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Ras-Dependent and -Independent Pathways Target the Mitogen-Activated Protein Kinase Network in Macrophages

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Mitogen-activated protein kinases (MAPKs) are activated upon a variety of extracellular stimuli in different cells. In macrophages, colony-stimulating factor 1 (CSF-1) stimulates proliferation, while bacterial lipopolysaccharide (LPS) inhibits cell growth and causes differentiation and activation. Both CSF-1 and LPS rapidly activate the MAPK network and induce the phosphorylation of two distinct ternary complex factors (TCFs), TCF/Elk and TCF/SAP. CSF-1, but not LPS, stimulated the formation of $p21^{ras} \cdot GTP$ complexes. Expression of a dominant negative *ras* mutant reduced, but did not abolish, CSF-1-mediated stimulation of MEK and MAPK. In contrast, activation of the MEK kinase Raf-1 was Ras independent. Treatment with the phosphatidylcholine-specific phospholipase C inhibitor D609 suppressed LPS-mediated, but not CSF-1-mediated, activation of Raf-1, MEK, and MAPK. Similarly, down-regulation or inhibition of protein kinase C blocked MEK and MAPK induction by LPS but not that by CSF-1. Phorbol 12-myristate 13-acetate pretreatment led to the sustained activation of the Raf-1 kinase but not that of MEK and MAPK. Thus, activated Raf-1 alone does not support MEK/MAPK activation in macrophages. Phosphorylation of TCF/Elk but not that of TCF/SAP was blocked by all treatments that interfered with MAPK activation, implying that TCF/SAP was targeted by a MAPK-independent pathway. Therefore, CSF-1 and LPS target the MAPK network by two alternative pathways, both of which induce Raf-1 activation. The mitogenic pathway depends on Ras activity, while the differentiation signal relies on protein kinase C and phosphatidylcholine-specific phospholipase C activation.

Mitogen-activated protein kinases (MAPKs) are serine/threonine kinases rapidly activated in cells stimulated by several extracellular agents.

A variety of mitogens lead to the formation of membrane-associated signalling complexes that deliver an essential signal via Ras to a protein kinase network involving the Raf-1 kinase, the MAPK activator MEK, and MAPKs (reviewed in references 7, 53, and 69). The Raf-1 kinase interacts directly with the $p21^{ras}$ proto-oncogene product (33, 44, 54, 82, 84, 85, 94). Activated Raf-1 in turn phosphorylates and activates MEK in vivo and in vitro (21, 39, 41, 48, 51, 82, 87). It has therefore been proposed that these kinases are arranged in a linear cascade, in which Raf-1 phosphorylates and activates MEK, which in turn phosphorylates and activates MAPK. MAPK carries the signal to the nucleus, where it phosphorylates transcription factors capable of mediating immediate-early gene induction (2, 30, 35–37, 42, 52, 65, 74). The hitherto best-defined nuclear target of MAPK is ternary complex factor (TCF), as exemplified by Elk-1, which binds and activates the serum response element (SRE) of the c-*fos* promoter in the context of a ternary complex with a serum response factor (SRF) dimer (30, 35–37, 42, 43, 52).

The general scheme of the Ras/Raf-1/MAPK pathway is extremely well conserved in higher eukaryotes (reviewed in reference 23). Nevertheless, a second entry point in this linear pathway was demonstrated by the cloning of a second MEK kinase, called MEKK. This enzyme is homologous to the yeast kinases Ste11 and Byr2 (50) and is activated in a Ras-dependent manner in epidermal growth factor (EGF)-treated PC-12 cells (49).

Although the majority of the studies in this field have concerned themselves with the role of the MAPK pathway in mitogenic signalling, the kinases in this network are also involved in signal transduction pathways leading to cellular differentiation. c-*raf* homologs have been shown to participate in the determination of terminal cell fate (3, 4, 62) and compound eye development (60, 63) in *Drosophila melanogaster*. Raf is also implicated in vulval development in *Caenorhabditis elegans* (34). A MEK-like protein acts downstream of Raf in the determination of terminal cell fate in *Drosophila* spp. (80), and *Drosophila* MAPK is involved in the determination of neural and terminal cell fate, as well as being involved in the differentiation of the wing veins (11). In mammalian cells, Raf-1 and MAPKs have been implicated in the Ras-dependent neuronal differentiation induced by nerve growth factor in the adrenal pheochromocytoma cell line PC-12 (31, 78, 89, 90).

Macrophages provide an excellent model system for the study of signal transduction events leading to cellular proliferation and differentiation or activation. We and others have previously shown that Raf-1, MEK (6, 67), and MAPK (67, 86) are activated upon treatment of macrophages with the mitogen colony-stimulating factor 1 (CSF-1) or with the differentiation or activating stimulus lipopolysaccharide (LPS) (1, 56). Both signals result in the phosphorylation of two distinct TCFs, identified, respectively, as TCF/Elk and TCF/SAP (37, 67).

In this study, we compare the early signal transduction events triggered by CSF-1 and LPS with the aim of identifying

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TABLE 1. Effect of a dominant negative Ras mutant on the nucleotides bound to p21^{ras}[a]

Cell line	% of GTP bound[b]			
	Without CSF-1		With CSF-1	
	Without dexamethasone	With dexamethasone	Without dexamethasone	With dexamethasone
BAC-1.2F5	0.12	0.16	10	9.6
BAC Asn 17	0.15	0.17	9.9	2.6
BAC Ras	0.16	0.3	9.2	9.3

[a] Quiescent, ^{32}P-labelled BAC-1.2F5, BAC Asn 17, and BAC Ras cells were deprived of CSF-1 in the presence or absence of dexamethasone (5×10^{-7} M) for 18 h prior to stimulation with 0.63 nM mouse recombinant CSF-1 (5 min). Nucleotides bound to Ras were analyzed by Ras immunoprecipitation followed by thin-layer chromatography. Results were quantitated in a PhosphorImager.

[b] The percentage of GTP on p21^{ras} was calculated as GTP/[(1.5 × GDP) + GTP] to adjust for the additional phosphate in GTP.

common denominators and divergence points in the MAPK regulatory network. Unlike CSF-1, LPS did not induce the formation of p21^{ras} · GTP complexes. CSF-1-induced MEK and MAPK activation was partially inhibited by the dominant negative *ras* mutant Asn 17 (15, 25, 26) that blocks MAPK activation in a number of different systems (22, 40, 68, 78, 90). Raf-1 activation by CSF-1 was completely Ras independent. Thus, Raf-1 may represent the common, Ras-independent MEK activator in CSF-1 and LPS signal transduction. LPS- and CSF-1-mediated signals differed in their dependence on phosphatidylcholine (PC)-specific phospholipase C (PLC) and protein kinase C (PKC). Sustained activation of Raf-1 by chronic tetradecanoyl phorbol acetate (TPA) treatment aimed at PKC down-regulation showed that activated Raf-1 alone was not sufficient to ensure MEK/MAPK activation in BAC-1.2F5 cells. In all instances, phosphorylation of TCF/Elk correlated strictly with MAPK activation, thus reinforcing its role as a nuclear target of MAPK. In contrast, phosphorylation of TCF/SAP was barely affected by Ras, PKC, or PC-PLC inactivation, implying that a distinct, MAPK-independent mechanism targets this TCF in vivo during macrophage proliferation and activation.

MATERIALS AND METHODS

Cell culture and stimulation. BAC-1.2F5 cells (55) and the stable cell lines BAC Asn 17 and BAC Ras, harboring the Ras-N17 or the c-Ha-*ras* construct expressed via the glucocorticoid-inducible mouse mammary tumor virus promoter (15), were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 20% L cell conditioned medium as a source of CSF-1 (76). Confluent cultures (10^7 cells per 100-mm-diameter tissue culture dish) were cultured in medium without CSF-1 for 16 h to up-regulate CSF-1 receptors and then were incubated for different periods of time with either 1 μg of bacterial LPS per ml (from *Escherichia coli* O111:B4 [Sigma, Deisenhofen, Germany]) or 0.63 nM mouse recombinant CSF-1 (46). In selected experiments, down-modulation of PKC was performed by treating cultures with 5×10^{-6} M TPA (Sigma) in dimethyl sulfoxide (14 mM, final concentration) or with dimethyl sulfoxide alone for 24 h. PKC inhibition was performed by treating the cells with different concentrations of bisindolylmaleimide (Calbiochem) for 30 min at 37°C prior to stimulation. Inhibition of PC-PLC activity was performed by preincubating the cells with 50 μg of the specific inhibitor xanthogenate tricyclodecan-9-yl (D609 [14, 72]; Kamyia Biomedical, Thousand Oaks, Calif.) per ml for 1 h before stimulation. Incubations were terminated by aspirating the medium and washing five times with ice-cold phosphate-buffered saline (PBS).

Cell lysis and immunoprecipitation. Cell lysis and immunoprecipitation were performed exactly as previously described (5).

A rabbit polyclonal antiserum raised against a carboxy-terminal peptide of v-*raf* (SP63, CTLTTSPRLPVF [71]) was used to immunoprecipitate Raf-1 molecules. MEK-1 and MEK-2 were immunoprecipitated with a rabbit polyclonal antibody (Affiniti, Nottingham, United Kingdom). Immune complexes were collected after incubation (1 h at 4°C) with protein A-Sepharose beads (Sigma). Raf-1 and MEK immune complex kinase assays were carried out exactly as described in reference 50.

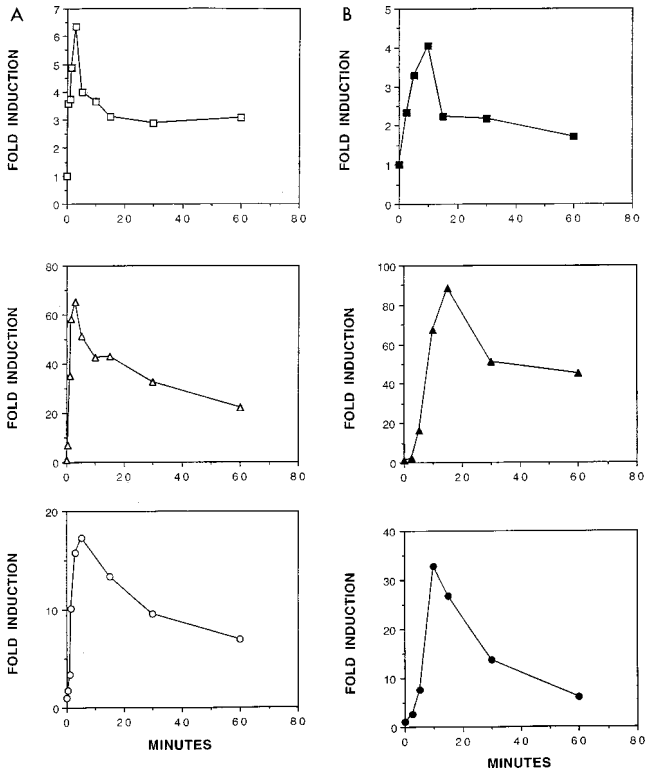

FIG. 1. Effect of CSF-1 (A) and LPS (B) on Raf-1, MEK, and MAPK activity. Quiescent BAC-1.2F5 cells were stimulated with either 0.63 nM mouse recombinant CSF-1 or with 1 μg of LPS per ml at 37°C for different times prior to solubilization. Raf-1 (□ and ■) and MEK-1 plus MEK-2 (△ and ▲) were immunoprecipitated from lysates normalized for protein content. The activity of the Raf-1 immunoprecipitates was assayed in an immune complex kinase assay by using bacterially expressed, catalytically compromised MEK-1 as a substrate. Catalytically compromised MEK-1 was expressed in bacteria with a histidine tag (His 6) and purified by affinity chromatography on nitrilotriacetic acid resin. MEK-1 plus MEK-2 immune complexes were tested for their ability to phosphorylate catalytically compromised GST–ERK-2. GST–ERK-2 was expressed in bacteria and purified by affinity chromatography on glutathione-Sepharose followed by the proteolytic cleavage of the ERK-2 moiety with thrombin. The activity of MAPKs (○ and ●) was measured in an in-gel kinase assay with myelin basic protein as a substrate. Autoradiograms were quantitated by densitometry. The kinase activity of immunoprecipitates (Raf-1, MEK) or lysates from uninduced cells has been arbitrarily defined as 1. The results are expressed as fold induction. The activation kinetics of ERK-1 and ERK-2, both visualized in the MAPK assay (Fig. 4C to 6C), were identical and are therefore shown as a single curve. A representative experiment, in which the activity of all kinases was assessed in the same lysates, is shown. Raf-1 immunoprecipitated from unstimulated BAC-1.2F5 cells consistently showed a low level of constitutive MEKK activity (Fig. 4A to 6A).

Autoradiograms were analyzed by densitometric scanning with a Bio-profil densitometer (Vilber Lourmat) by using version 5.07 of the Bio-1D software.

In-gel kinase assay. Samples containing 7.5 μg of protein were made to 62.5 mM Tris (pH 6.8)–2.3% sodium dodecyl sulfate (SDS)–5 mM EDTA–10% glycerol–100 mM dithiothreitol and heated at 85°C for 5 min before SDS-polyacrylamide gel electrophoresis (PAGE). The separating gels were polymerized with 0.2 mg of myelin basic protein (Sigma) per ml and after electrophoresis were denatured in 6 M guanidine-HCl, renatured, and assayed for kinase activity as described previously (16). Autoradiograms were quantitated by densitometry as described above.

Analysis of nucleotides bound to p21^{ras}. The fraction of p21^{ras} bound to GDP or GTP was determined as previously described (92). Confluent BAC-1.2F5 cells were incubated in phosphate-free Dulbecco's modified Eagle's medium supplemented with 10% dialyzed fetal calf serum in the absence of CSF-1 for 16 h and subsequently labelled by incubation for 4 h in phosphate-free medium containing 1/10 normal $NaHCO_3$ and 250 μCi of carrier-free *o*-[^{32}P]phosphate per ml (9,000 Ci/mmol; Amersham). Labelling media were supplemented with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer and 10% dialyzed fetal calf serum. After stimulation with CSF-1 or LPS, the cells were washed three times with ice-cold PBS and scraped in 1 ml of lysis buffer (20 mM Tris [pH

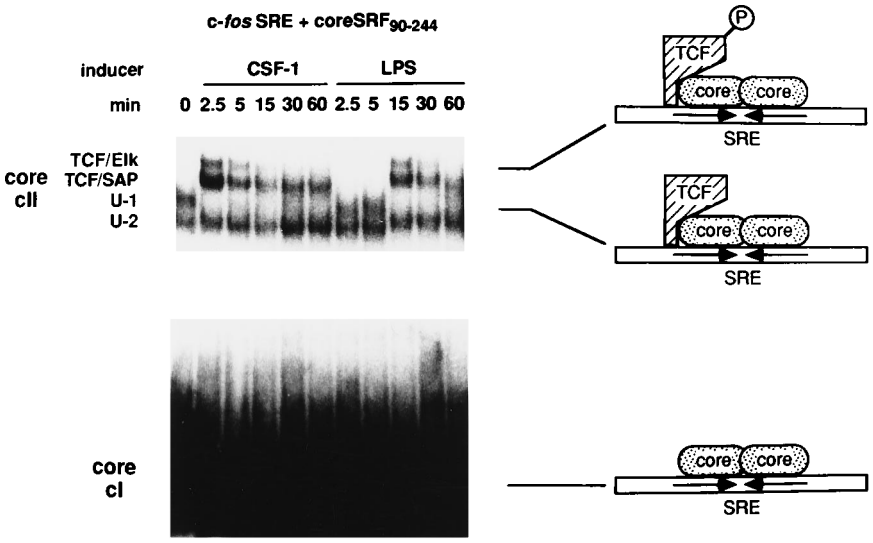

FIG. 2. Effect of CSF-1 and LPS on TCF binding. Whole-cell extracts of BAC-1.2F5 cells treated with CSF-1 or LPS as described in the legend to Fig. 1 were analyzed for their ability to form ternary complexes. The ^{32}P-labelled probe spans the c-*fos* SRE. The reaction mixtures were supplemented with coreSRF$_{90–244}$ (which comprises SRF residues 90 to 244) produced with recombinant vaccinia virus. The composition of the complexes is shown schematically on the side and described in the text. core cI, coreSRF dimer; core cII, ternary complexes; U-1 and U-2, uninduced ternary complexes; TCF/Elk and TCF/SAP, induced ternary complexes.

7.5], 150 mM NaCl, 20 mM $MgCl_2$, 1% Nonidet P-40, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1% aprotinin) supplemented with 2 μg of Ras antibody Y13-259 per ml. The extracts were mixed thoroughly and incubated at 4°C for 30 min. After centrifugation, the supernatants were mixed with 40 μl of protein A-Sepharose and 10 μg of rabbit anti-rat immunoglobulin G. After a 30-min incubation at 4°C, immunoprecipitates were collected by centrifugation and washed twice with lysis buffer and three times with PBS. The pellets were resuspended in 25 μl of 0.75 M KH_2PO_4 (pH 3.4)–5 mM EDTA–0.1 mM GDP–0.1 mM GTP and heated at 75°C for 5 min. Equal counts of the supernatants were spotted onto polyethyleneimine-cellulose thin-layer plates and developed with 1 M KH_2PO_4 (pH 4). Autoradiograms were analyzed by densitometric scanning as described above. In selected experiments (Table 1) a PhosphorImager (Molecular Dynamics) was used to quantitate the results.

Band shift assays. Reaction mixtures (7.5 μl each) contained the following components: 2.5 μg of poly(dI-dC) · (dI-dC), 250 ng of salmon sperm DNA, 5% glycerol, 66 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.35% Triton X-100, 0.05% low-fat milk, 15 mM dithiothreitol, 10 to 15,000 cpm of ^{32}P-labelled probe (0.2 ng/4 fmol), and 5 to 10 μg of protein, depending upon the extracts. After 30 min at room temperature, the entire reaction mixture was loaded on a 4% polyacrylamide (29:1 acrylamide/bis ratio) gel containing 0.5× Tris-borate-EDTA and run at 1 mA/cm for 3 to 4 h. Gels were dried and the complexes were visualized by autoradiography with intensifying screens. coreSRF$_{90–244}$ was produced in HeLa cells by using a recombinant vaccinia virus (38).

RESULTS

Activation of the Raf-1/MAPK pathway and phosphorylation of TCFs are early events in CSF-1 and LPS signal transduction. Quiescent BAC-1.2F5 cells were stimulated with either CSF-1 (Fig. 1A) or LPS (Fig. 1B) for different periods. Raf-1 kinase activity was measured by incubating Raf-1 immunoprecipitates with [γ-^{32}P]ATP and bacterially expressed, catalytically compromised MEK-1 as a substrate. MEK activity was similarly measured in immune complex kinase assays. The antiserum used in this assay recognizes MEK-1 and MEK-2 (91, 95, 96), both of which are expressed in BAC-1.2F5 cells (data not shown). Bacterially expressed, catalytically compromised ERK-2 was used as a MEK substrate. The activity of MAPK was visualized with an in-gel kinase assay. This technique allows the assessment of MAPK activity in whole-cell extracts. The extracts were resolved by SDS-PAGE in a gel containing myelin basic protein. After renaturation, the activities of ERK-1 and ERK-2 (13) were visualized by the transfer of radioactive phosphate to myelin basic protein. The two phosphorylated bands (see Fig. 4C to 6C) comigrate with proteins reacting with a MAPK-specific antiserum, as shown by Western blotting (immunoblotting); furthermore, activation measured in the MAPK assay strictly correlated with tyrosine phosphorylation of immunoprecipitated ERK-2 (data not shown). The activation kinetics of ERK-1 and ERK-2 were identical and are therefore shown as a single curve. Raf-1, MEK, and MAPKs were activated after CSF-1 and LPS treatment of BAC-1.2F5 cells (Fig. 1). The kinetics of CSF-1-mediated kinase activation and inactivation were markedly faster than those engendered by LPS. However, in both cases the kinetics were consistent with the proposed signalling order Raf-1→MEK→MAPK. Given the short stimulation times, it is important to mention here that the Raf-1 and MEK immune complexes and the extracts used in the MAPK assay were all derived from the same cell lysates.

To determine whether the signal from the MAPK network reached the nucleus, we analyzed the binding and therefore the phosphorylation of the transcription factor TCF at different times after CSF-1 or LPS stimulation. Band shift assays were used to measure the ability of whole-cell extracts to form ternary complexes with exogenously added coreSRF$_{90–244}$, which encompasses the domains necessary for DNA binding and interaction with TCF (57, 61), and a ^{32}P-labelled c-*fos* SRE probe. Changes in the mobility of such coreSRF-directed ternary complexes reflect the phosphorylation state of TCF, which in turn correlates tightly with the activity of the c-*fos* promoter (36, 97). In extracts prepared from unstimulated cells, the coreSRF ternary complexes appeared as two bands, labelled U-1 and U-2 (Fig. 2). U-1 and U-2 contain the uninduced forms of both TCF/Elk and TCF/SAP, as determined by their cross-reactivity with antisera specific for human Elk-1 and human SAP-1 (37). Stimulation led to the disappearance of the upper uninduced band (U-1) and caused a slight mobility change in the lower uninduced band (U-2). These changes corresponded to the appearance of two slower ternary complexes 2.5 min after CSF-1 treatment and 15 min after LPS treatment. The upper induced complex has been previously

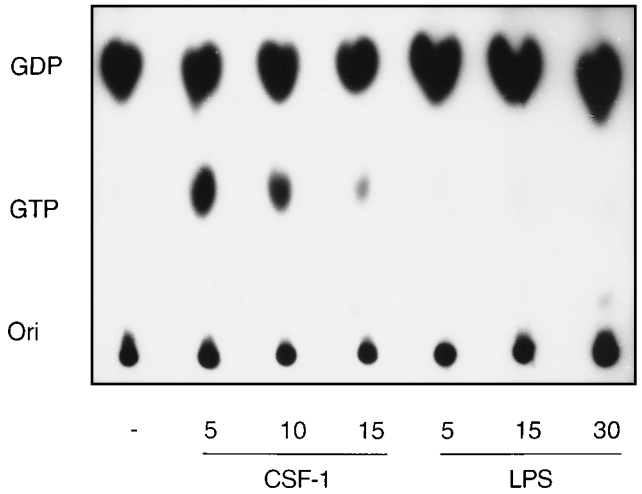

FIG. 3. Effect of CSF-1 and LPS treatment on the nucleotides bound to p21^{ras}. Quiescent, ^{32}P-labelled BAC-1.2F5 cells were stimulated with CSF-1 or LPS for different time periods. Nucleotides bound to Ras were analyzed by Ras immunoprecipitation followed by thin-layer chromatography. Ori, sample origin.

identified as hyperphosphorylated TCF/Elk (37), and its presence is correlated strictly with the kinetics of activation and deactivation of MAPK in both CSF-1- and LPS-induced extracts. The second inducible complex, previously identified as TCF/SAP on the basis of its reactivity with an antiserum against human SAP-1 (37), was also rapidly modified upon induction but persisted longer than TCF/Elk. Phosphorylation of this complex has been previously shown to be independent of MAPK activity (37).

Effect of CSF-1 and LPS stimulation on the formation of p21^{ras} · GTP complexes. CSF-1 stimulation causes an increase in the amount of GTP-bound p21^{ras} (Fig. 3 [92]) that correlates with the activation of the Raf-1/MAPK network. We compared the abilities of CSF-1 and LPS to induce the formation of p21^{ras} · GTP complexes in BAC-1.2F5 cells. Figure 3 shows that while CSF-1 stimulation yields the expected increase in p21^{ras} · GTP complexes, LPS treatment failed to induce significant changes in the ratio of GTP- versus GDP-bound p21^{ras} compared with that in unstimulated cells. Therefore, the activation of the Raf-1/MAPK network and the induction of TCF/Elk and TCF/SAP phosphorylation in LPS-treated cells are Ras-independent events.

Effect of a dominant negative *ras* mutant on CSF-1-mediated activation of Raf-1, MEK, and MAPK. We wondered whether Ras activation was indispensable for CSF-1-mediated Raf-1 activation or merely correlated with it. To establish this, we used stable cell lines that inducibly expressed either a dominant inhibitory *ras* mutant (15, 25, 26) (BAC Asn 17) or c-Ha-*ras* (BAC Ras) under the control of the glucocorticoid-inducible mouse mammary tumor virus promoter. Induction of the dominant negative Ras mutant by dexamethasone treatment caused a 74% reduction in the GTP loading of p21^{ras} after CSF-1 stimulation (Table 1). In the absence of dexamethasone, CSF-1 stimulation of all three kinases occurred normally (Fig. 4). Upon dexamethasone induction, however, CSF-1-mediated MEK activation was reduced (by 73%) in the BAC Asn 17 cell line and enhanced (1.5-fold) in the BAC Ras cells. Similarly, MAPK activation was reduced by 65% in the BAC Asn 17 cells and was slightly enhanced (1.2-fold) in the BAC Ras cells. Surprisingly, dexamethasone addition had no detrimental effect on Raf-1 activation in BAC Asn 17 cells (in fact

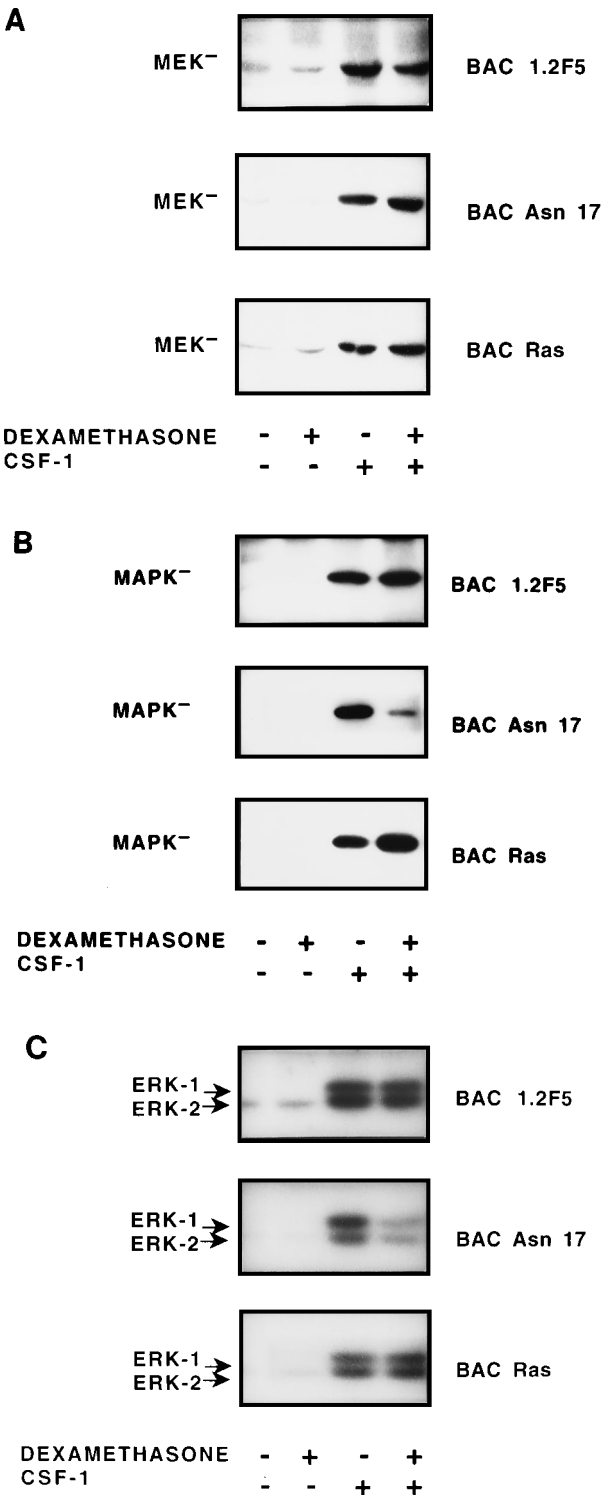

FIG. 4. Effect of a dominant negative Ras mutant on CSF-1 induced Raf-1, MEK, and MAPK activation. BAC-1.2F5, BAC Asn 17, and BAC Ras cells were deprived of CSF-1 in the presence or absence of dexamethasone (5×10^{-7} M) for 18 h prior to stimulation with either 0.63 nM mouse recombinant CSF-1 (5 min) or with 1 μg of LPS per ml (10 min). Cells were then solubilized and the activities of the different kinases were assessed as described in the legend to Fig. 1. (A) Raf-1 kinase activity was measured with catalytically compromised MEK-1 (MEK$^-$) as a substrate. (B) MEK activity measured with catalytically compromised ERK-2 (ERK-2$^-$) as a substrate. (C) MAPK activity in an in-gel kinase assay. ERK-1 and ERK-2 are indicated.

a 1.2-fold increase in activity was detected in the experiment shown), indicating that Ras does not play a role in this event. In parental BAC-1.2F5 cells, dexamethasone addition did not affect CSF-1-induced Raf-1, MEK, or MAPK activity.

Effect of PKC down-regulation and inhibition on CSF-1- and LPS-mediated activation of the Raf-1/MAPK pathway and on TCF phosphorylation. BAC-1.2F5 cells express the PKC isoforms δ, ε, and ζ (83). To determine whether TPA-sensitive isozymes were involved in CSF-1- or LPS-mediated kinase activation or TCF phosphorylation, BAC-1.2F5 cells were treated with TPA for 24 h to down-regulate PKC. Complete down-regulation of PKC δ and ε is evident already 6 to 8 h after TPA treatment (83). In contrast, TPA-mediated Raf-1 activation had not yet subsided 24 h after stimulation (Fig. 5A), and addition of CSF-1, LPS, or TPA itself did not further stimulate Raf-1 kinase activity. This sustained Raf-1 activation did not lead to the continued activation of MEK and MAPK (Fig. 5B and C). However, both MEK and MAPK were fully stimulated by the addition of CSF-1 to the TPA-treated cells, while activation of these kinases upon LPS stimulation or TPA restimulation was severely impaired.

Band shift analysis confirmed the putative role of TCF/Elk as a MAPK substrate (Fig. 5D). As with MAPK, TPA pretreatment abrogated LPS- but not CSF-1-induced TCF/Elk phosphorylation. On the other hand, neither LPS- nor CSF-1-induced TCF/SAP phosphorylation was affected by TPA pretreatment. As expected, TPA restimulation of PKC-depleted cells was unable to elicit either TCF/Elk or TCF/SAP phosphorylation.

To corroborate the results described above, BAC-1.2F5 cells were treated with the PKC inhibitor bisindolylmaleimide prior to stimulation with CSF-1, LPS, or TPA. Table 2 shows that pretreatment with the inhibitor repressed LPS- and TPA-mediated MEK/MAPK activation in a concentration-dependent manner. CSF-1-induced activation of these kinases was not affected. Bisindolylmaleimide did not impair Raf-1 activation by CSF-1, LPS, or, interestingly, TPA (Table 2). In particular, CSF-1-induced Raf-1 activation and LPS-induced Raf-1 activation were slightly enhanced by the inhibitor. Band shift analysis of TCF binding confirmed the results described in Fig. 5D (data not shown).

Effect of the PC-PLC inhibitor D609 on CSF-1- and LPS-mediated activation of the Raf-1/MAPK pathway and on TCF phosphorylation. PC-PLC has been recently identified as an important intermediate between Ras and Raf-1 in mitogenic signal transduction (14). CSF-1 has been shown to cause PC hydrolysis with biphasic kinetics, and PC-PLC has been shown to stimulate [^{3}H]thymidine incorporation and induction of gene expression in BAC-1.2F5 cells (92). To investigate whether PC-PLC activation was an important intermediate in the activation of the Raf-1/MAPK pathway, we treated quiescent BAC-1.2F5 cells with the specific PC-PLC inhibitor D609 prior to stimulation of the cells with either CSF-1 or LPS. Treatment with D609 selectively inhibits PC-PLC activation in fibroblasts (14) and, more germane to our study, in the human monocytic cell line U937 (72). In BAC-1.2F5 cells, D609 pretreatment reduced the LPS-induced, but not CSF-1-induced, activation of all three kinases (Fig. 6A to C). Band shift analysis of TCF binding again confirmed severe impairment of the LPS-mediated phosphorylation of TCF/Elk after D609 pretreatment, mirroring the reduction of MAPK activity. LPS-induced phosphorylation and CSF-1-induced phosphorylation of TCF/SAP were not affected by D609 pretreatment.

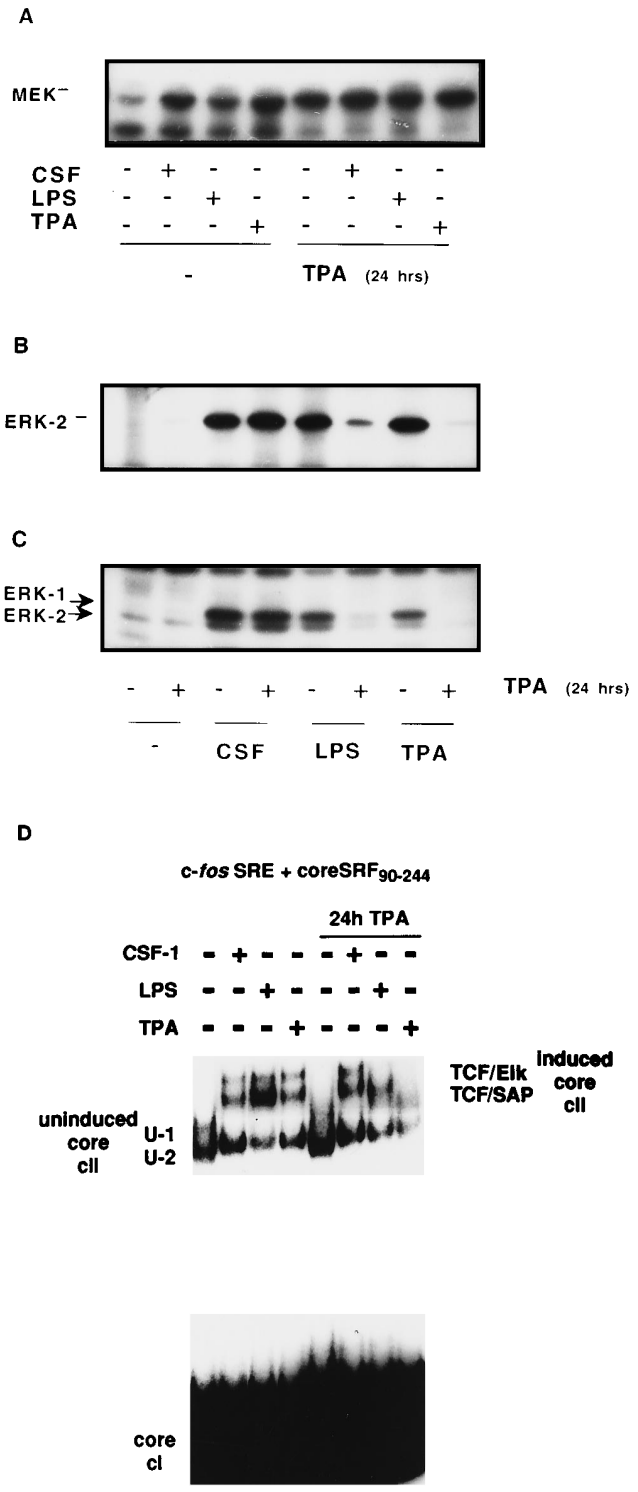

FIG. 5. Effect of TPA pretreatment on CSF-1- and LPS-induced kinase activity and TCF binding. BAC-1.2F5 cells were starved in the presence or absence of TPA for 24 h prior to stimulation with 0.63 nM mouse recombinant CSF-1 (5 min), with 1 μg of LPS per ml (10 min), or with 5×10^{-6} M TPA (10 min). The activities of the different kinases were measured as described in the legend to Fig. 1. (A) Raf-1 kinase activity was measured with catalytically compromised MEK-1 (MEK$^-$) as a substrate. (B) MEK activity measured with catalytically compromised ERK-2 (ERK-2$^-$) as a substrate. (C) MAPK activity in an in-gel kinase assay. ERK-1 and ERK-2 are indicated. (D) Band shift analysis of TCF binding was performed as described in the legend to Fig. 2. core cI, coreSRF dimer; core cII, ternary complexes; U-1 and U-2, uninduced ternary complexes; TCF/Elk and TCF/SAP, induced ternary complexes.

TABLE 2. Effect of the PKC inhibitor bisindolylmaleimide on CSF-1-, LPS- and TPA-induced kinase activity[a]

Kinase activity tested	% of stimulus-induced kinase activity with indicated concn of bisindolylmaleimide								
	CSF-1			LPS			TPA		
	0	1 μM	10 μM	0	1 μM	10 μM	0	1 μM	10 μM
Raf	100	116	182	100	120	152	100	112	101
MEK	100	97	105	100	69	23	100	1.48	0
MAPK	100	105	91	100	106	31	100	12	6

[a] Quiescent BAC-1.2F5 cells were treated with the PKC inhibitor bisindolylmaleimide (1 or 10 μM, 30 min) prior to stimulation with 0.63 nM mouse recombinant CSF-1 (5 min), with 1 μg of LPS per ml (10 min), or with 5×10^{-6} M TPA (10 min). The activity of the different kinases was measured as described in the legend to Fig. 1. CSF-1-, LPS- and TPA-induced kinase activity in the absence of inhibitor was defined as 100%. The results are expressed as the percentage of stimulus-induced kinase activity observed in the presence of bisindolylmaleimide at the concentrations indicated.

DISCUSSION

Ras-independent activation of Raf-1 by CSF-1 and LPS. The first step in LPS and CSF-1 signal transduction involves the activation of membrane-associated tyrosine kinases (19, 73, 77, 93). While the activated CSF-1 receptor stimulated the exchange of GDP for GTP on $p21^{ras}$, the kinases activated by LPS did not. Because LPS treatment still led to Raf-1 activation, a Ras-independent pathway is able to target Raf-1 in macrophages. To determine whether the CSF-1 signal actually required an active Ras upstream of Raf, a dominant inhibitory *ras* mutant and c-Ha-*ras* (15, 25, 26) were inducibly expressed in BAC-1.2F5 cells. The dominant negative *ras* mutant reduced MEK and MAPK activation by CSF-1 (described below). However, the mutant failed to inhibit CSF-1-mediated Raf-1 activation, and the inducible expression of c-Ha-*ras* failed to enhance it. Thus, both CSF-1-mediated activation and LPS-mediated activation of the Raf-1 kinase are Ras independent.

A common denominator in CSF-1 and LPS signal transduction is the fast activation of the *src* family kinases (19, 77). These kinases were found to bind to the CSF-1 receptor (19), as well as to the CD14 LPS receptor (77), and to play an important role in the transduction of the signal. In fact, CSF-1 receptors lacking the binding site for the *src* kinases are unable to transduce mitogenic signals in fibroblasts (19), and LPS-induced activation of the Raf-1/MAPK pathway (67, 86) and cytokine production (77) are blocked by the inhibition of tyrosine kinase activity with herbimycin or genistein. It is conceivable that kinases of the *src* family lie upstream of Raf-1 in CSF-1 and LPS signal transduction.

Raf-1 activation occurs in the absence of Ras activation in fibroblasts treated with the Ca^{2+} mobilizer thapsigargin (17) or with purified PC-PLC from *Bacillus cereus* (14). To our knowledge, however, this is the first report showing that a receptor tyrosine kinase activates Raf-1 independently of Ras. Our findings are at variance with recently published work showing that the same dominant negative Ras mutant reduces EGF-mediated Raf-1 activation (14). It is difficult to directly compare those data with ours, because the EGF receptor has not been shown to be associated with or to activate *src* family kinases. In fact, the expression of some of these kinases is restricted to cells of the myeloid lineage (9). Activation of $p21^{ras}$ and the *src* family kinases may represent alternative ways of regulating Raf-1 in different cell types. Consistent with this hypothesis, coexpression of Raf-1 with either $p21^{ras}$ or $pp60^{v\text{-}src}$ in insect Sf9 cells leads to Raf-1 autokinase and MEKK activity (24, 51, 88). Raf-1 activation by CSF-1 and LPS cannot result from direct phosphorylation by the *src* family kinases, because Raf-1 immunoisolated from CSF-1- and LPS-treated cells does not contain phosphotyrosine (6, 67). The fact that phosphopeptide maps of Raf-1 from CSF-1- or LPS-treated ^{32}P-labelled BAC-1.2F5 cells are superimposable suggests that these stimuli ultimately activate Raf-1 by the same modification (67).

CSF-1 and LPS activate MEK and MAPK by distinct mechanisms, and Raf-1 activation is not sufficient for the activation of MEK and MAPK. Raf-1 activation was a common feature of CSF-1 and LPS signal transduction, and the kinetics of activation were consistent with Raf-1 being upstream of MEK and MAPK (Fig. 1). However, other upstream activators influenced MEK and MAPK activation differentially in each pathway. For example, LPS activated these kinases via a Ras-independent pathway, while a dominant negative Ras mutant reduced CSF-1-mediated activation of these two kinases (Fig. 4). Depletion or inhibition of PKC strongly impaired MEK and MAPK induction in LPS- and TPA-treated cells but not in CSF-1-treated cells (Fig. 5B and C and Table 2). Interestingly, Raf-1 activation was neither inhibited in CSF-1-treated BAC Asn 17 cells nor was it affected by PKC depletion or inhibition prior to LPS treatment. On the contrary, pretreatment with bisindolylmaleimide slightly enhanced CSF-1- and LPS-mediated Raf-1 activation and did not block TPA activation. This raises the interesting hypothesis that a PKC isozyme might negatively regulate Raf-1 activity and that TPA-mediated activation of Raf-1 might be mediated by an alternative signal transduction pathway not involving PKCs. An attractive candidate would be the newly described protein kinase D, which binds phorbol esters with high affinity and has a pattern of substrate specificity clearly distinct from those of the PKCs (81).

LPS- but not CSF-1-induced Raf-1, MEK, and MAPK activation could also be reduced by pretreatment with D609. D609 has been reported to specifically inhibit PC-PLC in fibroblasts (14) and in human monocytes (72). The drug blocks Raf-1 activation in fibroblasts stimulated with serum or EGF, while exogenous PC-PLC activates Raf even in the presence of a dominant negative Ras mutant. In view of these results, PC-PLC has been positioned between Ras and Raf in the mitogenic signal transduction cascade (14). The lack of effect of D609 on CSF-1-induced Raf-1 activation is consistent with its apparent independence of Ras. At the same time, the failure to inhibit CSF-1-mediated MEK activation implies that the signal from Ras to MEK does not require PC-PLC activity. On the other hand, D609 causes a profound inhibition of LPS-mediated Raf-1 activation and a strong reduction in MEK and MAPK activation. Assuming that in BAC-1.2F5 macrophages the effect of D609 is as specific as that in U937 monocytes (72), this suggests that the pathways for Raf-1 activation on one hand and MEK/MAPK activation on the other hand bifurcate downstream of PC-PLC activation. It is conceivable that PC-PLC-derived diacylglycerol might activate PKC, which, as shown in Fig. 5A to C and Table 2, is important for LPS-

mediated MEK/MAPK activation but does not contribute to Raf-1 induction; at the same time, PC-PLC-derived diacylglycerol could be important in Raf-1 activation, either by binding to its amino-terminal regulatory domain (10) or by activating an upstream signal transduction component distinct from PKC (81). We currently favor the latter hypothesis, because diacylglycerol does not significantly increase the activity of recombinant Raf-1 (27).

Taken together, the data mentioned above show that, in BAC-1.2F5 cells, the pathways leading to Raf-1 and to MEK/MAPK activation diverge at the level of Ras in CSF-1-mediated signal transduction and at the level of PKC in LPS-stimulated signalling.

Raf-1 has been identified as a MEKK in vivo and in vitro (21, 39, 41, 48, 51, 79, 82, 88), albeit with a certain cell-type specificity (13, 28, 29, 32, 70, 90). Both MAPK activation (17) and MEK activation (29) have been observed in the absence of Raf-1 activation. In BAC-1.2F5 cells, we have not observed full MEK activation in the absence of Raf-1 activation under any of the conditions tested. As mentioned above, Raf-1 activation did not give rise to MEK/MAPK activation in cells expressing the Ras dominant negative mutant (Fig. 4) or in cells depleted of PKC by TPA pretreatment (Fig. 5A to C). Furthermore, expression of virally activated Raf in BAC-1.2F5 cells does not cause MEK activation unless the cells are stimulated with CSF-1 or LPS or treated with the phosphatase inhibitor okadaic acid (47). These results indicate that MEK in BAC-1.2F5 cells is the target of a negative regulator (possibly PP2A), which might be transiently inhibited by stimulation (18). Collectively, these data suggest that MEK integrates signals from different upstream activators. Raf-1 is to be considered a coregulator rather than a direct activator and cooperates with PKC-derived signals in the LPS pathway and $p21^{ras}$-derived signals in the CSF-1 pathway. Raf-1 and $p21^{ras}$ cooperate in activating MEK in insect cells (87) and in transforming NIH 3T3 cells (20), and PKC has been shown to cooperate with Raf-1 in fibroblasts (45). In agreement with this hypothesis, it has been shown recently that ERK-2 phosphorylation in EGF-stimulated fibroblasts represents the point of convergence of multiple Ras-dependent and -independent pathways (12).

Our data are also consistent with the alternative hypothesis that Raf-1 may not represent the MEKK activity induced by CSF-1 or by LPS in BAC-1.2F5 cells. At least two other protein kinases have the ability to phosphorylate and activate MEK in eukaryotes: the product of the proto-oncogene c-*mos*, which activates the MAPK pathway in *Xenopus* oocytes (59, 64), as well as in somatic cells (58, 75), and the kinases of the MEKK family, comprising mammalian MEKK and the yeast kinases BCK1, Byr2, and Ste11 (50). All of these MEKKs integrate signals from different upstream activators, including PKC (BCK1), Ste5 (Ste11), and Ras1 (Byr2). In particular, mammalian MEKK functions downstream of Ras in the EGF signal transduction pathway (49) and downstream of the PKC1 gene product in the yeast *Saccharomyces cerevisiae* (8). BAC-1.2F5 cells express MEKK (66). It is therefore conceivable that the Ras-dependent MEK/MAPK activation in CSF-1-treated cells and the PKC-dependent MEK/MAPK activation in LPS-stimulated cells might in fact be due to MEKK.

TCF/Elk and TCF/SAP are targeted by distinct mechanisms in CSF-1- and LPS-stimulated BAC-1.2F5 cells. Stimulation of BAC-1.2F5 cells led to the activation of two distinct TCFs, TCF/Elk and TCF/SAP (Fig. 2). Phosphorylation of TCF/Elk consistently correlates with the activation of the MAPK pathway (Fig. 4 to 6) (35–37, 42, 52). We have previously shown that the kinase responsible for TCF/SAP phosphorylation is Ras independent and is distinct from MAPK (37). In this paper, we

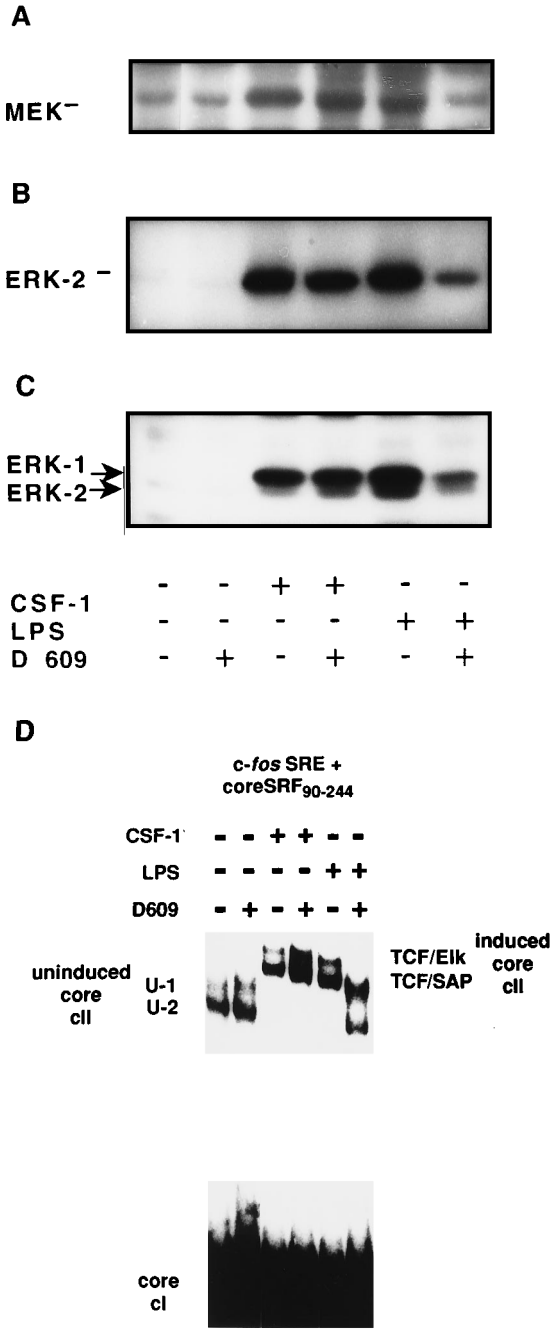

FIG. 6. Effect of D609 treatment on CSF-1- and LPS-induced kinase activity and TCF binding. Quiescent BAC-1.2F5 cells were treated with the PC-PLC inhibitor D609 (50 μg/ml, 1 h) prior to stimulation with either 0.63 nM mouse recombinant CSF-1 (5 min) or with 1 μg of LPS per ml (10 min). The activity of the different kinases was measured as described in the legend to Fig. 1. (A) Raf-1 kinase activity was measured with catalytically compromised MEK-1 (MEK^-) as a substrate. (B) MEK activity measured with catalytically compromised ERK-2 ($ERK\text{-}2^-$) as a substrate. (C) MAPK activity in an in-gel kinase assay. ERK-1 and ERK-2 are indicated. (D) Band shift analysis of TCF binding was performed as described in the legend to Fig. 2. core cI, coreSRF dimer; core cII, ternary complexes; U-1 and U-2, uninduced ternary complexes; TCF/Elk and TCF/SAP, induced ternary complexes.

investigated whether the pathways leading to TCF/Elk and TCF/SAP converged at any point in CSF-1 or LPS signal transduction. While confirming that TCF/Elk phosphorylation is linked to MAPK activation, our data show that TCF/SAP phosphorylation was unaffected by all of the inhibitors used in this study. Abrogation of TCF/SAP phosphorylation was observed only in LPS-stimulated cells after pretreatment with the tyrosine kinase inhibitor genistein (67) and in TPA-induced cells after PKC depletion by chronic TPA treatment or PKC inhibition by bisindolylmaleimide (Fig. 5D and data not shown). Together, these data indicate the existence of two divergent, specific pathways targeting distinct TCFs. This might seem redundant; however, the attractive possibility exists that TCF/SAP and TCF/Elk integrate different intracellular signals targeting the SRE and may in fact mediate the induction of different sets of immediate-early genes.

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