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Tumor necrosis factor α rapidly activates the mitogen-activated protein kinase (MAPK) cascade in a MAPK kinase kinase-dependent, c-Raf-1-independent fashion in mouse macrophages

BRENT W. WINSTON*, CAROL A. LANGE-CARTER*, ANNE M. GARDNER*, GARY L. JOHNSON*†, AND DAVID W. H. RICHES*†‡¶

*Division of Basic Sciences, Department of Pediatrics, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO 80206; †Department of Pharmacology and ‡Department of Biochemistry, Biophysics, and Genetics and Division of Pulmonary Sciences, Department of Medicine, University of Colorado Health Sciences Center, Denver, CO 80262

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ABSTRACT **Tumor necrosis factor α (TNFα) is bound by two cell surface receptors, CD120a (p55) and CD120b (p75), that belong to the TNF/nerve growth factor receptor family and whose signaling is initiated by receptor multimerization in the plane of the plasma membrane. The initial signaling events activated by receptor crosslinking are unknown, although activation of the mitogen-activated protein kinase (MAPK) cascade occurs shortly after ligand binding to CD120a. In this study, we investigated the upstream kinases that mediate the activation of the 42-kDa MAPK $p42^{mapk/erk2}$ following crosslinking of CD120a in mouse macrophages. Exposure of mouse macrophages to TNFα stimulated a time-dependent increase in the activity of MAPK/ERK kinase (MEK) that temporally preceded peak activation of $p42^{mapk/erk2}$. MEKs, dual-specificity threonine/tyrosine kinases, act as a convergence point for several signaling pathways including Ras/Raf, MEK kinase (MEKK), and Mos. Incubation of macrophages with TNFα was found to transiently stimulate a MEKK that peaked in activity within 30 sec of exposure and progressively declined toward basal levels by 5 min. By contrast, under these conditions, activation of either c-Raf-1 or Raf-B was not detected. These data suggest that the activation of the MAPK cascade in response to TNFα is mediated by the sequential activation of a MEKK and a MEK in a c-Raf-1- and Raf-B-independent fashion.**

Tumor necrosis factor α (TNFα) plays a vital role in host responses to bacteria and obligate intracellular parasites, in addition to initiating many aspects of the inflammatory response (1). TNFα is produced predominantly by macrophages, which also respond rapidly to TNFα by expressing a variety of gene products that contribute to the role of these cells in host defense (1–4). We and others have shown that crosslinking of the TNF receptor CD120a (p55) by TNFα itself or by receptor-specific agonistic polyclonal antibodies stimulates an increase in tyrosine phosphorylation of the mitogen-activated protein kinase (MAPK) $p42^{mapk/erk2}$ and a concomitant activation of its catalytic activity (refs. 5 and 6; unpublished data). MAPKs, in turn, have been shown to phosphorylate a number of important protein substrates including $p90^{rsk}$ (8), c-Myc (9), c-Jun (10), and cytosolic phospholipase A_2 (11). Thus, delineating the mechanism of activation of $p42^{mapk/erk2}$ by TNFα is of central importance to understanding the regulation of macrophage functions by this cytokine.

MAPKs are activated by phosphorylation on residues Thr^{183} and Tyr^{185} by a dual-specificity MAPK/ERK kinase (MEK) (12) which lies immediately upstream of MAPK in the signaling cascade. At least three different MEKs have been described (13, 14) and appear to act as the convergence point of at least three distinct signaling pathways that lead to the activation of the MAPK cascade (15). Most extensively studied is the activation of MEK by c-Raf-1 (16, 17), which couples through c-Ras both to receptors with intrinsic tyrosine kinase activity, such as the epidermal growth factor receptor (EGFR) on fibroblasts (18, 19), and to receptors lacking tyrosine kinase activity, such as surface IgM on B cells (20). A second route of activation of MEK has been described in *Xenopus* oocytes and is mediated by the germ-line-specific kinase Mos (21). Recent work has identified two additional MEK kinases (MEKKs) which are distinct from both c-Raf-1 and Mos. A 73-kDa MEKK, described as a mammalian homologue of the yeast pheromone-responsive kinases Ste11 and Byr2, has been suggested, based on complementation analyses in yeast, to mediate signaling between non-tyrosine kinase and tyrosine kinase receptors and MEK (15). In addition MEKKs of ≈56 kDa have been suggested to mediate the activation of MEK in response to stimulation of adipocytes with insulin (22). The goal of the present study was to investigate the upstream kinases that mediate the activation of $p42^{mapk/erk2}$ in mouse macrophages following stimulation with TNFα. Stimulation of macrophages with TNFα activated MEK independent of c-Raf-1. This activation of MEK was associated with the rapid and transient activation of a MEKK activity that was immunoprecipitated by antibodies directed against p73 MEKK and that catalyzed the *in vitro* phosphorylation of kinase-inactive MEK1.

MATERIALS AND METHODS

Materials. C3H/HeJ mice were bred at the National Jewish Center Biological Resource Center. Monoclonal anti-MEK1 antibody was purchased from Transduction Laboratories (Lexington, KY). Anti-c-Raf-1 and anti-Raf-B antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Affinity-purified rabbit polyclonal anti-mouse MEKK (p73) antibody was prepared by immunizing rabbits with a recombinant portion of the N-terminal domain of MEKK (23). Histidine-tagged recombinant kinase-inactive MEK1 ($rMEK_{kd}$), wild-type kinase-active MEK1 ($rMEK_{wt}$), and ki-

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Abbreviations: TNFα, tumor necrosis factor α; MAPK, mitogen-activated protein kinase; $p42^{mapk/erk2}$, 42-kDa mitogen-activated protein kinase; $rMAPK_{wt}$, recombinant wild-type kinase-active MAPK; MEK, MAPK/ERK kinase; $rMEK_{kd}$, recombinant kinase-inactive MEK; $rMEK_{wt}$, recombinant wild-type kinase-active MEK; MEKK, MEK kinase (MAPK kinase kinase); EGFR, epidermal growth factor receptor; PAF, platelet-activating factor; CSF-1, colony-stimulating factor 1.

¶To whom reprint requests should be addressed.

nase-active $p42^{mapk/erk2}$ ($rMAPK_{wt}$) were expressed in *Escherichia coli* and purified as described (18). Recombinant mouse TNFα was generously provided by Genentech. Colony-stimulating factor 1 (CSF-1) was purchased from Upstate Biotechnology (Lake Placid, NY). Platelet-activating factor (PAF) was obtained from Avanti Polar Lipids. [γ-^{32}P]ATP (>3000 Ci/mmol; 1 Ci = 37 GBq) was purchased from New England Nuclear. IP-20 (TTYADFIASGRTGRRNAIHD, a peptide inhibitor of protein kinase A) and EGFR-(662–681) (RRELVEPLTPSGEAPNQALLR) peptides (24, 25) were synthesized by Macromolecular Resources (Fort Collins, CO).

Macrophage Isolation and Culture. Bone marrow-derived macrophages were cultured from femoral and tibial bone marrow (26). The growth medium was Dulbecco's modified Eagle's medium containing penicillin (100 units/ml), streptomycin (100 μg/ml), 10% (vol/vol) heat-inactivated fetal bovine serum, and 10% (vol/vol) L929 cell conditioned medium (as a source of CSF-1). Bone marrow cells were cultured at a density of 2.4×10^5 cells/cm^2 at 37°C in a 10% (vol/vol) CO_2 atmosphere for 5–6 days. Macrophages were stimulated in growth medium as described in *Results*.

Resolution of MEK by Ion-Exchange FPLC. Macrophage monolayers were scraped into ice-cold lysis buffer [20 mM Mes (pH 6.0) containing 70 mM β-glycerophosphate, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM Na_3VO_4, 2 mM $MgCl_2$, 0.5% (vol/vol) Triton X-100, leupeptin (2 μg/ml), and aprotinin (2 μg/ml)]. Nuclear material was pelleted by centrifugation at 14,000 × *g* for 15 min at 4°C. The supernatant was loaded onto a Mono S HR 5/5 FPLC column (Pharmacia) equilibrated in 20 mM Mes (pH 6.0) containing 70 mM β-glycerophosphate, 1 mM EGTA, 1 mM dithiothreitol, and 0.1 mM Na_3VO_4 and was eluted at a flow rate of 60 ml/hr with a linear gradient of 0–250 mM NaCl in equilibration buffer. One-milliliter fractions were collected and aliquots were tested for MEK catalytic activity as described below. In addition, trichloroacetic acid-precipitated fractions were analyzed for MEK protein by SDS/PAGE followed by nitrocellulose immunoblotting with anti-MEK1 antibody.

Determination of MEK Catalytic Activity. MEK catalytic activity was quantified in Mono S column fractions by a coupled assay (15, 18) based on the ability of MEK to activate $rMAPK_{wt}$, thereby stimulating its ability to phosphorylate a synthetic peptide substrate, EGFR-(662–681) (24, 25). Thirty microliters from each Mono-S column fraction was mixed with 10 μl of reaction mixture to yield final concentrations of 40 mM Hepes (pH 7.2), 25 mM β-glycerophosphate, 50 μM Na_3VO_4, 10 mM $MgCl_2$, 100 μM ATP, 25 μg of IP-20 [a synthetic peptide inhibitor of protein kinase A (24)] per ml, 0.5 mM EGTA, 200 μM EGFR-(662–681), 30 μg of $rMAPK_{wt}$ per ml, and 10 μCi of [γ-^{32}P]ATP (>3000 Ci/mmol). Assays were also run in the absence of $rMAPK_{wt}$ to identify endogenous MAPK activity. After incubation for 20 min at 30°C, reactions were stopped by the addition of 10 μl of 25% (wt/vol) trichloroacetic acid. Forty-five microliters of each sample was spotted onto Whatmann P81 filter paper disks, washed three times in 75 mM phosphoric acid and once in acetone, and dried before β scintillation counting.

***In Vitro* Assay of Immunoprecipitated Kinases.** Macrophage monolayers were rinsed in ice-cold 20 mM Hepes-buffered saline (pH 7.4) and lysed on ice in a modified RIPA lysis buffer, 50 mM Tris·HCl, pH 7.2/150 mM NaCl/0.1% (wt/vol) SDS/0.5% (wt/vol) sodium deoxycholate/1% (vol/vol) Triton X-100/10 mM $Na_4P_2O_7$/25 mM β-glycerophosphate/2 mM Na_3VO_4 with aprotinin at 2.1 μg/ml, for c-Raf-1 immunoprecipitations. The lysis buffer used in MEKK immunoprecipitations was 10 mM Tris·HCl, pH 7.4/1% Triton X-100/5 mM EDTA/50 mM NaCl/50 mM NaF/0.1% bovine serum albumin/1 mM phenylmethanesulfonyl fluoride/2 mM Na_3VO_4 with aprotinin at 20 μg/ml. The lysates were centrifuged at 14,000 × *g* for 10 min at 4°C and the supernatants were precleared with 15 μl of protein A-Sepharose. Antibody (3 μl of anti-MEKK, 9 μl of anti-c-Raf-1, or 9 μl of anti-Raf-B) was then added to the precleared lysates along with 12 μl of protein A-Sepharose and the mixtures were rotated for 2.5 hr at 4°C. The immunoprecipitates were then washed twice with modified RIPA lysis buffer and twice with PAN buffer (10 mM Pipes, pH 7.0/100 mM NaCl with aprotinin at 21 μg/ml). After the last wash the pellets were resuspended in 40 μl of PAN to bring the mixture up to 60 μl and then used in an *in vitro* kinase assay with kinase buffer (20 mM Pipes, pH 7.2/10 mM $MnCl_2$ with aprotinin at 20 μg/ml), 20 μCi of [γ-^{32}P]ATP, and ≈200 ng $rMEK_{kd}$ as substrate in a final volume of 80 μl. Raf kinase reaction mixtures were incubated at 30°C for 15 min and MEKK reaction mixtures were incubated for 40 min. Phosphorylated MEK1 was localized on SDS/polyacrylamide gels by electrophoresing a sample of $rMEK_{wt}$ which had been allowed to undergo autophosphorylation in the same reaction mixture. The kinase reactions were terminated by the addition of 20 μl of 5× Laemmli sample buffer containing 100 mM dithiothreitol, and the mixtures were boiled for 5 min, separated by SDS/10% PAGE under reducing conditions, and transferred to nitrocellulose for autoradiography and Western analysis. ^{32}P-labeled phosphoproteins were detected by autoradiography using Kodak X-Omat AR5 film and quantified with a PhosphorImager (Molecular Dynamics).

Western Blotting. Samples were separated in SDS/10% polyacrylamide gels and transferred onto nitrocellulose membranes. The blots were washed in Tris-buffered saline (20 mM Tris, pH 7.6/137 mM NaCl) with 0.05% (vol/vol) Tween 20 (TBST), blocked overnight with 5% (wt/vol) bovine serum albumin or 3% (wt/vol) fat-free dry milk in TBST, and probed with antibodies according to Towbin *et al.* (27). Bound antibody was detected by enhanced chemiluminescence (Amersham).

RESULTS

Activation of MEK by TNFα. Previous work has shown $p42^{mapk/erk2}$ to be maximally activated 10–15 min after stimulation of macrophages with a saturating concentration of TNFα (5). Therefore, to investigate activation of MEK, macrophages were incubated in medium alone or with TNFα (40 ng/ml) for 10 min before lysis and determination of MEK catalytic activity by a coupled assay (see *Materials and Methods*). Two peaks of kinase activity were detected after Mono S ion-exchange chromatography of macrophage lysates (Fig. 1*A*). The first peak which was eluted immediately in fractions 1–4 represents endogenous MAPK activity and was detected in the absence of exogenous $MAPK_{wt}$. The 4- to 5-fold stimulation of endogenous MAPK activity in response to TNFα is consistent with previous data (ref. 5; unpublished data). The second peak of MAPK activity, fractions 16–20 at ≈125–175 mM NaCl (Fig. 1*A*), contained catalytically active MEK as shown by the detection of MAPK activity in the presence but not in the absence of $rMAPK_{wt}$ and by the localization of MEK1 protein in these fractions by immunoblotting with anti-MEK1 antibody (Fig. 1*B*). MEK activity was not detected in lysates of unstimulated macrophages, although MEK1 was detected by immunoblotting of these fractions. The time course of MEK activation by TNFα was examined by stimulating mouse macrophages with a fixed concentration of TNFα (40 ng/ml) for up to 15 min. MEK activation of $rMAPK_{wt}$ was detected in fractions 16–20 of TNFα-stimulated (40 ng/ml) murine macrophages at 5 min and peaked at 10 min before declining toward baseline activity at 15 min (Fig. 1 *A* and *C*). Thus, stimulation of macrophages with TNFα transiently activated MEK with a time course that preceded the previously reported peak activation of $p42^{mapk/erk2}$ at 10–15 min (ref. 5; unpublished data).

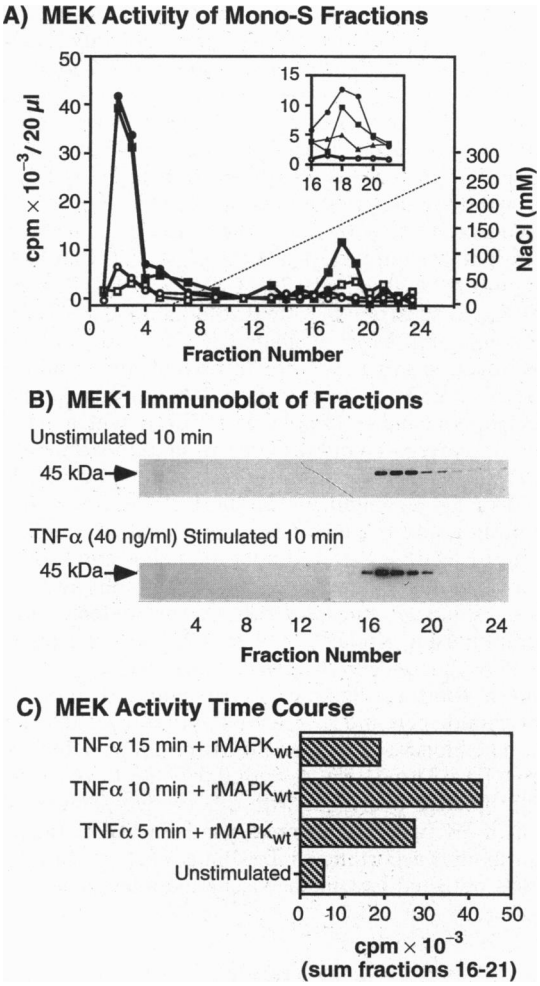

FIG. 1. MEK activity in TNFα-stimulated murine macrophages. (*A*) MEK activity in Mono S FPLC fractions of unstimulated (open symbols) or TNFα-stimulated (40 ng/ml) (solid symbols) macrophage lysates at 10 min. The first peak (fractions 1–4) represents endogenous MAPK activity which is detected in the absence of exogenous $rMAPK_{wt}$. The second peak (fractions 16–20 at ≈125–175 mM NaCl) represents MEK activity. ○, Unstimulated, without $rMAPK_{wt}$; ●, TNFα, without $rMAPK_{wt}$; □, unstimulated, with $rMAPK_{wt}$; ■, TNFα, with $rMAPK_{wt}$. (*Inset*) MEK activity time course of unstimulated macrophages (10 min without $rMAPK_{wt}$) (○) and of macrophages stimulated with TNFα for 5 min (■), 10 min (●), or 15 min (▲) before assay with $rMAPK_{wt}$. (*B*) Immunoblot of Mono S fractions with monoclonal anti-MEK1 antibody. (*C*) MEK activity time course obtained by integrating the area under the MEK activity curve in fractions 16–21 of *Inset* in *A*.

Activation of MEK by TNFα Occurs Independently of c-Raf-1 or Raf-B. We next investigated whether the activation of MEK by TNFα was mediated by the kinase c-Raf-1. Specific kinase activity of c-Raf-1 was measured toward a catalytically inactive form of MEK1 ($rMEK_{kd}$) in which Lys^{97} was mutated to Met by site-directed mutagenesis, thereby interrupting the ATP binding site (18). Macrophage monolayers were incubated in growth medium alone or were stimulated with TNFα (40 ng/ml) for up to 10 min, the cells were lysed, and c-Raf-1 was immunoprecipitated for *in vitro* kinase assay using $rMEK_{kd}$ as substrate in the presence of [γ-^{32}P]ATP (15, 18). Autophosphorylation of $rMEK_{wt}$ was used to localize the phosphorylated $rMEK_{kd}$ on the autoradiograph of the SDS/polyacrylamide gel. Basal activity of c-Raf-1 was detected in unstimulated macrophages and showed a modest and transient increase at 2–5 min (Fig. 2*A*). However, we did not detect any difference in the activity of c-Raf-1 in TNFα-stimulated macrophages compared with time-matched unstimulated controls

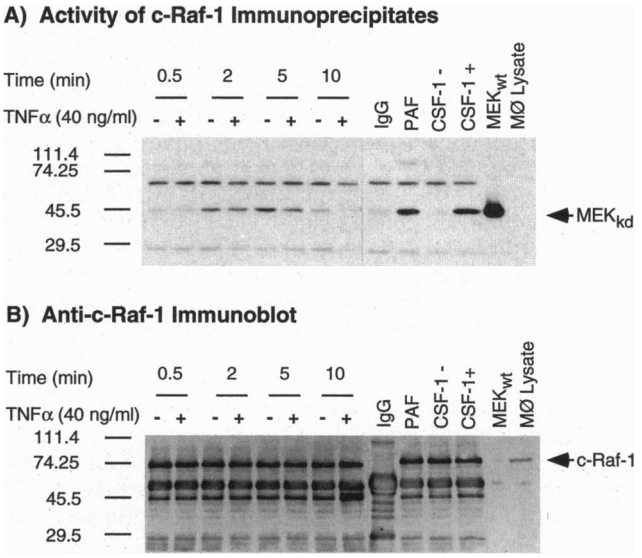

FIG. 2. c-Raf-1 activity in TNFα-stimulated murine macrophages. c-Raf-1 was immunoprecipitated from lysates of unstimulated and TNFα-stimulated (40 ng/ml) macrophages (Mϕ) at 0.5, 2, 5, and 10 min. (*A*) Immunoprecipitates were subjected to *in vitro* kinase reaction using $rMEK_{kd}$ as a substrate and the proteins were separated by SDS/PAGE. Immunoprecipitation with nonimmune rabbit IgG provided a negative control. Positive controls were PAF (1 μM) and CSF-1 (1000 units/ml) stimulation of CSF-1-deprived cells at 2 min. *In vitro* autophosphorylation of purified $rMEK_{wt}$ was used to localize $rMEK_{kd}$. (*B*) Anti-c-Raf-1 immunoblot of the immunoprecipitates in *A*.

at any time point studied. Immunoblotting with anti-c-Raf-1 antibody revealed equivalent amounts of c-Raf-1 protein with the predicted molecular mass of 72 kDa in all samples, indicating that the enzyme was efficiently immunoprecipitated with this antibody but not by nonimmune rabbit IgG (Fig. 2*B*). In addition, c-Raf-1 was readily detected in macrophage lysates (Fig. 2*B*). In contrast to the lack of effect of TNFα on c-Raf-1, exposure of macrophages to PAF (1 μM) or to CSF-1 (1000 units/ml added to cells starved for CSF-1 for 18 hr) stimulated an increase in c-Raf-1 activity in macrophage lysates, as previously reported (28). The presence of Raf-B, a high molecular weight form of c-Raf-1, has been detected in a number of cell lines (29, 30), and activation of this kinase has been reported to be initiated in PC12 cells stimulated with platelet-derived growth factor, epidermal growth factor, and nerve growth factor (30). However, we did not detect Raf-B protein in lysates of mouse macrophages by immunoblotting, nor did we detect activation of this kinase in immunoprecipitates from lysates of unstimulated or TNFα-stimulated macrophages (data not shown), although Raf-B protein and activation have been readily detected in PC12 cells with the same method and reagents (23). Thus, the activation of MEK by TNFα occurs in a c-Raf-1- and Raf-B-independent fashion.

Activation of MEKK by TNFα. We next determined whether MEKK was activated in macrophages following stimulation with TNFα. Macrophage monolayers were stimulated with TNFα (40 ng/ml) or incubated in growth medium alone for up to 5 min, lysed, and subjected to immunoprecipitation with an affinity-purified rabbit polyclonal antibody raised against a portion of the N-terminal domain of p73 MEKK (15). The immunoprecipitates were washed and subjected to an *in vitro* kinase reaction using $rMEK_{kd}$ as substrate in the presence of [γ-^{32}P]ATP for 40 min. Autophosphorylation of $rMEK_{wt}$ was used to localize phosphorylated $rMEK_{kd}$. TNFα stimulated maximal activation of MEKK at 30 sec as detected by an increase in phosphorylation of $rMEK_{kd}$ compared with unstimulated cells (Fig. 3*A*). The time course of MEKK activa-

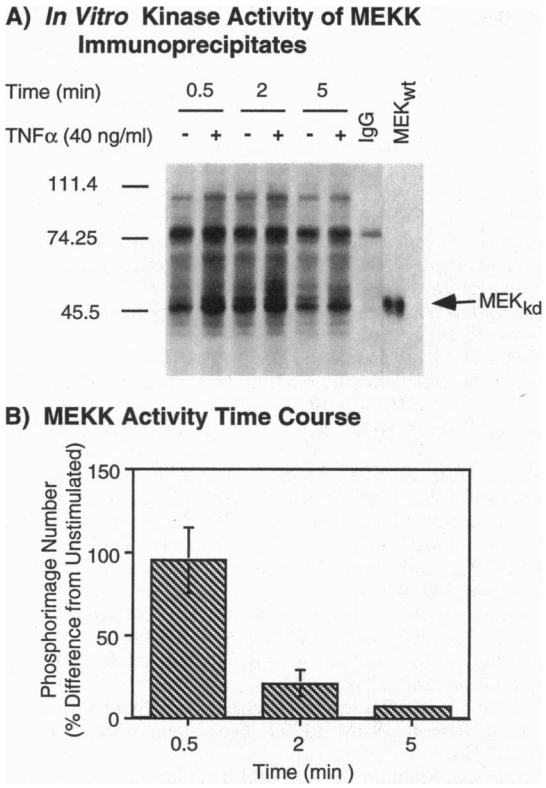

FIG. 3. Time course of activation of MEKK in response to TNFα. Affinity-purified rabbit polyclonal anti-MEKK antibody was used to immunoprecipitate MEKK from unstimulated and TNFα-stimulated (40 ng/ml) murine macrophages at 0.5, 2, and 5 min. (*A*) Immunoprecipitates were subjected to *in vitro* kinase reaction using rMEK$_{kd}$ as substrate. *In vitro* autophosphorylation of purified rMEK$_{wt}$ was used to localize rMEK$_{kd}$. (*B*) Time course of MEKK activation by TNFα (40 ng/ml). MEKK activity is described as percent difference from unstimulated based on phosphorimage number (mean ± SEM of four experiments).

tion by TNFα was measured by PhosphorImager scanning analysis of the phosphorylated rMEK$_{kd}$ band at each time point (Fig. 3*B*). Peak MEKK activity was detected at 30 sec and declined to 8% by 5 min. We were unable to obtain data at times earlier than 30 sec because of inaccuracies in stimulating and lysing the cell monolayers in these brief time intervals. Immunoblots of the immunoprecipitated MEKK with the same anti-MEKK polyclonal antibody that was used for immunoprecipitation revealed several bands of 70–100 kDa (Fig. 4*A*) that were not detected in control immunoprecipitations using nonimmune rabbit IgG. Immunoblotting with anti-c-Raf-1 antibody also failed to reveal any immunoreactive protein in anti-MEKK immunoprecipitates, although c-Raf-1 was readily detected in macrophage lysates and in a c-Raf-1 immunoprecipitate used as a positive control (Fig. 4*B*). Thus, these data suggest that a family of immunologically related MEKKs exists in macrophages and that one or more of these enzymes were transiently activated in response to TNFα.

DISCUSSION

Despite the molecular cloning of the TNFα receptors CD120a (p55) and CD120b (p75) almost 5 years ago, the mechanism of signal transduction by these receptors has only recently begun to be clarified. The use of specific antibodies directed against the extracellular domains of the two receptors and the observed species differences in ligand binding have led to the conclusion that the majority of cellular responses elicited by TNFα are initiated by signaling through CD120a (p55) (31,

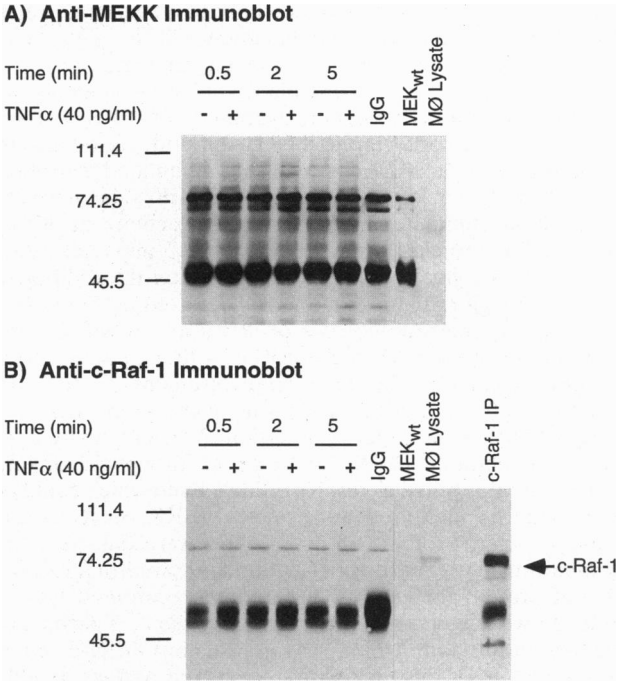

FIG. 4. Anti-MEKK and anti-c-Raf-1 immunoblots of anti-MEKK immunoprecipitates of the macrophage lysates seen in Fig. 3. (*A*) Rabbit polyclonal anti-MEKK antibody (the same antibody that was used for immunoprecipitation) was used to immunoblot for MEKK. Nonimmune rabbit IgG and macrophage (Mϕ) lysate were used as controls. (*B*) Immunoblotting the same samples with anti-c-Raf-1 antibody failed to reveal immunoreactive c-Raf-1 in the MEKK immunoprecipitated lysates. The macrophage (Mϕ) whole-cell lysate and anti-c-Raf-1 immunoprecipitate (IP) lanes (positive controls) identify immunoreactive c-Raf-1.

32). Studies in fibroblasts (5), HL-60 cells (6), and mouse bone marrow-derived macrophages have indicated that activation of p44$^{mapk/erk1}$ and/or p42$^{mapk/erk2}$ is one of the early signaling events initiated by TNFα, and signaling has been shown to be stimulated by crosslinking of CD120a (p55) (unpublished data). The present results have extended these findings (*i*) by showing that activation of MEK, an upstream kinase previously shown to be capable of phosphorylating and activating p42$^{mapk/erk2}$, precedes peak activation of p42$^{mapk/erk2}$ and (*ii*) by demonstrating the rapid and transient TNFα-dependent activation of a MEKK activity capable of phosphorylating rMEK$_{kd}$. In addition, activation of MEK was initiated in a c-Raf-1- and Raf-B-independent fashion. These findings are consistent with the idea that TNFα initiates a kinase cascade in which MEKK activation stimulates activation of MEK, thereby leading to the tyrosine phosphorylation and activation of p42$^{mapk/erk2}$.

MEKs are a convergence point of signaling by multiple growth factor receptor (33) or oncogene (15) activators of MAPKs. Activation of MEKs is mediated by at least three distinct groups of kinases: c-Raf-1 (34–36) and Raf-B (30), Mos (21), and MEKKs (15, 22). Although TNFα is a growth factor for fibroblasts and could conceivably signal MEK activation in a fashion analogous to other growth factors, cytokine receptor signaling (such as that by the interferon α/β and γ receptors) has been shown to be quite different from that of the growth factors (37). In the present study, we did not detect activation of c-Raf-1 in response to stimulation of mouse macrophages with TNFα. By contrast, under the same conditions, CSF-1 and PAF activation of c-Raf-1 was readily detected. We also analyzed macrophage lysates for the presence and possible activation of Raf-B in response to TNFα. However, neither Raf-B kinase nor activation of Raf-B was de-

tected either in unfractionated macrophage lysates or in Raf-B immunoprecipitates, indicating the absence of this higher molecular weight Raf isoform in mouse macrophages.

In contrast to the Raf independence of TNFα signaling, we detected a rapid and transient activation of a MEKK that was specifically immunoprecipitated by an antibody raised against the murine 73-kDa MEKK and which stimulated the phosphorylation of a kinase-inactive MEK1 ($rMEK_{kd}$) in an *in vitro* assay. TNFα stimulated maximal MEKK activity at 30 sec which declined to close to basal activity by 5 min. Activation of MEK was maximally stimulated 10 min after the addition of TNFα, although initial activation was detected at 5 min. It is not clear why the time courses of activation of MEKK and MEK do not overlap more extensively, although possible explanations include (*i*) the potential involvement of additional signaling proteins or (*ii*) a progressive increase in MEK catalytic activity by autophosphorylation. Western analysis of immunoprecipitated MEKK with an affinity-purified anti-MEKK antibody failed to resolve a single band which could be unambiguously identified as a single MEKK, since several immunoreactive proteins of 70–100 kDa were detected. Immunoblotting with anti-c-Raf-1 antibody showed that c-Raf-1 was not present in the MEKK immunoprecipitated lysates. Although we have no definitive data at present, it is conceivable that the antibody, which was raised against the N-terminal portion of MEKK, may recognize conserved regions of additional MEKKs. cDNA cloning has identified three additional MEKKs of similar molecular weights (G.L.J., unpublished observations) and work in adipocytes has identified a MEKK activity (I-MEKK) that is rapidly and transiently activated in response to insulin and which by gel filtration has an apparent molecular mass of 56 kDa (22).

The rapid activation and inactivation/decay of MEKK by TNFα are analogous to the rise and fall of I-MEKK activity following insulin stimulation in adipocytes (22) and suggest that this transient MEKK activation may be close to the initiating events in signal transduction by the TNFα receptor CD120a (p55). Tartaglia and colleagues (7, 31) have shown that signaling is impaired in a series of deletional and point mutants of the intracellular domain of CD120a (p55). Their findings strongly suggest that a signaling protein(s) binds to multiple contact residues presented within the folded structure of the C-terminal half of the intracellular domain, and recent work has identified two phosphoproteins, pp95 and pp120, that are constitutively associated with the receptor (D.W.H.R., unpublished observations). However, based on the molecular weights of these phosphoproteins, it seems unlikely that they represent currently known MEKKs. Thus, it seems likely that at least one additional protein—e.g., an adapter protein—is involved in the activation of MEKK following crosslinking of CD120a (p55).

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