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Tumor Necrosis Factor α Stimulates AP-1 Activity through Prolonged Activation of the c-Jun Kinase*

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John K. Westwick‡§, Christoff Weitzel‡, Audrey Minden¶, Michael Karin¶, and David A. Brenner‡‖

From the ‡Departments of Medicine and Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina 27599 and the §Program in Molecular Pathology and ¶Department of Pharmacology, University of California at San Diego School of Medicine, La Jolla, California 92093

Tumor necrosis factor α (TNFα) has multiple biological functions including the prolonged activation of the collagenase and c-*jun* genes, which are regulated via their AP-1 binding sites. We show that incubating human fibroblasts with TNFα induces prolonged activation of JNK, the c-Jun kinase, which phosphorylates the transactivation domain of c-Jun. Furthermore, an immune complex kinase assay specifically demonstrates that TNFα stimulates the activity of JNK1, the recently described predominant form of JNK. TNFα also produces a small and transient increase in extracellular signal-regulated kinase (ERK) activity and no measured increase in Raf-1 kinase activity. On the other hand, epidermal growth factor causes a prolonged activation of Raf-1 kinase and ERK activity and a smaller, more transient activation of JNK, whereas the phorbol ester phorbol 12-myristate 13-acetate causes a small stimulation of Raf-1 kinase and a pronounced stimulation of ERK activity. The activation of JNK by TNFα does not correlate with Raf-1 or ERK activity. The kinetics of Raf-1, ERK, and JNK induction by epidermal growth factor, phorbol 12-myristate 13-acetate, or TNFα indicate distinct mechanisms of activation in human fibroblasts.

Tumor necrosis factor α (TNFα),[1] a potent cytokine produced mainly by activated monocytes, has multiple biological functions (1). TNFα actions are initiated by binding to two distinct TNFα receptors of 55–60 kDa (TNF R-55 or TNFR1) and 75–80 kDa (TNF R-75 or TNFR2), which have different affinities for TNFα and may mediate different downstream responses (2–8). The binding of TNFα to its receptors triggers the activation of several second messengers through stimulation of protein kinase C, sphingomyelinase, and phospholipase A_2 (9–12). Exposure to TNFα also results in NFκB activation (13, 14) and stimulation of AP-1 activity (9). The final nuclear targets for TNFα include genes containing AP-1 binding sites, such as collagenase and c-*jun*, and genes containing NFκB binding sites, such as the human immunodeficiency virus-long terminal repeat and a variety of cytokine genes (9, 14, 15).

Incubating cultured fibroblasts with TNFα results in prolonged activation of the collagenase and c-*jun* genes as compared with incubating with the phorbol ester PMA (9). The stimulation of c-*jun* transcription by TNFα is independent of *de novo* protein synthesis (9). TNFα also causes a transient activation of the c-*fos* gene. The products of the c-*fos* and c-*jun* genes are components of the transcription factor AP-1 (16, 17).

The transcriptional activity of the c-Jun protein is regulated by its phosphorylation status. Phosphorylation of c-Jun at Thr-231, Ser-243, and Ser-249 near its DNA binding domain inhibits its DNA binding and therefore reduces transcriptional activity (17, 18). Phosphorylation of two of these sites (Thr-231 and Ser-249) is catalyzed *in vivo* and *in vitro* by casein kinase II (18). Ser-243 is the major site of phosphorylation by ERKs (19–21). On the other hand, phosphorylation of c-Jun in its activation domain at Ser-63 and Ser-73 increases its transcriptional activity (22–24) but does not affect its DNA binding activity (25). Phosphorylation of the amino-terminal activation domain is catalyzed by a specific Jun nuclear kinase (JNK), which has been recently purified, and one of its constituents, JNK1, has been cloned (26, 27).

In this study, the pathways in human diploid fibroblasts by which TNFα stimulates c-Jun are compared with those stimulated by EGF and the phorbol ester PMA. PMA functions in part by causing dephosphorylation of residues adjacent to the DNA binding domain in the carboxyl-terminal half of c-Jun (17). EGF functions through multiple signal transduction pathways, which include the activation of Ras, Raf, and extracellular signal-regulated kinases (ERK1 and ERK2) (reviewed in Refs. 19 and 28).

EXPERIMENTAL PROCEDURES

Cell Culture—MRC-5 human fibroblast cells at the 14th passage were obtained from ATCC (CCL 171) and cultured in minimum essential medium with Earle's salts (Life Technologies, Inc.) containing 10% fetal bovine serum (Gemini), 0.5 mg/ml L-glutamine, 180 units/ml penicillin G, and 180 μg/ml streptomycin (Irvine Scientific).

Materials—Receptor grade EGF (Sigma) and TNFα (R & D Systems) were used at a final concentration of 10 ng/ml. PMA (Sigma) was used at a final concentration of 60 ng/ml.

Plasmids—The luciferase reporter gene, 2XTRELuc, is described in Ref. 29 (gift of K. Chien, University of California, San Diego). pSVGal-Jun (amino acids 1–246 of c-Jun linked to the Gal4 DNA binding domain), pSVGal-Jun(AA) (with serine 63 and serine 73 mutated to alanine), GST-Jun (amino acids 1–223 of c-Jun), and GST-Jun(AA) (with serine 63 and serine 73 mutated to alanine) are described in Ref. 26. Gal4-ElkC (Elk-1 codons 307–428) was provided by R. Treisman (30). RSVc-Jun is described in Ref. 31. Ras N17 was a gift from C. Der. The kinase inactive MEK-1 expression construct was a gift from G. L. Johnson.

Transfections and Luciferase Assays—MRC-5 cells were transfected by the calcium phosphate coprecipitation technique as previously described (32). Briefly, 5 μg of reporter gene were cotransfected with 0.5 μg of expression vector where indicated. Cells were incubated in complete media for 4 days after removing the precipitates, and then the indicated

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‖ To whom correspondence should be addressed: Dept. of Medicine, CB 7080, University of North Carolina, Chapel Hill, NC 27599. Tel.: 919-966-2513; Fax: 919-966-6842; E-mail: dab@med.unc.edu.

[1] The abbreviations used are: TNFα, tumor necrosis factor α; ERK, extracellular signal-regulated kinase; EGF, epidermal growth factor; PMA, phorbol 12-myristate 13-acetate; JNK, Jun nuclear kinase; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis.

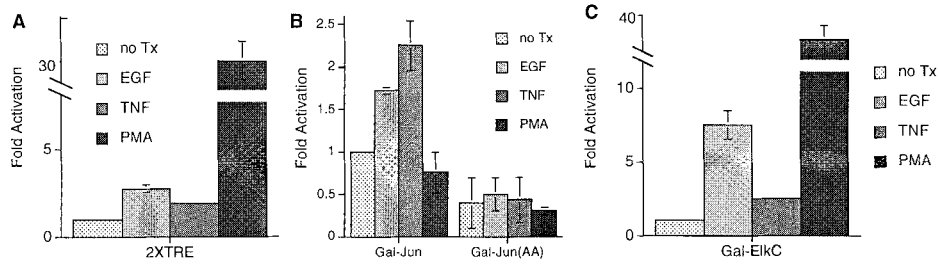

FIG. 1. **TNFα, EGF, and PMA stimulate AP-1 transcriptional activity.** *A*, MRC-5 cells were transfected with a luciferase reporter gene containing two consensus AP-1 binding sites (2XTRE). Following transfection, cultures were incubated until confluent (4 days) and then stimulated for 5 h with TNFα, EGF, or PMA as indicated. *B*, MRC-5 cells were transfected with 5XGal-Luc plus the Gal-Jun expression vector or the mutated version, Gal-Jun(AA), and treated as in *A*. *C*, MRC-5 cells were transfected with the luciferase reporter gene containing 5 Gal4 binding sites (5XGal-Luc) and the expression vector Gal-ElkC. Cells were treated as in *A*. The graphs represent the mean values from two to four experiments performed in duplicate. *Tx*, treatment.

factors were added. 5 h after factor addition, extracts for luciferase and protein assays were prepared as described (32). All luciferase values were normalized for extract protein concentration.

Cellular Extracts—Extracts were prepared as described in Ref. 33. The nuclear isolation step was omitted for the preparation of whole cell extracts.

Cell Labeling, Immunoprecipitation, and Phosphopeptide Mapping—Subconfluent 100-mm plates of MRC-5 cells were labeled with 0.6 mCi/ml ^{32}P (orthophosphate) or 150 μCi/ml Tran^{35}S-label (ICN). After 2.5 h, EGF or TNFα was added to the media, and cells were incubated an additional 30 min before washing with phosphate-buffered saline and lysis in radioimmune precipitation buffer. Immunoprecipitation of endogenous c-Jun protein, transfer to nitrocellulose, and two-dimensional phosphopeptide mapping were performed as described (32). No differences in c-Jun protein content between treated and untreated cells were detected on [^{35}S]methionine-labeled gels (data not shown). Plates were exposed to film (Kodak XAR) for 10 days at -85 °C.

Gel Mobility Shifts Assays—Electrophoretic mobility shift assays were carried out as previously described (29) using 10 μg of MRC-5 nuclear extract prepared at various times following cell stimulation. An oligonucleotide representing the consensus AP-1 binding site from the human collagenase gene (34) was synthesized on a Millipore Cyclone Plus synthesizer and used as probe. Polyclonal rabbit anti-c-Jun antiserum or normal rabbit serum was added where indicated, and reactions were continued another 20 min before loading onto 4% non-denaturing polyacrylamide gels. The anti-c-Jun antisera had no effect on binding when assayed with a binding site for HNF-1 (data not shown).

In Vitro Kinase Assays—The solid-phase Jun kinase assays were carried out as described (26) using a GST-c-Jun (1–223) fusion protein coupled to glutathione-agarose beads as substrate. 50 μg of cellular extract were used as the kinase source. The mutated version (GST-c-Jun Ala 63,73) and GST protein alone were used as control substrates. Preparation of recombinant kinase inactive MEK-1, immunoprecipitation of Raf-1, and Raf-1 immune complex kinase assays were performed as described (35). For the immune complex JNK1 assay, 50 μg of cellular extracts were immunoprecipitated with a specific antiserum raised against recombinant JNK1 (27), and the kinase assay was performed as described for Raf-1 with the addition of 20 μM ATP, using GST-c-Jun as substrate. Phosphorylated proteins were resolved on 10% SDS-PAGE, radioactivity was visualized by autoradiography, and all gels were quantified by scintillation counting of excised bands.

In-gel ERK Assay—Activation of ERK1 and ERK2 was determined using myelin basic protein as the substrate in an in-gel kinase assay as described, using 50 μg of each cell extract (36). The position of the ERKs was confirmed by comparison with both Rainbow molecular weight markers (Amersham Corp.) and to low molecular weight protein markers (Life Technologies, Inc.).

RESULTS

TNFα Stimulates AP-1 Activity and c-Jun Phosphorylation—To assess the effect of TNFα, PMA, or EGF on AP-1 transcriptional activity, MRC-5 fibroblasts were transfected with a luciferase reporter gene driven by AP-1 binding sites (2XTRE). As assessed by this assay, treatment with TNFα, EGF, or PMA stimulates the endogenous AP-1 activity of the cells (Fig. 1*A*), as previously demonstrated (9, 37), with PMA being the most potent activator.

The c-Jun transactivation domain is stimulated by its phosphorylation at Ser-63 and Ser-73 (as discussed below). To functionally assess the effect of TNFα, EGF, or PMA on the transactivation domain, we transfected MRC-5 cells with an expression construct encoding the activation domain of c-Jun fused to the DNA binding domain of the Gal4 protein. This Gal4-Jun expression vector was cotransfected with a reporter gene containing five copies of the Gal4 DNA binding domain linked to the luciferase reporter gene (5XGal-Luc). Treatment with TNFα enhances the c-Jun transcriptional activity, resulting in an increase of reporter gene activity ($p < 0.05$, Fig. 1*B*). Treatment with TNFα does not cause an increase in luciferase activity in the presence of a mutated expression vector in which Ser-63 and Ser-73 are changed to alanines (Fig. 1*B*). EGF treatment stimulates luciferase activity to a lesser degree ($p < 0.05$), and PMA has no significant effect. Cotransfection with the dominant negative Ras N17 mutant does not inhibit TNFα stimulation of the reporter gene (data not shown).

Although ERK activation is critical in multiple signaling pathways, its role in TNFα signaling is unknown. To functionally assess ERK activation by TNFα, EGF, or PMA, we transfected the fibroblasts with an expression construct encoding the activation domain of the transcription factor Elk-1 fused to the DNA binding domain of the Gal4 protein. Phosphorylation of Elk-1 by ERKs stimulates the Elk-1 activation domain (30). This Gal-ElkC expression vector is cotransfected with the 5XGal-Luc reporter gene. Treatment with PMA or to a lesser degree EGF enhances the Elk-1 transcriptional activity (Fig. 1*C*). TNFα weakly stimulates Elk-1 transcriptional activity, consistent with its minimal stimulation of ERK activity, as discussed below.

To assess the role of the c-Jun protein in stimulated AP-1 activity, we investigated the effect of agonist treatment on its post-translational modification. Since TNFα induces c-*jun* transcription in the absence of *de novo* protein synthesis (9), we assessed the effect of TNFα on c-Jun phosphorylation, the only known modification that affects c-Jun activity (16). Treatment of fibroblasts with either TNFα or EGF for 30 min increased [^{32}P]phosphate incorporation into c-Jun relative to untreated cells (Fig. 2*A*). Previous studies have demonstrated that the phosphorylated forms of c-Jun are present in both bands seen in SDS-PAGE gels (38). Two-dimensional phosphopeptide maps demonstrated that treatment with TNFα increased phosphorylation of Ser-63 and Ser-73 of c-Jun as indicated by the increased intensity of the *X* and *Y* phosphopeptides (Fig. 2*B*). Previous studies demonstrated that *X* and *Y* contain Ser-73 and Ser-63 (22, 23). No effect on the carboxyl-terminal sites was detected. In comparison, treatment with EGF (Fig. 2*B*) increased phosphorylation of the *X* phosphopeptide of c-Jun to a lesser extent than TNFα treatment and induced no phosphorylation of the *Y* peptide. On the other hand, EGF treatment increases the intensity of the *b* and *c* carboxyl-terminal phos-

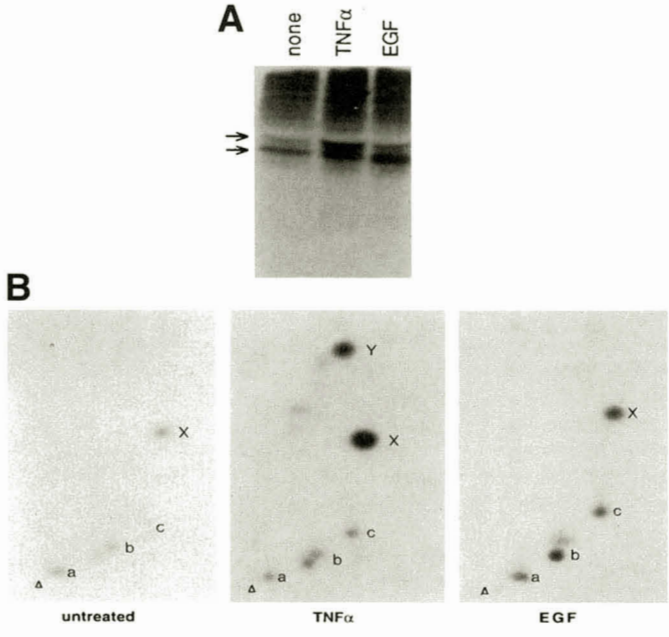

FIG. 2. **TNFα and EGF modulate phosphorylation of the c-Jun protein.** *A*, cultures of MRC-5 cells were labeled with 0.6 mCi/ml ^{32}P (orthophosphate) prior to stimulation with the indicated factors for 30 min. Proteins immunoprecipitated with a c-Jun-specific antisera were subjected to SDS-PAGE. The *arrows* indicate the c-Jun protein. *B*, c-Jun protein from *A* was eluted, trypsinized, and subjected to two-dimensional phosphopeptide mapping. *Spots* representing specific peptides from the amino-terminal end of c-Jun (*X*, *Y*) and the carboxyl-terminal end (*a*, *b*, *c*) are indicated.

phopeptides compared with untreated and TNFα-treated cells. This is consistent with activation of ERKs 1 and 2 by EGF (39). The c-Jun phosphorylation pattern of PMA-treated fibroblasts has been previously shown to consist of decreased phosphorylation of sites adjacent to the DNA binding domain (17).

PMA Increases AP-1 DNA Binding Activity—Next, we performed gel mobility shift assays with a radiolabeled AP-1 binding site probe incubated with nuclear extracts prepared from untreated and treated fibroblasts. Treatment with the phorbol ester PMA causes a rapid and prolonged increased DNA binding (Fig. 3*A*), which has previously been demonstrated to result from dephosphorylation of c-Jun serine residues near the DNA binding domain as well as increased Fos synthesis (17). In comparison, treatment with EGF or TNFα results in a smaller increase in the level of the AP-1 binding complexes (Fig. 3, *A* and *B*). To demonstrate that c-Jun contributes to the AP-1 complex binding to this probe, extracts from TNFα-treated cells at a variety of time points were used in mobility shift assays in the presence or absence of c-Jun-specific antiserum. The disruption of the AP-1 DNA complex by c-Jun antiserum (Fig. 3*C*) demonstrates that c-Jun is a major constituent of the AP-1 complex in control and TNFα-treated cells.

TNFα Induces a Prolonged Activation of JNK—To determine whether TNFα-induced phosphorylation of c-Jun results from either increased JNK activity or decreased phosphatase activity, we used a previously described solid-state assay to measure JNK activity (26). Immobilized GST-c-Jun protein (consisting of the activation domain of c-Jun linked to GST) was incubated with fibroblast extracts prepared at different time points following stimulation with EGF, TNFα, or PMA. TNFα treatment results in a biphasic induction of c-Jun kinase activity with an early maximal stimulation of greater than 23-fold, followed by a prolonged plateau stimulation persisting for at least 19 h (Fig. 4*A* and data not shown). On the other hand, treatment with PMA or EGF results in a smaller, transient stimulation of JNK activity with rapid return to basal levels (Fig. 4*A*). Quan-

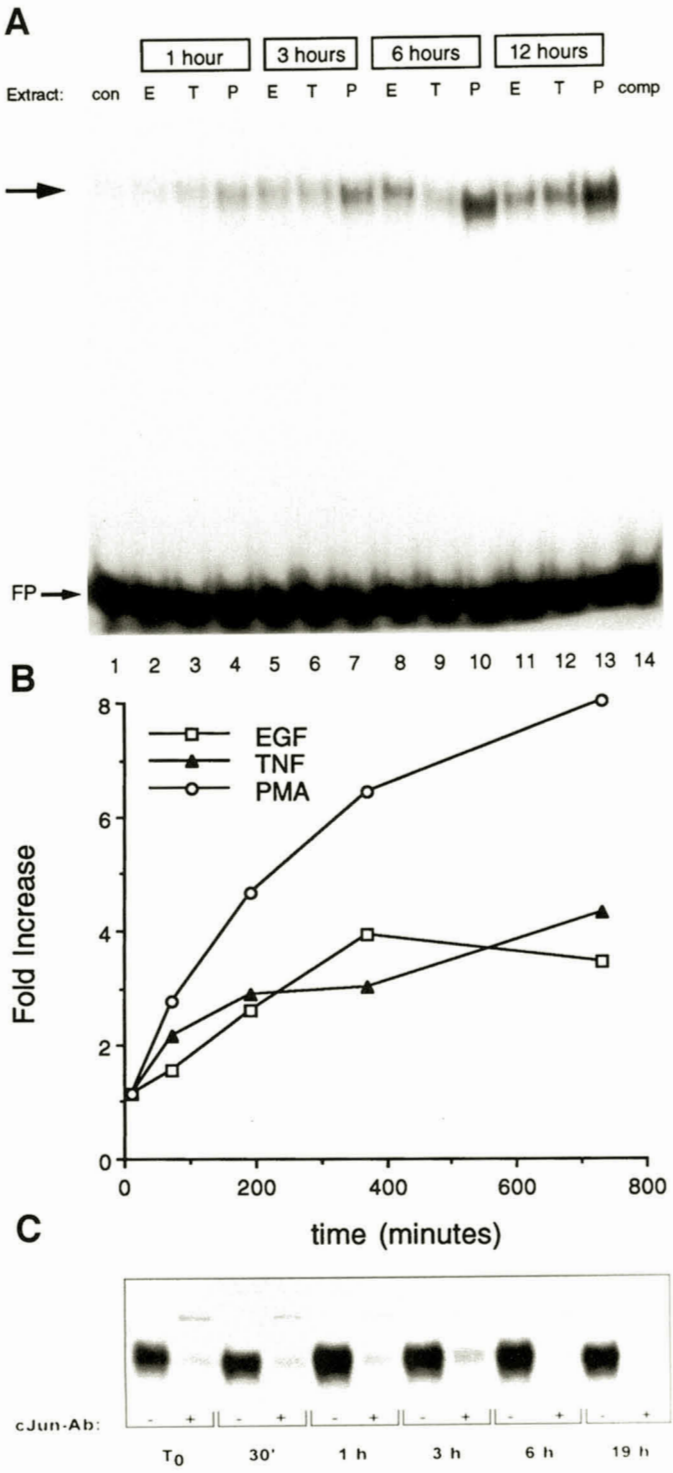

FIG. 3. **Effect of cytokine treatment on AP-1 DNA binding activity in MRC-5 cells.** *A*, AP-1 DNA binding activity was assayed with a radiolabeled human collagenase probe in gel mobility shift assays. Extracts from untreated and treated MRC-5 cells were incubated with an excess of probe on ice for 20 min, followed by non-denaturing gel electrophoresis. Shifted complexes are indicated by the *large arrow*. *T*, TNFα; *E*, EGF; *p*, PMA; *Fp*, free probe; *con*, control cells; *comp*, competitor. *B*, quantitation of *A* expressed as -fold increase over unstimulated levels. *C*, contribution of c-Jun to the AP-1 complex following TNFα treatment. Extracts, prepared at the indicated times after TNFα treatment, were incubated with an excess of probe with or without subsequent incubation with a c-Jun-specific antisera (+) or normal rabbit serum (–) and subjected to electrophoresis as in *A*. Specific shifted complexes, which were disrupted by the anti-c-Jun antisera, are shown. *c-Jun-Ab*, c-Jun antiserum.

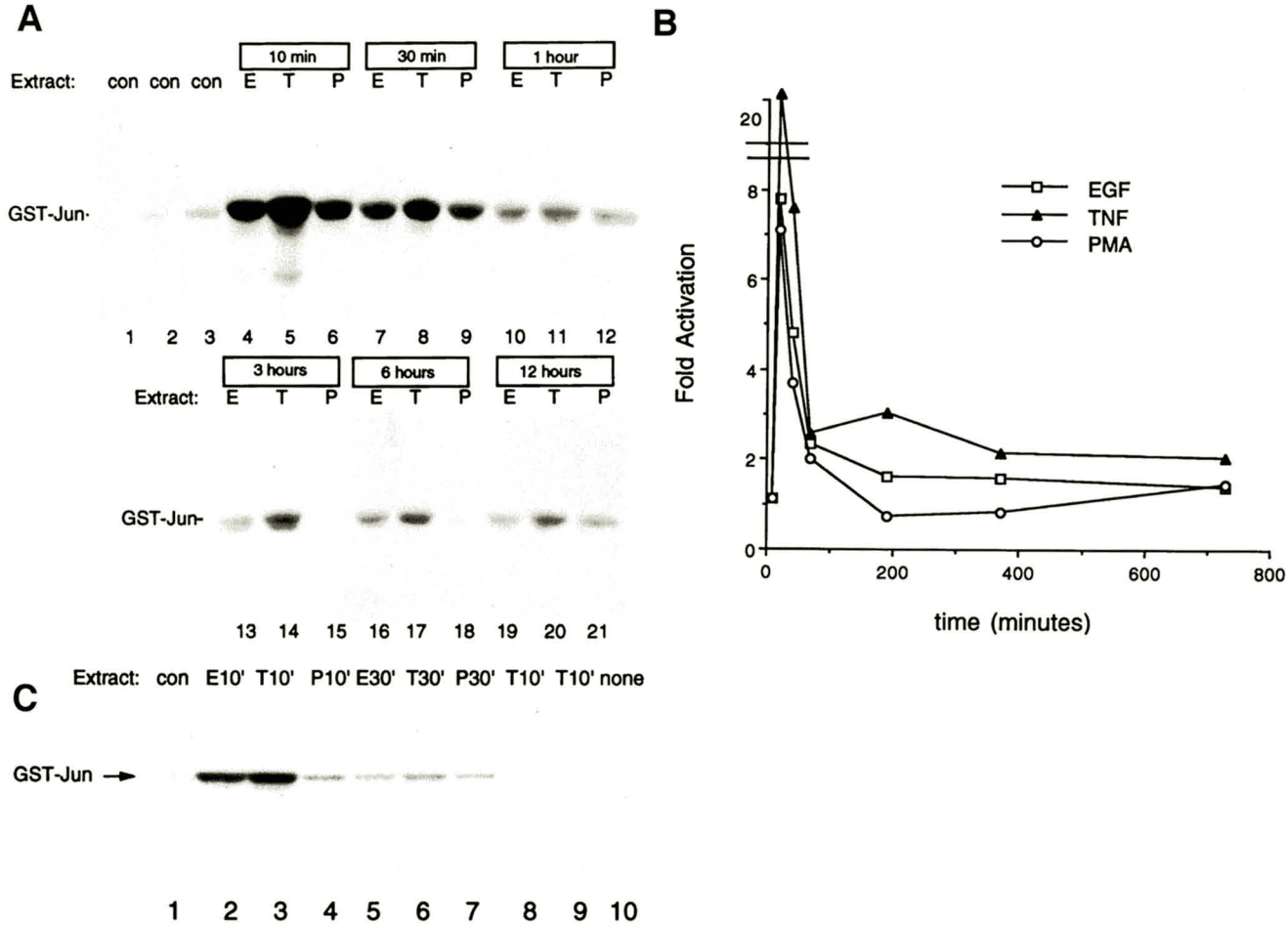

FIG. 4. **Activation of JNK following cytokine treatment.** *A*, recombinant GST (*lane 1*), GST-c-Jun (*lanes 3–21*), or mutated GST-Jun(AA) (*lane 2*) were incubated with 50 μg of extract from untreated cells (*con*) or cells treated for the indicated time period with TNFα (*T*), EGF (*E*), or PMA (*P*). Phosphorylation was assessed by incorporation of [γ-^{32}P]ATP in the c-Jun protein, followed by SDS-PAGE and autoradiography. The *single band* represents the GST-c-Jun protein. The experiment was repeated with three separate extract preparations with $p < 0.04$ for TNFα *versus* control or EGF for all time points. *B*, quantitation of *A* expressed as -fold activation over unstimulated levels. *C*, an immune complex kinase assay using JNK1-specific antiserum was performed with cell extracts from control cells or cells treated for the indicated times with TNFα, EGF, or PMA.

titation of a representative experiment is graphically depicted (as -fold increase over base-line levels) in Fig. 4*B*.

To directly assess the effect of agonist treatment on JNK1 activity, an immune complex kinase assay was performed using JNK1-specific antiserum. Although all three agonists stimulate JNK1 activity compared with control cells, TNFα treatment results in the greatest stimulation, followed by EGF and then PMA (Fig. 4*C*). After 10 min of incubation, JNK1 activity is stimulated 9.4-fold by TNFα, 5.8-fold by EGF, and 3.1-fold by PMA.

TNFα Does Not Induce Raf-1 Kinase and Transiently Induces ERKs—Although recent studies have demonstrated a signal transduction pathway in which EGF receptor binding to its ligand sequentially activates Ras, Raf-1, and ERKs (19, 28), the signal transduction pathways for TNFα are largely unknown. Therefore, we assessed Raf-1 kinase activity in MRC-5 fibroblasts after incubation with EGF, TNFα, or PMA. As expected, EGF induces a large and prolonged activation of Raf-1 kinase (Fig. 5, *A* and *B*). PMA produces a lesser stimulation while TNFα does not stimulate Raf-1 kinase activity at any measured time point.

Finally, we examined whether ERKs are activated in parallel with JNK. Using an in-gel myelin basic protein kinase assay with the same extracts assayed for JNK and Raf-1 kinase activity, a completely different temporal pattern of stimulation is observed. TNFα-treated extracts contain elevated ERK activity only at the earliest time points, while PMA- or EGF-treated extracts demonstrate markedly elevated ERK activity at early time points followed by prolonged activity that remained greater than 2-fold over base-line levels for 3 h (Fig. 6, *A* and *B*). Improved resolution by a longer electrophoresis time revealed the appearance of two bands consistent with the stimulation of ERK1 and ERK2 activity (data not shown). The different effects of TNFα, PMA, and EGF on JNK and ERK activity were seen in several experiments using different extract preparations.

DISCUSSION

TNFα actions are initiated by binding to two distinct monomeric TNFα receptors, TNF R-55 and TNF R-75, which are found in nearly all cells (5–8). Most of the downstream signaling events initiated by TNFα receptor binding has been attributed to the TNF R-55 (2, 4, 40). A knockout mouse in which the TNF R-55 gene is disrupted is unable to mediate proper responses to microorganisms, including endotoxic shock and clearance of Listeria monocytogenes (4, 40). The role of TNF R-75 is more controversial, but biological activities have been attributed to it, including cytotoxicity and cytokine secretion by T cells and stimulation of TGFα mRNA levels (3, 41–43). Neither TNFα receptor has intrinsic kinase activity, and our understanding of the signal transduction pathways activated by TNFα are incomplete.

TNFα increases the levels of multiple second messengers. TNFα has been demonstrated to activate phosphatidylcholine-

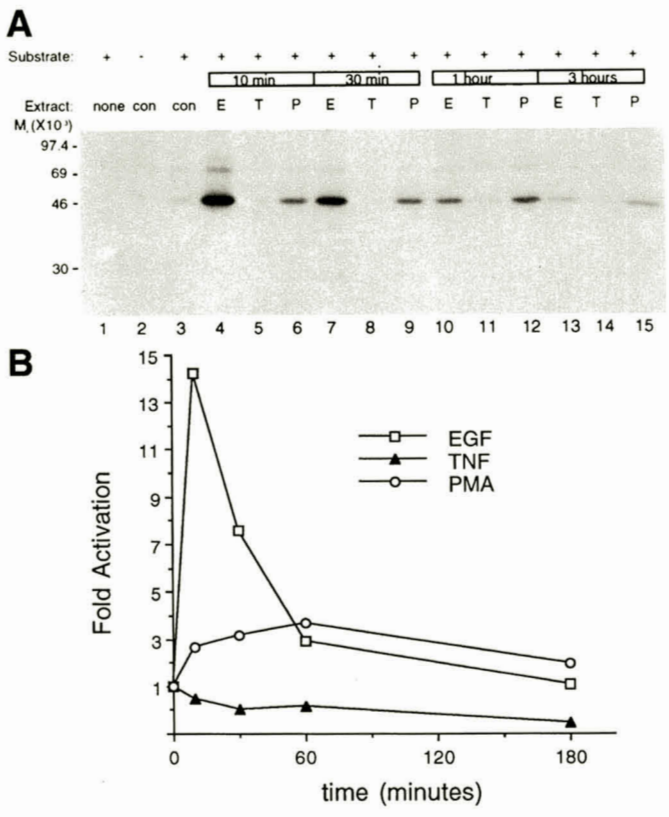

FIG. 5. **TNFα treatment does not stimulate Raf-1 kinase activity.** *A*, extracts from EGF- (*E*), TNFα- (*T*), and PMA- (*P*) treated cells were used as the kinase source in a c-Raf-1 immune complex *in vitro* kinase assay. Kinase inactive MEK-1 was used as substrate. Substrate alone (*lane 1*) and immunoprecipitated extract alone (*lane 2*) were included as controls. The prominent band migrating at 46 kDa corresponds to the size of recombinant MEK-1. *con*, control cells. *B*, quantitation of *A* expressed as -fold activation over unstimulated levels.

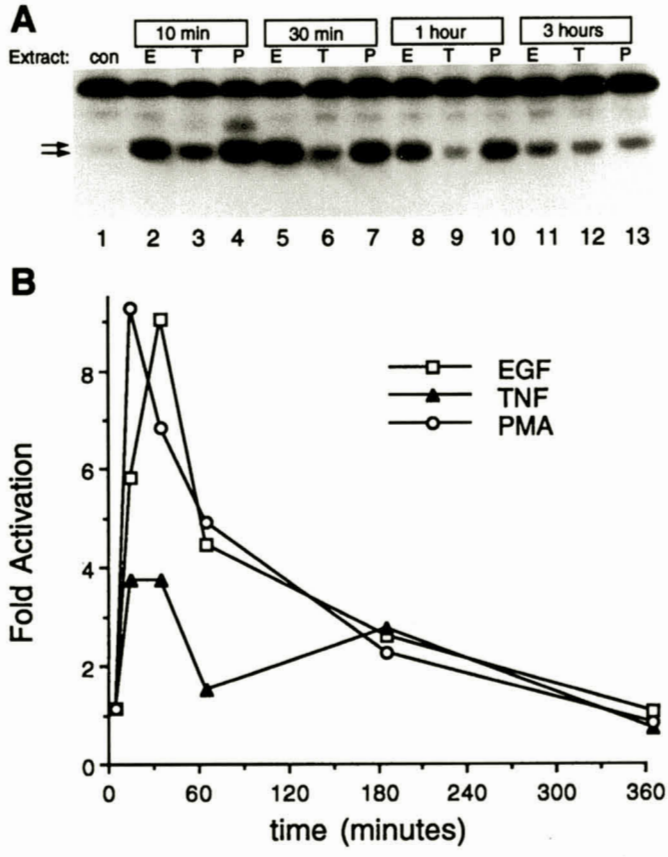

FIG. 6. **Activation of ERKs following cytokine treatment.** *A*, 50 μg of the indicated extracts were electrophoresed on gels containing 0.5 mg/ml myelin basic protein. An in-gel kinase assay with [γ-^{32}P]ATP was performed, and phosphorylated myelin basic protein was visualized by autoradiography. *Arrows* represent p42 and p44 ERKs. Positions of ERKs were confirmed with two different size standards on the gel (data not shown). Similar results were obtained in three independent experiments. *B*, quantitation of *A* (ERKs) expressed as -fold activation over unstimulated levels.

specific phospholipase C, which in turn produces diacylglycerol (2). Diacylglycerol is a classical second messenger that activates protein kinase C, and, as expected, protein kinase C is activated by TNFα (9). In addition, TNFα activates sphingomyelinase, which releases the second messenger ceramide (2), and some of the TNFα biological activities have been attributed to elevated ceramide levels (2, 44, 45). Furthermore, TNFα activates phospholipase A_2, which produces the second messenger arachidonic acid with the subsequent synthesis of leukotrienes and prostaglandins (2, 10). Finally, TNFα is reported to inhibit protein phosphatase activity (46), as many of the effects of TNFα are mimicked by the phosphatase inhibitor, okadaic acid (47).

TNFα induces the transcription factor NFκB (13, 14, 44), which requires the TNF R-55 as demonstrated by the inhibition of this pathway in the TNF R-55 knockout mouse (4). TNFα activates NFκB by causing the rapid degradation of IκB, and the released cytoplasmic NFκB then translocates to the nucleus to activate gene transcription (11).

TNFα also stimulates the activity of transcription factor AP-1, which results in increased expression of the c-*jun* and collagenase genes (9) and decreased expression of the elastin gene (48). The present study demonstrates that TNFα causes a prolonged activation of JNK, a small transient activation of ERK, and no increased activation of Raf-1 kinase. This results in the phosphorylation of the c-Jun activation domain with enhanced c-Jun transcriptional activity and little change in c-Jun DNA binding activity.

This study demonstrates that the kinetics for the activation of JNK and ERKs by TNFα, EGF, and PMA are different. Recent studies indicate that JNK1, the 46-kDa protein that contributes the predominant c-Jun amino-terminal kinase activity, is biochemically and immunologically distinct from ERK1 and ERK2 (26). Furthermore, JNK1, although a member of the mitogen-activated protein kinase group, is distantly related to ERKs 1 and 2 (27). Finally, overexpression and activation of ERKs 1 and 2 fail to enhance the transcriptional activation of c-Jun by Ras or Raf (32). Altogether, these results imply that JNK and ERKs are distinct proteins and are regulated by different, possibly parallel, signal transduction pathways. Therefore, the activities of JNK and ERKs can be independently regulated.

The signal transduction pathway by which TNFα activates JNK is unknown. Since oncogenic Ras proteins activate JNK and enhance c-Jun transactivation activity (22, 32), the role of Ras and Raf in TNFα signal transduction needs to be assessed. A dominant negative mutant Ras (H-Ras N17) has been used to demonstrate the requirement for endogenous activation in signal pathways, including insulin and platelet-derived growth factor activation of ERK2 (49) and UV induction of NFκB (50). However, H-Ras N17 alone failed to inhibit EGF stimulation of ERK2 in murine fibroblast cell lines, which required simultaneous inhibition of additional pathways (51). Since H-Ras N17 fails to inhibit TNFα stimulation of c-Jun transcriptional activity, this implies a Ras-independent pathway for TNFα signaling.

Activated Ras stimulates ERKs in a variety of cell types (49, 52–54). Ras directly interacts with the amino-terminal regula-

tory domain of Raf-1, which in turn leads to phosphorylation and activation of MEK-1 and MEK-2 in a variety of cells, including fibroblasts (55–58). Recent work demonstrates that Raf activity is a necessary component for activation of ERK by oncogenes, serum, and PMA (59). Our study confirms the activation of Raf and ERKs by PMA and EGF. However, TNFα does not stimulate Raf-1 kinase activity and only transiently stimulates ERK activity, presumably through a Raf-independent pathway such as MEKK (60). Thus, this study provides evidence that TNFα activates JNK through a Ras-, Raf-, and ERK-independent pathway.

Addendum—While this paper was under review, Kyriakis *et al.* (61) reported a family of c-Jun kinases that includes JNK1 and is activated by TNFα.

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