspended in kinase buffer plus 50 μM ATP (1 to 4 cpm/fmol) and 0.3 mg of substrate per ml. The substrates for JNK/SAPK, p38, MEK1 and MEK2, and MEK3 and MEK4 were glutathione *S*-transferase (GST)-cJun(1-221), GST-ATF21(1-254) (from M. Karin), His-ERK2K52R (8), and histidine-tagged p38 (22), respectively. The reactions were carried out for 40 min at 30°C. Samples were centrifuged, supernatants were added to Laemmli buffer, and the mixtures were boiled for 5 min. Aliquots of the reaction mixtures were loaded on SDS–10% polyacrylamide gels for electrophoresis (as described above). The gels were dried and were exposed to X-ray film. Radioactivity was quantitated with an automated β-detection and imaging (AMBIS) device.

LPS-induced MEK6 activity. RAW 264.7 cells were transfected with 3× HA-tag-MEK6-SRα3 by using Lipofectamine (Gibco) and were divided onto six plates and grown overnight. The cells were then starved in Dulbecco modified Eagle medium containing 0.5% fetal calf serum for 18 h prior to treatment with LPS at 1 μg/ml (or with diluent). MEK6 was immunoprecipitated from equal amounts of protein by using an anti-HA antibody, and an in vitro kinase assay was performed as described above, using histidine-tagged p38 as a substrate. Where indicated, MEK6 kinase activity was determined following immunoprecipitation from 400 μg of RAW 264.7 cell lysate by using rabbit polyclonal antiserum Q806 and histidine-tagged p38 as a substrate as described above.

CAT assays. RAW 264.7 cells were transfected with the Profection Mammalian Transfection System-DEAE Dextran (Promega). Cells were cotransfected with the TNF-α transcriptional construct, the TNF-α translational construct, or the control CMV-CAT construct along with either the SAPKβ K-A, the SAPKβ, or the pCMV5 constructs. Transfected cells were split into the desired number of plates and were allowed to grow overnight. The plates were then stimulated with LPS at 1 μg/ml or were treated with diluent for 6 or 16 h (depending on the experiment, as indicated below). Where indicated, dexamethasone (or dexamethasone diluent [control]) was added to the cells 1 h prior to LPS stimulation

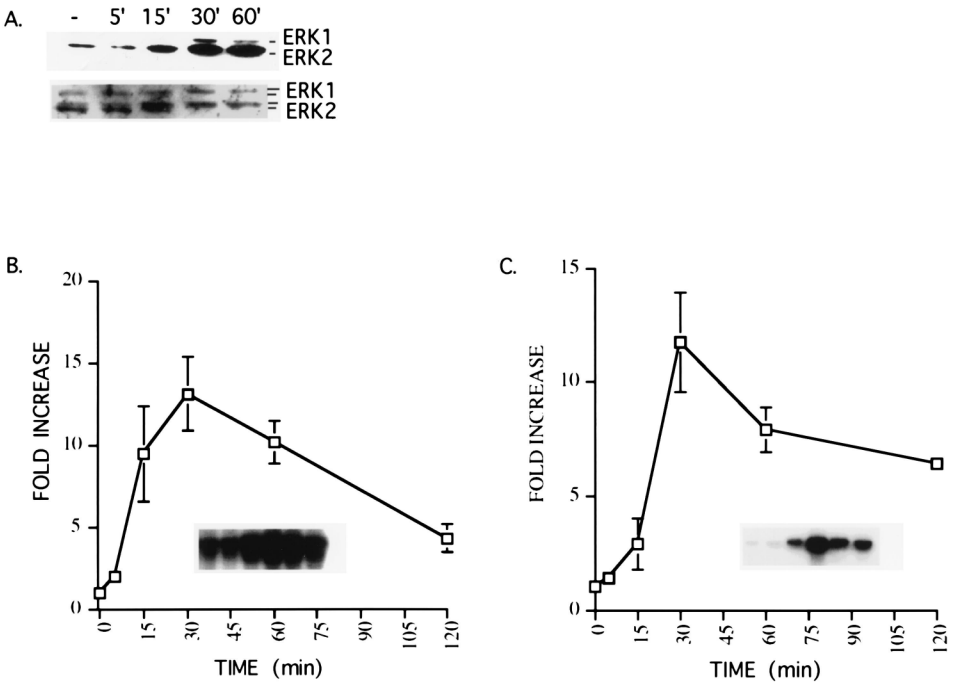

FIG. 1. LPS activates ERK1 and ERK2, p38, and JNK/SAPK. RAW 264.7 cells were starved for 18 h and were treated with LPS (1 μg/ml) or diluent (control) for the times indicated above the lanes. (A) Equal amounts of lysates were subjected to SDS-PAGE for Western blotting with an antibody which recognizes only activated ERK1 and ERK2 (top). The filter was stripped of antibody and was reprobed with an antibody (Y691) which recognizes both active and inactive ERK1 and ERK2 (bottom). Lane −, untreated. (B and C) Equal amounts of lysates were used to immunoprecipitate either p38 or JNK/SAPK, respectively, for in vitro kinase assays using GST-ATF2(1-254) or GST-cJun(1-221), respectively, as substrates. Kinase activities were measured with an AMBIS radioanalytic device. The data points are the averages of two separate experiments, with the ranges indicated. Insets are autoradiographs from a representative experiment.

for a final concentration of 10 μM. The cells were then harvested for the assay of CAT as previously described (15).

Northern blotting. RAW 264.7 cells were cotransfected with the TNF-α translational reporter and either pCMV5 or SAPKβ K-A as described above. The cells were allowed to grow overnight and were stimulated with LPS (1 μg/ml). Six hours poststimulation, RNA was harvested by using Trizol (Gibco) according to the protocol provided by the manufacturer. Equal amounts of RNA were subjected to electrophoresis on a 1.2% agarose–formaldehyde gel. The RNA was blotted onto Hybond-N (Amersham) nylon sheets. The blots were placed in prehybridization solution (50% formamide, 4× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 5× Denhardt's solution, 0.05 M sodium phosphate, 500 μg of sonicated salmon sperm DNA) for 3 h at 42°C. The blots were hybridized for 18 h at 42°C with a CAT cDNA probe (10^6 cpm/ml; CAT cDNA provided by B. Beutler) which had been labeled with [α-^{32}P]dCTP (NEN) by using a Boehringer Mannheim Random Primed Labeling kit. The blots were washed twice with 2× SSC–0.1% SDS for 5 min at room temperature, once with 0.5× SSC–0.1% SDS for 15 min at 55°C, and twice with 0.1× SSC–0.1% SDS for 30 min at 65°C and were exposed to Kodak X-Omat film. The blots were stripped of probe by adding boiling 0.1% SDS and placing them on a rotating platform at room temperature for 10 min. The blots were then reprobed with a β-actin probe (β-actin cDNA provided by T. Wilkie, University of Texas Southwestern Medical Center) as described above.

RESULTS

LPS stimulates activation of MAPK pathways. It has previously been shown that ERK1 and ERK2, JNK/SAPK, and p38 become activated in response to LPS (13, 16, 21, 40, 50). We set out to confirm these results in our system. A Promega antibody recognizes the dually phosphorylated forms of ERK1 and ERK2 (Thr-183 and Tyr-185). Phosphorylation of these two residues is required for and correlates with activation of ERK1 and ERK2 (9). Using the Promega antibody, we demonstrate via Western blotting of lysates of LPS-stimulated RAW 264.7 cells that LPS induces dual phosphorylation (and thus activation) of both ERK1 and ERK2 (Fig. 1A, top). Immunoblotting with Y691, which recognizes both active and inactive forms of ERK1 and ERK2, confirmed that similar amounts of ERK proteins were present in all samples (Fig. 1A, bottom). The activation of the ERKs was also detected by the shift in ERK mobility, noticed only in the samples in which active ERKs were detected with the Promega antibody 20291, as seen in Fig. 1 (compare top and bottom of Fig. 1A). We tested activation of p38 and JNK/SAPK using in vitro kinase assays. As can be seen in Fig. 1B and C, p38 and JNK/SAPK become activated following LPS treatment of RAW 264.7 cells. The kinetics of activation of all MAPK family members are similar and unusually slow, with peak activity occurring approximately 30 min poststimulation, and all MAPKs remain active for at least 2 h. Thus, we confirm activation of three MAPK pathways in RAW 264.7 cells in response to LPS.

LPS induces activation of MEK1, -2, -3, -4, and -6. The immediate upstream activators of the MAPK family members are the MEKs. In other systems, MEK1 and MEK2 activate ERK1 and ERK2 (10, 42, 43), while MEK3 and MEK6 are believed to be the upstream activators of p38 (12, 22, 46). MEK4 has been shown to phosphorylate both JNK/SAPK and p38 (12, 30, 39) and is implicated in the JNK/SAPK pathway (53). We tested the ability of LPS to induce activation of the MEKs, in order to confirm and establish the kinases upstream of the MAPK family members that become activated in response to LPS. As shown in Fig. 2A and B, MEK1 and MEK2 become activated following stimulation with LPS, with similar kinetics. MEK3 and MEK6 both become activated in response to LPS and are able to phosphorylate p38 (Fig. 2C and E). However, the kinetics of activation of these kinases are different, with MEK3 activity peaking at 30 min poststimulation and MEK6 demonstrating higher levels of kinase activity with increasing times poststimulation. As previously shown (40),

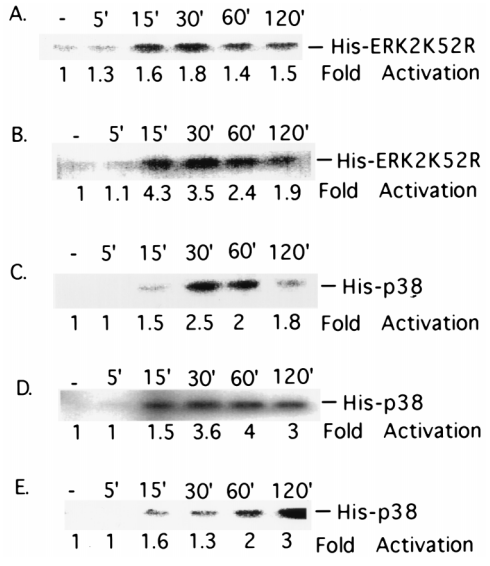

FIG. 2. LPS-induced activation of MEK1, -2, -3, -4, and -6. RAW 264.7 cells were starved for 18 h and were treated with LPS (1 μg/ml) or diluent for the times indicated above the lanes. (A to D) Equal amounts of lysates were used to immunoprecipitate MEK1, MEK2, MEK3, and MEK4, respectively, for in vitro kinase assays using His-ERK2K52R (for MEK1 and MEK2) and His-p38 (for MEK3 and MEK4) as substrates. (E) RAW 267.4 cells were transfected with an expression vector encoding an HA-tagged MEK6. The cells were split into six plates. At 24 h following transfection, cells were starved for 18 h and were treated with LPS (1 μg/ml) or diluent for the indicated times. Equal amounts of lysates were used to immunoprecipitate MEK6 with an anti-HA antibody. Immunoprecipitates were subjected to in vitro kinase assays using His-p38 as a substrate. The data are representative of at least two separate experiments (for each MEK). Lanes −, untreated.

MEK4 becomes activated in response to LPS with kinetics similar to those of MEK1, -2, and -3 (Fig. 2D). The fact that MEK1, -2, -3, -4, and -6 become activated in RAW 264.7 cells in response to LPS confirms and establishes the kinases upstream of the MAPK family members involved in LPS signaling.

Dexamethasone inhibits LPS-induced JNK/SAPK activity. Glucocorticoids are among the most important anti-inflammatory agents, having a range of anti-inflammatory activities. The glucocorticoid dexamethasone is known to block the production of various cytokines, including TNF-α (4). Dexamethasone inhibits TNF-α production specifically at the level of translation (4, 19). Conversely, pentoxifylline, a phosphodiesterase inhibitor, was shown to inhibit transcription of the TNF-α gene with no effect on TNF-α translation (19). We tested the ability of dexamethasone and pentoxifylline to interfere with LPS stimulation of ERK1 and ERK2, p38, or JNK/SAPK not only to establish a potential mechanism by which these agents act to inhibit TNF-α biosynthesis but also to determine if MAPK family members are involved in the regulation of TNF-α production. The amounts of dexamethasone and pentoxifylline used were the same as those shown to be effective for blocking TNF-α translation or transcription, respectively (19). Dexamethasone was able to inhibit LPS-induced JNK/SAPK activity by 70% (compared to that of untreated cells) (Fig. 3A), and this effect was dose dependent (Fig. 3B). A similar reduction of LPS-induced JNK/SAPK activity was observed following treatment with cortisone, hydrocortisone, and triamcinolone; thus, the inhibitory effect on LPS induction of JNK/SAPK activity is not unique to dexamethasone but is universal to the family of glucocorticoids (data not shown). β-Estradiol and progesterone had no effect on LPS induction of JNK/SAPK activity (Fig. 3B), thus demonstrating that the inhibitory effect on LPS induction of JNK/SAPK is specific for glucocorticoids. The ERK and p38 MAPKs were unaffected by dexamethasone (Fig. 3A). Pentoxifylline was unable to block LPS stimulation of ERK, p38, or JNK/SAPK activity (Fig. 3A). A 1-h incubation with the various agents prior to LPS addition was used since it had been shown that 1 h with either dexamethasone or pentoxifylline was sufficient to inhibit LPS induction of TNF transcription or translation (19). To further characterize the antagonistic effect of dexamethasone on LPS induction of JNK/SAPK activity, we varied the time of preincubation with dexamethasone. The effect of dexamethasone occurs rapidly, causing an inhibition of LPS-induced JNK/SAPK activity by 15 min, the earliest time examined (Fig. 3C). Longer incubations with dexamethasone do not enhance the inhibitory effect of dexamethasone on LPS induction of JNK/SAPK activity.

Dexamethasone inhibits a novel JNK/SAPK activator. To define the site of action of dexamethasone, we tested whether dexamethasone was able to inhibit LPS induction of MEK3, -4, or -6 activity. As shown in Fig. 4A, dexamethasone was not able to inhibit LPS induction of MEK3, -4, or -6 activity. There are at least two separate pathways, discernible by the type of cell stimulus, which lead to the activation of JNK/SAPK (2, 33, 35). JNK/SAPK activators such as NaCl and sorbitol do not mediate their JNK/SAPK-stimulatory effects through MEK4/SEK-1, whereas anisomycin induction of JNK/SAPK is MEK4/SEK-1 dependent (33, 35). Thus, dexamethasone may act to inhibit LPS induction of JNK/SAPK activity by inhibiting another activator of JNK/SAPK that is different from MEK4/SEK-1. If this is so, we might predict that dexamethasone would also inhibit JNK/SAPK activation by sorbitol. Thus, we tested dexamethasone effects on sorbitol induction of JNK/SAPK. As shown in Fig. 4B (top), dexamethasone significantly inhibits sorbitol-induced JNK/SAPK but does not inhibit JNK/SAPK activity induced by anisomycin. Sorbitol is able to activate MEK4; however, dexamethasone had no effect on sorbitol or anisomycin induction of MEK4/SEK-1 activity (Fig. 4B, bottom). These results indicate the existence of a novel (MEK4/SEK-1-independent) pathway leading to JNK/SAPK activation which is blocked by dexamethasone. These results also suggest a potential role for JNK/SAPK in translational regulation of TNF-α production and suggest that the mechanism by which glucocorticoids inhibit TNF-α translation may be mediated through an effect on JNK/SAPK activity.

SAPKβ K-A blocks LPS induction of TNF-α translation. We determined the involvement of JNK/SAPK in transcriptional and translational regulation of TNF-α production directly by examining the effect of a kinase-defective mutant of JNK/SAPK on the LPS-induced TNF-α transcriptional or translational reporter. As shown in Fig. 5A and B, SAPKβ K-A blocked the LPS-induced derepression of the translational blockade normally imposed by the TNF-α 3′-UTR but did not block the LPS-induced activation of the TNF-α promoter. SAPKβ K-A did not inhibit CAT mRNA transcription in cells cotransfected with the translational reporter (Fig. 5C), thus indicating that SAPKβ K-A acts specifically at the level of translation. SAPKβ K-A did not change transcription driven by the CMV promoter or by a cyclic AMP-responsive element (CAT activity was measured from lysates of cells cotransfected with control constructs), thus further demonstrating the specificity of the inhibitory effect of SAPKβ K-A on the TNF-α translational reporter (data not shown). SAPKβ K-A had no effect on LPS-induced activation of epitope-tagged ERK2 or p38 that was cotransfected with SAPKβ K-A (data not shown).

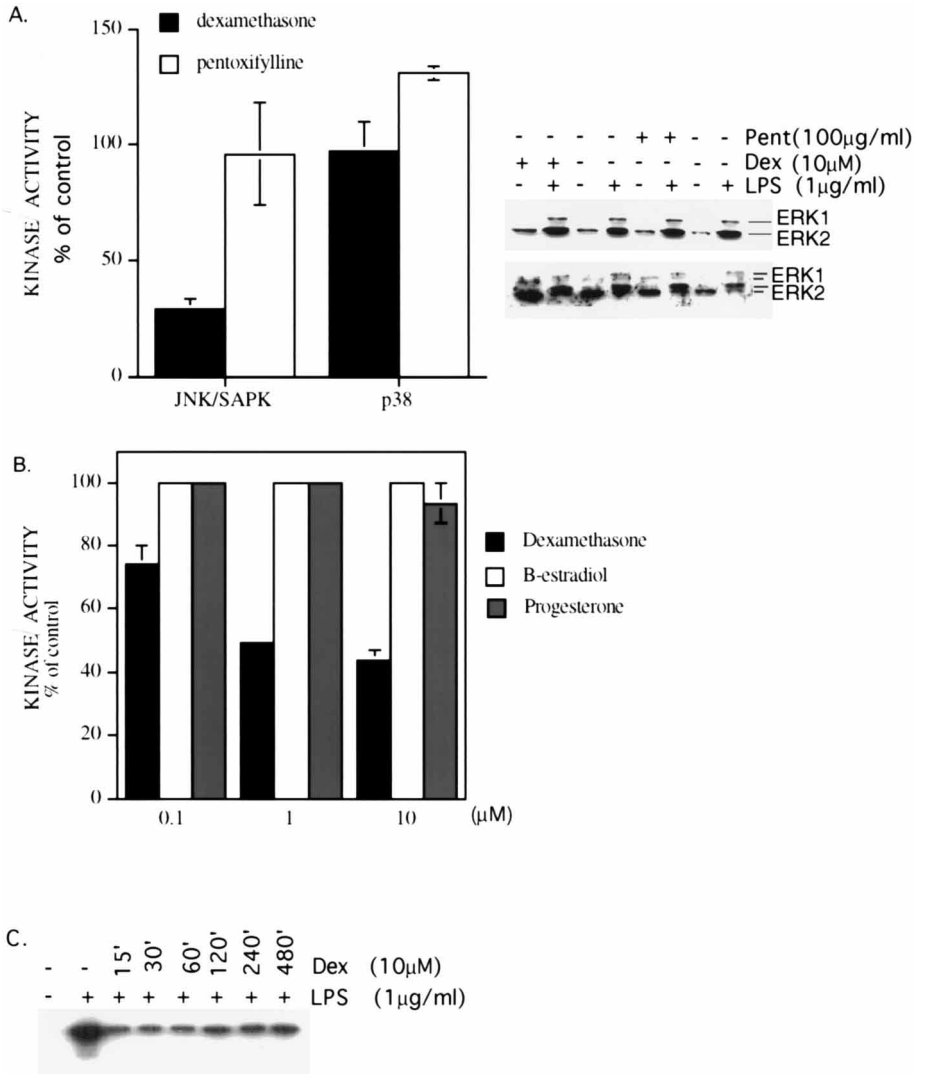

FIG. 3. Dexamethasone inhibits LPS induction of JNK/SAPK activity. (A) RAW 264.7 cells were starved for 18 h and were treated with dexamethasone (Dex), pentoxifylline (Pent), or diluent for 1 h, as indicated above the lanes. The cells were then stimulated with LPS or diluent for 30 min and lysed, and equal amounts of lysates were used to immunoprecipitate JNK/SAPK or p38 for in vitro kinase assays. Data are represented as percentages of the kinase activity demonstrated in control cells (pretreated with the respective diluent and stimulated with LPS) and are the averages of two separate experiments with the range indicated (left). Equal amounts of lysates were run on SDS-PAGE for Western blotting of ERK1 and ERK2 using an antibody which selectively recognizes active ERKs (right, top). The filter was stripped of antibody and was reprobed with an antibody (Y691) which recognizes both active and inactive ERK1 and ERK2. Active ERKs migrate at a lower rate than nonactive ERKs (right, bottom). (B) RAW 264.7 cells were starved for 18 h and were treated with increasing amounts of dexamethasone, β-estradiol, or progesterone (as indicated) or diluent (control) for 1 h prior to a 30-min stimulation with LPS (1 μg/ml) or diluent (control). The cells were lysed, and equal amounts of lysates were used to immunoprecipitate JNK/SAPK for in vitro kinase assays. Kinase activity was determined with an AMBIS radioanalytic device. Data represent LPS-induced JNK/SAPK activity (percentages of control values) and are the averages of two separate experiments, with the indicated range. (C) RAW 264.7 cells were starved for 18 h and were treated with dexamethasone (10 μM) or diluent (control) for the indicated times prior to a 30-min stimulation with LPS (1 μg/ml) or diluent (control). The cells were lysed, and equal amounts of lysates were used to immunoprecipitate JNK/SAPK for in vitro kinase assays. The autoradiograph displays phosphorylated GST-cJun and is representative of two separate experiments.

Wild-type SAPKβ was without effect on the TNF-α transcriptional, translational, or control reporter (data not shown).

Overexpression of wild-type SAPKβ overcomes dexamethasone suppression of the LPS-induced response by the TNF-α translational reporter. Dexamethasone inhibits LPS-induced TNF-α translation by 60 to 80%, as deduced from studies of TNF-α production and from use of the TNF-α 3′-UTR reporter in our studies (4, 19). Because dexamethasone also blocked LPS-induced JNK/SAPK activity, we tested whether overexpression of wild-type SAPKβ would overcome the dexamethasone-induced inhibition of TNF-α translation. As shown in Fig. 6, dexamethasone caused a 55% inhibition of the LPS-induced response by the TNF-α 3′-UTR translational reporter. Strikingly, overexpression of wild-type SAPKβ was able to restore LPS induction of the response by the TNF-α translational reporter to stimulated levels (Fig. 6). Thus, we propose that dexamethasone blocks TNF-α translation through its effects on JNK/SAPK.

DISCUSSION

The signal transduction pathway utilized by LPS to induce TNF-α biosynthesis is a subject of great importance; however, it is one about which little is known. Three separate MAPK

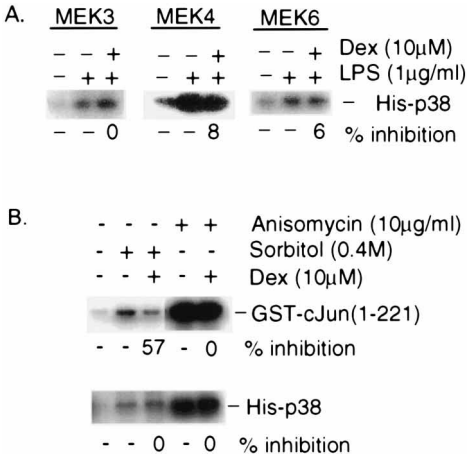

FIG. 4. Dexamethasone inhibits sorbitol-induced JNK/SAPK activity. (A) RAW 264.7 cells were starved for 18 h and were treated with dexamethasone (Dex) or diluent as indicated above the lanes for 1 h prior to a 30-min (for MEK3 and MEK4) or 60-min (for MEK6) stimulation with LPS or diluent. The cells were lysed, and equal amounts of lysates were used to immunoprecipitate either MEK3, MEK4, or MEK6 for in vitro kinase assays. Kinase activity was determined with an AMBIS radioanalytic device. Percent inhibition of MEK activity compared to that of a control is shown. The data are representative of at least two separate experiments. (B) RAW 264.7 cells were starved for 18 h and were treated with dexamethasone or diluent as indicated above the lanes for 1 h prior to a 30-min stimulation with sorbitol, anisomycin, or diluent. The cells were lysed, and equal amounts of lysates were used to immunoprecipitate either JNK/SAPK (top) or MEK4 (bottom) for in vitro kinase assays. Kinase activity was determined with an AMBIS radioanalytic device. Percent inhibition of JNK/SAPK activity compared to that of a control is shown. The data are representative of at least two separate experiments.

pathways containing either ERK2, p38, or JNK/SAPK become activated in response to LPS (13, 15, 16, 40, 50). While the observation that the three kinases are activated by LPS has been previously reported, only p38 and upstream regulators of ERKs, Ras and Raf, have been shown to be directly involved in regulation of TNF-α production (15, 30). Thus, we set out to further characterize the biochemical pathways leading to TNF-α production as well as to establish signaling molecules directly involved in regulation of TNF-α biosynthesis, using the murine macrophage cell line RAW 264.7. We demonstrate that LPS activates ERK1 and ERK2, p38, and JNK/SAPK in our system, all having similar kinetics of activation. Thus, we have confirmed the activation of the MAPK family members by LPS and established the starting point for our investigation. We next determined whether the kinases found to be upstream of the MAPKs in other systems, the MEKs, also become activated in response to LPS. We confirm that LPS activates MEK1 and MEK4 (upstream regulators of ERK1 and ERK2 and of JNK/SAPK, respectively). We demonstrate that LPS also activates MEK2, -3, and -6 (upstream regulators of ERK1 and ERK2 and of p38, respectively). Thus, we have established two tiers of the MAPK module for the MAPK family members ERK1 and ERK2, p38, and JNK/SAPK which become activated in response to LPS (Fig. 7).

In addition, we demonstrate direct involvement of JNK/SAPK in regulation of TNF-α biosynthesis at the level of translation. Expression of the kinase-dead mutant SAPKβ, SAPKβ K-A, blocked LPS-induced derepression of the translational blockade normally imposed by the TNF-α 3′-UTR.

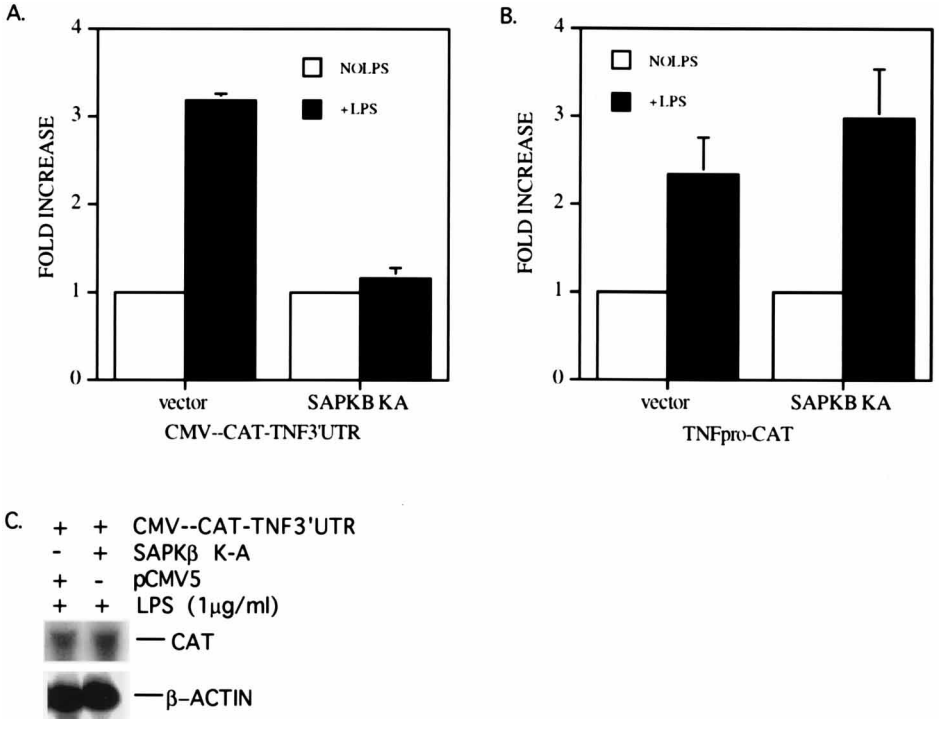

FIG. 5. Release of the translational blockade imposed by the TNF-α 3′-UTR is prevented by a dominant inhibitor of SAPKβ. RAW 264.7 cells were cotransfected with either pCMV5 (empty vector) or an expression vector encoding a dominant inhibitor of SAPKβ (SAPKβ K-A) and either the TNF-α translational reporter (A) (CMV-CAT-TNF3′UTR) or the TNF-α transcriptional reporter (B) (TNFpro-CAT). The transfected cells were split into two plates. At 24 h following transfection, the cells were treated with LPS (1 μg/ml) or diluent. After 16 h, cells were harvested for assessment of CAT activity. The fold increase in CAT activity in stimulated versus unstimulated cells was determined with an AMBIS radioanalytic device. The data are the averages of two separate experiments, with the ranges indicated. RNA from RAW 264.7 cells cotransfected with the TNF-α translational reporter and either pCMV5 or SAPKβ K-A was electrophoresed on a formaldehyde-containing gel and was blotted onto nylon sheets (C). The blots were hybridized with either a radioactive CAT cDNA probe (top) or a radioactive β-actin probe (bottom). CAT and β-actin mRNA transcripts are indicated. The data are representative of three separate experiments.

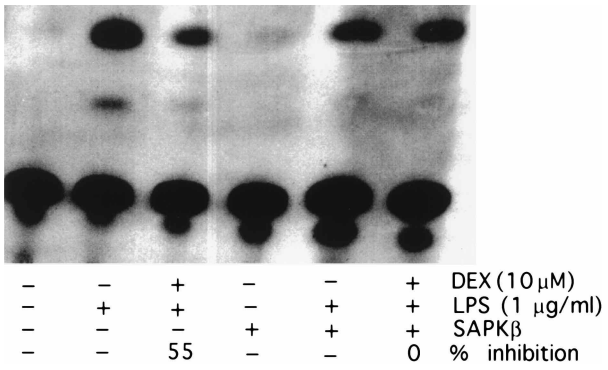

FIG. 6. SAPKβ is able to overcome the dexamethasone-induced suppression of TNF-α translation. RAW 264.7 cells were cotransfected with the TNF-α translational reporter and either pCMV5 (empty vector) or SAPKβ. The transfected cells were split into three plates (for each transfection type). At 24 h following transfection, the cells were pretreated with dexamethasone (DEX) or diluent (as indicated below the lanes) for 1 h prior to a 6-h stimulation with LPS (1 μg/ml) or diluent. Cells were harvested for assessment of CAT activity (measured with an AMBIS radioanalytic device). The data are representative of three separate experiments.

The molecular mechanism of this blockade remains unknown. This effect was not due to a lack of CMV-driven transcription of CAT mRNA, since SAPKβ K-A did not inhibit transcription of CAT mRNA in cells cotransfected with the translational reporter, nor did SAPKβ K-A have any effect on CAT activity from a control CMV promoter-driven CAT reporter which is identical to the TNF-α translational reporter except that it does not contain the TNF-α 3′-UTR sequence. Previous studies have demonstrated that JNK/SAPK regulates transcription by activating the transcription factor AP-1 (25). Our studies expand the role of JNK/SAPK by demonstrating that it regulates translational events as well.

Glucocorticoids are among the most potent and clinically important immunosuppressant drugs and inhibit many proinflammatory events, such as TNF-α production, by ill-defined mechanisms. Pretreatment of animals with glucocorticoids prevents LPS-induced shock in animals (32). Previous studies demonstrated that the glucocorticoid dexamethasone inhibits TNF-α translation (4, 19). However, as with other anti-inflammatory actions of glucocorticoids, the mechanism for this inhibition is unknown. Our results suggest a potential mechanism. We demonstrate that dexamethasone inhibits LPS induction of JNK/SAPK activity but not that of ERK1 and ERK2, p38, or MEK3, -4, and -6. We also show that overexpression of wild-type SAPKβ overcomes the dexamethasone-induced inhibition of the response of a TNF-α translational reporter following LPS stimulation. These results support our hypothesis that JNK/SAPK is involved in translational regulation of TNF-α production and offer a mechanism for dexamethasone inhibition of cytokine production, specifically through the inhibition of JNK/SAPK.

Dexamethasone is able to inhibit sorbitol-induced but not anisomycin-induced JNK/SAPK activation. This indicates that there is an as-yet-undefined upstream activator of JNK/SAPK, the activity or activation of which is inhibited by dexamethasone. Thus, LPS, like sorbitol, uses primarily a MEK4-independent pathway to activate JNK/SAPK, which may be the site of action of dexamethasone. The dexamethasone-insensitive residual JNK/SAPK activity induced by LPS (Fig. 3A) is probably due to LPS induction of MEK4 activity, since MEK4 is resistant to the inhibitory effects of dexamethasone. It is possible that the major role of LPS-induced MEK4 activity is to activate p38, since both p38 and JNK/SAPK become phosphorylated and activated by MEK4 (12, 30, 39).

The fact that dexamethasone did not inhibit LPS induction of p38 or the ERKs does not imply that these kinases are not involved in the regulation of TNF-α production. p38 was identified because it binds to a compound which blocked TNF-α production at the level of translation (30) and because it is activated in response to LPS (21). Thus, p38 also appears to be required for translational regulation of TNF-α. Furthermore, upstream components in the ERK pathway, Ras and Raf, are required for TNF-α production at the level of both transcription and translation (15); however, direct evidence supporting a role for ERKs themselves in TNF-α production has not been reported.

The regulation of TNF-α biosynthesis appears to be quite complex. LPS-induced signaling pathways leading to TNF-α biosynthesis are depicted in Fig. 7. This model incorporates

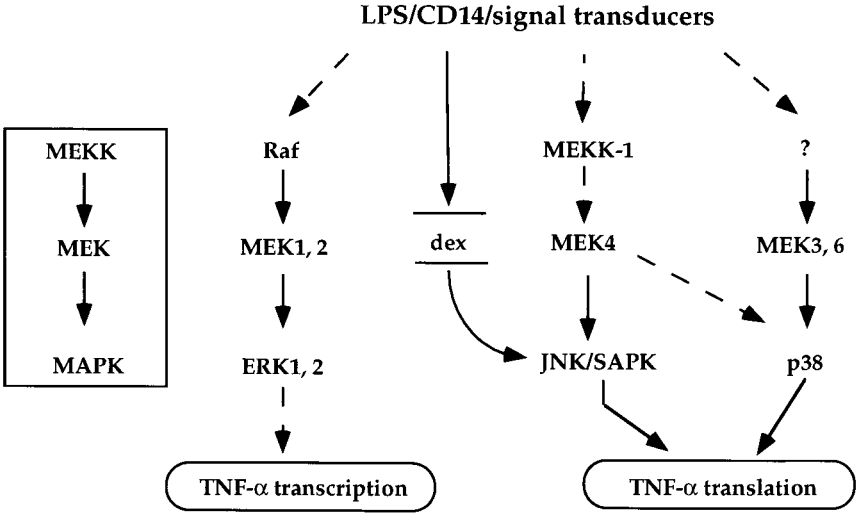

FIG. 7. LPS-induced signaling pathways leading to TNF biosynthesis. The MAPK module (boxed) and members of the various MAPK pathways are indicated. Relationships supported by data in the literature as well as data presented in this paper (solid arrows) and unknown mechanisms or relationships (broken arrows) are shown.

data from the literature, as well as data described in this paper. We propose a potential role for the ERKs in transcriptional regulation of TNF-α and a requirement for the p38 and JNK/SAPK pathways for regulation of TNF-α biosynthesis at the level of translation; however, the relative contributions of the pathways are currently unknown.

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