Macrophage Colony-Stimulating Factor Induces the Expression of Mitogen-Activated Protein Kinase Phosphatase-1 Through a Protein Kinase C-Dependent Pathway

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*J Immunol* 1999; 163:2452-2462; http://www.jimmunol.org/content/163/5/2452
Macrophage Colony-Stimulating Factor Induces the Expression of Mitogen-Activated Protein Kinase Phosphatase-1 Through a Protein Kinase C-Dependent Pathway

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M-CSF triggers the activation of extracellular signal-regulated protein kinases (ERK)-1/2. We show that inhibition of this pathway leads to the arrest of bone marrow macrophages at the G0/G1 phase of the cell cycle without inducing apoptosis. M-CSF induces the transient expression of mitogen-activated protein kinase phosphatase-1 (MKP-1), which correlates with the inactivation of ERK-1/2. Because the time course of ERK activation must be finely controlled to induce cell proliferation, we studied the mechanisms involved in the induction of MKP-1 by M-CSF. Activation of ERK-1/2 is not required for this event. Therefore, M-CSF activates ERK-1/2 and induces MKP-1 expression through different pathways. The use of two protein kinase C (PKC) inhibitors (GF109203X and calphostin C) revealed that M-CSF induces MKP-1 expression through a PKC-dependent pathway. We analyzed the expression of different PKC isoforms in bone marrow macrophages, and we only detected PKCβ, PKCe, and PKCζ. PKCζ is not inhibited by GF109203X/calphostin C. Of the other two isoforms, PKCe is the best candidate to mediate MKP-1 induction. Prolonged exposure to PMA slightly inhibits MKP-1 expression in response to M-CSF. In bone marrow macrophages, this treatment leads to a complete depletion of PKCβ, but only a partial down-regulation of PKCe. Moreover, no translocation of PKCβ or PKCζ from the cytosol to particulate fractions was detected in response to M-CSF, whereas PKCe was constitutively present at the membrane and underwent significant activation in M-CSF-stimulated macrophages. In conclusion, we remark the role of PKC, probably isoform ε, in the negative control of ERK-1/2 through the induction of their specific phosphatase.

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M-CSF induces macrophage proliferation, and it is the major and specific growth factor for this cell type (3). M-CSF is recognized by a specific receptor encoded by the protooncogene c-fms (4). The first step in transduction of the M-CSF signal is the activation of the tyrosine kinase domain of the receptor. As a consequence, the receptor becomes autophosphorylated on several tyrosine residues (4, 5). These residues are recognized by distinct tyrosine kinases, including members of the Src family (6, 7), which then phosphorylate a large number of intracellular substrates, and Tyk2, a Janus kinase that phosphorylates and activates transcription factors from the STAT family (8, 9). M-CSF also activates phosphatidylinositol 3-kinase (PI3-K) (10), which subsequently generates phosphatidylinositol (3, 4, 5) trisphosphate (PIP₃), a molecule that acts as a second messenger that activates p21Ras and some protein kinase C (PKC) isoforms (11, 12).

M-CSF also induces the production of diacylglycerol (DAG) and the subsequent activation of PKC (13, 14). The PKC family consists of multiple isoforms that are classified in three main groups (conventional, novel, and atypical PKCs) depending on their primary structure and activation requirements (15, 16). Conventional PKCs, i.e., , α, βI, βII, and γ, require both calcium (Ca²⁺) and DAG/phorbol esters for activation and phosphorylase as a cofactor. Novel PKCs require DAG/phorbol esters and phosphatidylinositol 3-kinase, but do not depend on Ca²⁺ for activation; they include the isoforms δ, ε, θ, η, and μ. Atypical PKCs, represented by isoforms ξ and α, cannot be activated by Ca²⁺ or DAG/phorbol esters, but are regulated by PIP₃, ceramide, and phosphatidic acid (17–20).

M-CSF triggers the activation of the Raf/MEK/ERK pathway in macrophages (21, 22). Raf-1, a serine/threonine protein kinase, phosphorylates and activates the threonine/tyrosine protein kinase MEK-1 (23), which, in turn, phosphorylates and activates extracellular signal-regulated protein kinase-1 (ERK-1) and ERK-2 (24). These are proline-directed serine/threonine protein kinases, also known as p44- and p42-mitogen-activated protein kinases (-MAPK), respectively (25). Active ERKs phosphorylate and regulate several cellular proteins, including additional protein kinases,
cytoskeletal components, phospholipase A₂, and nuclear transcrip-
tion factors, such as Elk1/TCF and c-Jun, which regulate the ex-
pression of immediate early genes (25, 26).

The negative regulation of ERK activity may be mediated by the
members of a family of dual specificity tyrosine phosphatases,
including MAPK phosphatase-1 (MKP-1) (27, 28). Phosphoryla-
tion on both tyrosine and threonine residues is required for ERK
activity (29). MKP-1 dephosphorylates both phosphotyrosine and
phosphothreonine residues on ERK-1 and -2 both in vitro and in
vivo, thus suggesting that this phosphatase is crucial for keeping
the balance between ERK phosphorylation and dephosphorylation
(27, 28). Recent reports have also shown the capability of MKP-1
to dephosphorylate and inactivate other MAPKs, including JNK/ SAPK and p38/RK (30). In fibroblasts, overexpression of MKP-1
inhibits ERK-regulated reporter gene expression, Ras-induced
DNA synthesis, and growth factor-induced entry into the S phase
(31, 32). The expression of MKP-1 constitutes a mechanism of
control and attenuation of mitogenic signaling pathways.

Although several cell types require ERK activation to proliferate
in response to a number of growth factors (reviewed in Ref. 33),
only a correlation between ERK activation and M-CSF signal
transduction has been found in macrophages, and the exact role of
ERK-1/2 in the proliferation of these cells is still unclear. In fact,
in the macrophage cell line BAC1.2F5, v-Raf conferred M-CSF-
dependent growth without ERK activation (34). On the other
hand, the extent of ERK activation appeared to be similar in both
proliferating and poorly proliferating macrophages, thus suggest-
ing that ERK activation was not sufficient to induce macrophase
proliferation (22). In the present report we show with certainty that
blockage of the activation of ERK-1/2 inhibits M-CSF-induced
proliferation of bone marrow macrophages, leading to a growth
arrest of these cells at the G₁ phase of the cell cycle. This blockage
was not accompanied by programmed cell death.

The time course of ERK activation determines the fate of sev-
eral cell responses, including cell proliferation (35–37). Our stud-
ies show that in bone marrow macrophages, M-CSF induces the
transient expression of MKP-1, which correlates with most of the
dephosphorylation and inactivation of ERK-1/2. Thus, the induct-
ion of this phosphatase seems to be an important mechanism for
the negative control of ERK activation in this system. Although the
induction of MKP-1 has been also described in M-CSF-stimulated
BAC1.2F5 cells (38), the signaling mechanisms that control the
expression of this phosphatase in response to M-CSF are unknown.
In this report we show that activation of ERK-1/2 is not required
for the induction of MKP-1. Instead, the expression of this phos-
phatase is induced by M-CSF through a PKC-dependent pathway.
Of all the PKC isoforms detected in macrophages, the main can-
tidate to mediate MKP-1 expression is PKCe. This suggests an
important role for PKC, putatively isoform ε, in the negative con-
trol of ERK activity through the induction of its specific phosphatase.

Materials and Methods

Macrophages

Bone marrow macrophages were obtained from 6- to 10-wk-old BALB/c
mice (Charles River Laboratories, Wilmington, MA) as previously de-
scribed (39). The cultures were cultured in 150-mm plates in DMEM (Sigma,
St. Louis, MO) supplemented with 20% PBS (Sigma) and 30% t. cell-
conditioned medium as a source of M-CSF. Once macrophages were 60–
80% confluent, they were deprived of t. cell-conditioned medium for 14–16
treatments to render the cells quiescent and then were subjected to different
treatments.

Reagents

Recombinant M-CSF was a gift from DNAX (Palo Alto, CA). In some experiments we used t. cell-conditioned medium as the source of this
growth factor. We used selective inhibitors/activators to either block or
activate specific signal transduction pathways. Bisindolylmaleimide I
(GF109203X), PMA, and calphostin C were purchased from Calbiochem
(St. Louis, MO) and calphostin C was light sensitive. 2′,3′-O-(di-
acetate) cAMP was purchased as a powder from Calbiochem (San Diego, CA). Bisindolylmaleimide I (GF109203X) and 1,2-sn-dioley-
glycerol were obtained from Sigma. PD98059 was purchased from New England Biolabs (Beverly, MA).

Proliferation assay

Cell proliferation was measured as previously described (40, 41) with mi-
ner modifications. Quiescent cells (10⁵) were incubated for 24 h in 24-well
plates (3424 MARK II, Costar, Cambridge, MA) in 1 ml of medium with
different concentrations of M-CSF. The medium was aspirated and re-
placed with 0.5 ml of medium containing [3H]thymidine (1 μCi/ml; ICN,
Costa Mesa, CA). After 4–6 h of incubation at 37°C, the medium was
removed, and the cells were fixed in ice-cold 70% methanol. After three
washes in ice-cold 10% TCA, the cells were solubilized in 1% SDS and 0.3
M NaOH at room temperature. Radioactivity was counted by liquid scin-
tillation using a 1400 Tri-Carb Packard scintillation counter (Packard,
Downers Grove, IL). Each point was performed in triplicate, and the results
were expressed as the mean ± SD.

Analysis of DNA content with 4′,6′-diamidino-2-phenylindole
(DAPI)

Cells (10⁶) were resuspended and fixed in ice-cold 70% ethanol. The cells
were then washed in PBS; resuspended in 0.2 ml of a solution containing
150 mM NaCl, 80 mM HCl, and 0.1% Triton X-100; and incubated at
0–4°C for 10 min. Afterward, 1 ml of a solution containing 180 mM
Na₂HPO₄, 90 mM citric acid, and 2 μg/ml DAPI (pH 7.4), was added to
each sample. After incubating the cells at 4°C for 24 h, their fluorescence
was measured with an EPICS Elite flow cytometer (Coulter, Miami, FL).
For this analysis, we used a UV laser with an excitation beam of 25 mW
at 333–364 nm, and fluorescence was collected with a 525-nm band-pass
filter. Cell doublets were gated out by comparing the pulse area vs the pulse
width. Cells (12,000) were counted for each histogram, and cell cycle
distributions were analyzed with the Multicycle program (Phoenix Flow
Systems, San Diego, CA).

Chromatin fragmentation assay

Fragmentation of DNA due to internucleosomal cleavage was determined
using a commercial ELISA kit (Cell Death Detection ELISA Kit plus,
Boehringer Mannheim, Indianapolis, IN). Briefly, the cells were harvested
and washed in ice-cold PBS. The cells were then lysed in 0.5 ml of lysis
buffer (50 mM Tris-HCl, 10 mM EDTA, and 1% SDS, pH 8.0) at 4°C, and
the lysates were centrifuged (15,000 × g) to separate high m.w. DNA
(pellet) from cleaved low m.w. DNA (supernatant). The DNA
present in the supernatants was analyzed in ELISA plates following the
commercial kit instructions. Each point was performed in triplicate, and the
results were expressed as the mean ± SD.

RNA extraction and Northern blot analysis

The cells were washed twice in PBS, and total RNA was extracted as
previously described (42). Total RNA samples (20 μg) were separated on
1% agarose gels containing formaldehyde and transferred to nylon mem-
branes (GeneScreen, DuPont-New England Nuclear, Boston, MA). For
MKP-1 mRNA detection, we obtained a probe corresponding to the full-
length cDNA of MKP-1 by digesting pBS/MKP-1 (provided by Dr. R.
Bravo, Bristol-Myers Squibb, Princeton, NJ) with HindIII. To detect the
L32 transcript, we used the EcoRI/HindIII fragment of pGEM1/L32 as a
probe (43). The probes were labeled with [α-32P]dCTP (ICN). The mem-
branes were hybridized in a solution containing 20% formamide, 5× Den-
hart’s solution, 5× SSC, 10 mM EDTA, 1% SDS, 25 mM Na₂HPO₄, 25
mM NaH₂PO₄, and 0.2 mg/ml salmon sperm DNA for 18 h at 65°C. Af-
terward, the membranes were subjected to three washes of 5 min each at
room temperature in 2× SSC/0.1% SDS, and one more wash at 65°C for
20 min in 0.1× SSC/0.1% SDS. The membranes were finally exposed to
Kodak X-AR films (Eastman Kodak, Rochester, NY). The bands of interest
were quantified with a Molecular Analyst (Bio-Rad, Richmond, CA).

Protein extraction and Western blot analysis

The cells were washed twice in cold PBS and lysed on ice with a lysis
solution containing 1% Triton X-100, 10% glycerol, 50 mM HEPES (pH
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leupeptin, 1 mM EDTA and 2 mM EDTA were incorporated in the lysis solution. Insoluble material was removed by centrifugation at 13,000 \( \times \) g for 8 min at 4°C. The protein concentration of the samples was determined by the Bio-Rad protein assay. Proteins from cell lysates (50–100 \( \mu \)g) were heated at 95°C in Laemmli SDS loading buffer, separated by 10% SDS-PAGE, unless stated otherwise. The membranes were blocked in 2% BSA in Tris buffer saline-0.5% Tween-20 (TBS-T) for 3 h at room temperature. To recognize PKC isozymes, the incubation was performed for 1 h at room temperature. To recognize PKC isoforms, the incubation with primary Abs (provided by Dr. P. J. Parker, Imperial Cancer Research Fund, London, U.K.) was performed overnight at 4°C. After three washes of 15 min each in TBS-T, the membranes were incubated for 1 h with peroxidase-conjugated anti-rabbit IgG Ab (Cappel-Organon Technik, Durham, NC). After three washes with TBS-T, enhanced chemiluminescence detection was performed (Amersham), and the membranes were exposed to x-ray films (Amersham). The bands of interest were quantified by densitometric analysis.

Determination of the ERK phosphorylation state by mobility shift assay

This assay was performed as described for the Western blot analysis with slight modifications (44). Proteins from cell lysates (50–100 \( \mu \)g) were subjected to 7.5% SDS-PAGE to allow efficient separation of phosphorylated and dephosphorylated forms of ERKs. The blocking of the membrane was conducted in 5% nonfat dry milk in TBS-T for at least 1 h at room temperature. Incubations with anti-ERK-1/2 primary Ab (1/10,000; provided by Dr. M. J. Weber, University of Virginia School of Medicine, Charlottesville, VA) and with peroxidase-conjugated anti-mouse IgG Ab (1/5000; Cappel) were performed in TBS-T for 1 h each at room temperature.

Determination of ERK activity by in-gel kinase assay

This assay was performed as previously described (45). Briefly, 50 \( \mu \)g of total protein was separated by 12.5% SDS-PAGE in the presence of 0.1 mg/ml of myelin basic protein (MBP; Sigma) co-polymerized in the gel. After electrophoresis, SDS was removed by washing the gel with two changes of 20% 2-propanol in 50 mM Tris-HCl (pH 8.0) for 1 h at room temperature. The gel was then incubated with 50 mM Tris-HCl (pH 8.0) containing 5 mM 2-ME (buffer A) for 1 h at room temperature. The proteins were denatured by incubating the gel with two changes of 6 M guanidine-HCl (pH 10) for 1 h at room temperature and then renatured by incubating with five changes of buffer A containing 0.04% Tween-20 for 16 h at 4°C. To perform the phosphorylation assay, the gel was washed in deionized water and equilibrated in 40 mM Tris-HCl (pH 7.4) containing 2 mM DTT, 0.1 mM EDTA, 15 mM MgCl\(_2\), and 300 \( \mu \)M sodium orthovanadate for 30 min at 25°C and then incubated for 1 h in the same solution containing 50 \( \mu \)M ATP and 100 \( \mu \)Ci \([\gamma ^{32} P]ATP\) (ICN). The reaction was stopped by washing the gel with 5% TCA containing 10 mM sodium pyrophosphate to inhibit phosphatase activity. The gel was dried, exposed to x-ray films (Kodak), and quantitated with a Bio-Rad Molecular Analyser.  

Determination of PKC translocation

PKC translocation was determined as previously described (46) with some modifications. The cells were lysed by scraping in cold hypotonic buffer T10 (10 mM Tris-HCl (pH 7.5), 1 mM EGTA, and 10 mM NaCl) containing protease inhibitors and 100 \( \mu \)M sodium orthovanadate. The cell lysates were centrifuged (100,000 \( \times \) g) for 30 min at 4°C, and the supernatants were collected (cytosolic fraction). The pellets were resuspended in cold T10 buffer containing 1% Triton X-100 and homogenized with a Dounce homogenizer on ice (15–20 strokes; Kontes, Vineland, NJ). To allow PKC extraction from the cell membrane, the samples were incubated for 1 h at 4°C and then centrifuged (100,000 \( \times \) g) for 30 min at 4°C. The supernatants were collected (plasma membrane fraction), and the pellets were resuspended in cold T10 containing 1% SDS, repeatedly passed through a 19-gauge needle, and heated at 100°C for 5 min. Insoluble material was removed by centrifugation (13,000 \( \times \) g) for 10 min, and the supernatants were collected (cytoskeleton fraction). Samples from each fraction (25 \( \mu \)g of protein) were boiled at 95°C in loading buffer and separated by 8% SDS-PAGE. The proteins were electrophoretically transferred to Hybond-ECL membranes (Amersham) and immunoblotted with Abs against each PKC isoform.

Measurement of PKCe activity

This assay was performed as previously described (47) with modifications. Specific Abs against PKCe (Life Technologies, Grand Island, NY) were used to immunoprecipitate this isoform from subcellular fractions (2 \( \mu \)g of Ab/150 \( \mu \)g of total protein in a total volume of 300 \( \mu \)l). Incubation was conducted for 2 h at 4°C. Immunocomplexes were separated by addition of 75 \( \mu \)l of 20% protamine and cell slurry, incubated 2 h at 4°C, and pelleted. The pellets were washed twice with RIPA buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 2 mM EDTA, and 1 mM EGTA) supplemented with protease inhibitors and 1 mM sodium orthovanadate and once with preincubation buffer (50 mM \( \beta \)-glycerolphosphate, 10 mM MgCl\(_2\), 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, protease inhibitors, 1 mM sodium orthovanadate) and then resuspended in reaction buffer (preincubation buffer supplemented with 100 \( \mu \)M ATP, 33 \( \mu \)M L-2,3-diolyeoleoylglycerol, 40 \( \mu \)g/ml L-\( \alpha \)-phosphatidylserine, 1 \( \mu \)M \([^{32}P]PKC\), and 5 \( \mu \)Ci \([\gamma ^{32} P]ATP\). Ser\(^{25}\)-substituted peptide obtained from the pseudosubstrate region of PKC (Calbiochem, La Jolla, CA) was used as the substrate for the phosphorylation assay, because it represents an appropriate substrate for measuring PKCe activity (48). The reaction was conducted for 10 min at 30°C. Each sample was spotted on a phosphocellulose filter (Whatman 3 MM, Clifton, NJ) and subjected to five washes of 30 min each in 5% TCA and 10 mM sodium pyrophosphate. Radioactivity was counted by liquid scintillation using a 1400 Tri-Carb Packard scintillation counter.

Results

The following studies were performed with bone marrow-derived macrophages because they constitute a homogeneous population of primary macrophages that become quiescent in the absence of M-CSF (40, 41). One of the earliest events in the signaling response to M-CSF is the induction of ERK activity (21, 22). We were interested in studying whether this activation was necessary for the proliferative response of macrophages to M-CSF. Activation of the ERK pathway was blocked by incubating the cells with the MEK inhibitor PD98059 (Fig. 1A). In the presence of M-CSF, bone marrow macrophages proliferated in a dose-dependent manner, as measured by thymidine incorporation (Fig. 1B). This method gives an efficient indication of macrophage proliferation as previously determined (41). Macrophages preincubated with PD98059 were unable to proliferate in response to M-CSF, thus showing that activation of the MEK/ERK pathway is necessary for M-CSF-dependent macrophage proliferation. We also analyzed the cell cycle distribution of M-CSF-stimulated macrophages in the presence or the absence of PD98059. To do this, we stained the cells with DAPI, a fluorescent dye that specifically binds to DNA, and we measured the cellular DNA content by flow cytometric analysis. Cultures of bone marrow macrophages growing in M-CSF-containing medium (control cells) showed a random distribution among the different phases of the cell cycle, with 51% of cells in G0/G1, 30% in S, and 17% in G2/M (Fig. 1C). When these cells were deprived of M-CSF for 24 h (starving cells), the tetraploid peak corresponding to the G0/M phase was drastically reduced, whereas a subdiploid peak representing apoptotic cells (Sub-G1) appeared. When macrophages growing in the presence of M-CSF were also incubated with PD98059 over a period of 24 h, they turned out to be distributed homogeneously (74% of total cells) in the G0/G1 phase of the cell cycle, with no peaks of apoptotic cells. This suggests that the blockage of M-CSF-induced ERK activation causes a growth arrest of macrophages at the G0/G1 phase of the cell cycle and that this growth arrest is not due to induction of apoptosis. To support this observation, the induction of apoptosis was further analyzed using a cell death detection kit, which measures DNA fragmentation caused by internucleosomal cleavage. Apoptosis was observed in cells deprived of M-CSF for 24 h (positive control), but not in cells growing in the presence...
of M-CSF or treated with PD98059 (Fig. 1D). These results show that blockage of ERK activation inhibits M-CSF-induced proliferation by inducing a growth arrest of macrophages at the G1 phase of the cell cycle without causing apoptosis.

The time course of ERK activation appears to be determinant in the control of some cellular responses involving cellular differentiation or proliferation. Although a transient and acute peak of ERK activation is induced by a number of growth factors, prolonged activation of these kinases has been recently associated with differentiation and cell cycle arrest (35–37, 49, 50). Therefore, to induce a normal proliferative response, the time course of ERK activity must be finely controlled. Two different assays were used to study the time course of ERK activation in bone marrow macrophages stimulated with M-CSF. The phosphorylation state of these kinases was determined by a mobility shift assay. The addition of M-CSF to quiescent macrophages induced the rapid phosphorylation of ERK-1/2 (Fig. 2A), which decreased progressively after 30 min of stimulation. Moreover, the activity of ERK-1/2 was analyzed by an in-gel kinase assay, using myelin basic protein as the substrate. ERK activation was detected as soon as 2–3 min after M-CSF treatment and, again, decreased progressively after 20 min of stimulation (Fig. 2B). Some residual activity was observed at prolonged times of M-CSF treatment.

MKP-1 has been involved in the dephosphorylation and inactivation of ERK-1/2 in vitro and in vivo (27, 28). To study whether ERK dephosphorylation in our system could be mediated by the expression of MKP-1, we analyzed the induction of this phosphatase in bone marrow macrophages stimulated with M-CSF. The expression of MKP-1 was not detected in quiescent cells. The induction of the transcript was detectable 10 min after M-CSF stimulation, reached a maximum at 30 min, and then decreased progressively (Fig. 2C). The expression of the protein was detected after 20 min, peaked at 60–75 min, and returned to basal levels within 2 h of stimulation with M-CSF (Fig. 2D). MKP-1 expression correlated with the inactivation by dephosphorylation of ERK-1/2 during the macrophage response to M-CSF (Fig. 2E), thus suggesting that synthesis of this phosphatase is an important mechanism induced by M-CSF to control the time course of activation of the ERK pathway.

We next analyzed the mechanisms that mediate the induction of MKP-1 in response to M-CSF. In fibroblasts, transcription of the MKP-1 gene is under the control of the ERK pathway (51). For this reason, we studied whether the activation of ERK-1/2 was required for the induction of this phosphatase by M-CSF. Fig. 3 shows that blockage of the ERK pathway with PD98059 did not alter MKP-1 expression at the level of either mRNA or protein synthesis, thus indicating that activation of ERK-1/2 is not strictly required for MKP-1 induction in M-CSF-stimulated macrophages. Instead, M-CSF must activate other signaling pathways that ensure MKP-1 expression.
M-CSF also activates the enzyme PI3-K (10). Although the exact role of this enzyme in the macrophage response to M-CSF is not clear (reviewed in Ref. 52), in our hands, the pretreatment with wortmannin, an inhibitor of PI3-K, led to a 40–70% inhibition of the incorporation of \[^{3}H\]thymidine in macrophages stimulated with subsaturant doses of M-CSF (200–600 U/ml; data not shown). Therefore, we also studied the implication of this pathway in the induction of MKP-1. However, wortmannin did not alter the expression of MKP-1 in response to M-CSF (Fig. 3, B and C). This assay was also performed with subsaturant concentrations of the growth factor without obtaining any inhibitory effect of wortmannin (data not shown). Taken together, these results indicate that activation of PI3-K is not required for the induction of MKP-1 by M-CSF.

The enzyme PKC has been also involved in the signal transduction of M-CSF (13, 14). PKC may mediate the activation of the transcriptional complex AP-1 (53–55), which, in turn, recognizes and activates transcription from gene promoters containing \(\alpha\)-tetradecanoylphorbol 13-acetate (TPA) response elements (TREs) (56, 57). Because a TRE site has been described at position 2450 bp in the promoter of the MKP-1 gene (58), we studied the involvement of PKC in the induction of MKP-1 in M-CSF-stimulated macrophages. The cells were preincubated with the PKC inhibitor GF109203X (59) before adding M-CSF. GF109203X inhibited the expression of MKP-1 in a dose-dependent manner (Fig. 4, A and B). Eighty percent inhibition of MKP-1 expression was observed when macrophages were pretreated with 5 \(\mu\)M GF109203X. Recently, it has been shown that GF109203X can also inhibit the activation of two other molecules, Rsk-2 and p70 56 kinase (60). However, it is unlikely that blockage of MKP-1 expression is due to the inhibition of any of these proteins for two reasons. First, Rsk-2 is an enzyme that lies immediately

![FIGURE 2](http://www.jimmunol.org/)

The expression of MKP-1 correlates with the dephosphorylation of ERK-1/2 during the macrophage response to M-CSF. Quiescent bone marrow macrophages were incubated with saturating amounts of M-CSF (1200 U/ml) for the indicated periods of time. A, ERK-1/2 phosphorylation was analyzed by a mobility shift assay. Because the primary Ab used has more affinity toward ERK-2 than ERK-1, the ECL-stained membrane was subjected to two different times of exposure to clearly study the time course of phosphorylation of both proteins. Phosphorylated and unphosphorylated forms of ERK-1/2 are indicated. B, ERK-1/2 activation was analyzed with an in-gel kinase assay. C, MKP-1 mRNA expression was studied by Northern blotting. Expression of the ribosomal gene L32 was used as a control for RNA loading and transfer. D, Detection of the MKP-1 protein was performed by Western blotting (150 \(\mu\)g/lane). Expression of the \(\beta\)-actin protein was measured as a control for loading and transfer. E, Comparison of the time courses of ERK activity and expression of the MKP-1 protein. All experiments in this figure were repeated, with identical results.
downstream of ERK-1/2 and is activated by the same agonists that activate this pathway (61). As shown above, specific blockage of this cascade with PD98059 does not inhibit MKP-1 induction by M-CSF (Fig. 3A). Second, rapamycin, a selective inhibitor for p70 S6 kinase (62) does not modify the levels of MKP-1 mRNA in response to M-CSF (data not shown). These results suggest that inhibition of MKP-1 expression by GF109203X is due to selective blockage of PKC. To confirm this, we also used calphostin C (63), a PKC inhibitor not related to GF109203X. As shown in Fig. 4C, the induction of MKP-1 by M-CSF was also inhibited by

FIGURE 3. MKP-1 induction by M-CSF does not require the activation of either the MEK/ERK cascade or the enzyme PI3-K. Quiescent macrophages were preincubated with PD98059 (50 μM), vehicle (0.1% DMSO), or wortmannin (100 nM) for 1 h and then stimulated or not with M-CSF (1200 U/ml). A and B, MKP-1 expression after 30 min of M-CSF stimulation was analyzed by Northern blotting. L32 expression was measured as a control of loading and transfer. The images are representative of two independent experiments. C, The expression of the MKP-1 protein after 1 h of M-CSF stimulation was analyzed by Western blotting.

FIGURE 4. The inhibition of PKC blocks the expression of MKP-1 and extends the time course of ERK activation in response to M-CSF. A, Quiescent macrophages were either left untreated or preincubated with different concentrations of GF109203X (GF) for 1 h and then stimulated with M-CSF (1200 U/ml) for 30 min. The expression of MKP-1 mRNA was analyzed by Northern blotting and is represented as a percentage of maximal expression. B, The macrophages were treated or not with GF109203X (GF, 5 μM) for 1 h and then stimulated with M-CSF (1200 U/ml) for 1 h. The expression of the MKP-1 protein was studied by Western blotting. C, The cells were preincubated or not with calphostin C (100 nM) for 1 h and then stimulated or not with M-CSF (1200 U/ml) for 30 min. The expression of MKP-1 was determined by Northern blotting. D, The macrophages were stimulated with M-CSF for different periods of time in the presence or the absence of GF109203X (GF). The time course of ERK-1/2 activation was studied by an in-gel kinase assay.
calphostin C. Taken together, these results indicate that a PKC-dependent pathway mediates MKP-1 induction in M-CSF-stimulated macrophages.

We also measured the time course of ERK activation in macrophages pretreated with GF109203X. The initial activation of ERK1/2 was not altered by the presence of the PKC inhibitor (Fig. 4D), thus indicating that PKC activity is not required for ERK activation by M-CSF. However, the extent of ERK activation was significantly more prolonged in macrophages in which MKP-1 expression had been inhibited by preincubation with GF109203X. This observation further supports the involvement of PKC in the negative regulation of ERK activity by inducing the expression of the phosphatase MKP-1 in M-CSF-stimulated macrophages.

Atypical isotypes of PKC are not sensitive to inhibition by GF109203X (64). Therefore, our results suggest that either a classical or a novel isoform of PKC mediates the induction of MKP-1 by M-CSF. By using polyclonal Abs raised against isoform-specific peptides, we studied the expression of isoenzymes α, βI, ε, δ, γ, θ, η, λ/ι, and ζ in bone marrow macrophages. We detected the expression of PKCβI and PKCe was performed by Western blotting (80 μg of total protein/lane). None of the other isoforms mentioned above was detected under our experimental conditions. We did not assay the presence of PKCβII. However, βI and βII result from differential splicing of the same transcript, and in most cases, one of the two isoforms is mainly expressed in a certain tissue (reviewed in Ref. 15). In support of the absence of PKCδ, rottlerin (1–20 μM), a specific inhibitor of this isoform, had no effect on the induction of MKP-1 by M-CSF (data not shown). Of the three isoforms detected in our system, only PKCβI and PKCe are sensitive to inhibition by GF109203X.

To assess the involvement of these two isoforms, the cells were

**FIGURE 5.** Prolonged treatment with PMA leads to a slight inhibition of the M-CSF-induced expression of MKP-1. A. Macrophages express PKC isoforms βI, ε, and ζ. The expression of PKC isoforms was analyzed by immunoblotting of total cell lysates (80 μg of total protein/lane) from quiescent macrophages. To assess the specificity of each Ab the membranes were incubated with the primary Ab in the presence or the absence of the specific peptides used to raise that Ab. B. Quiescent macrophages were incubated in the presence or the absence of PMA (100 ng/ml) for 12 h. The detection of PKCβI and PKCe was performed by Western blotting (80 μg of total protein/lane). C. Quiescent cells were incubated in the presence or the absence of PMA (100 ng/ml) for 12 h and then stimulated with M-CSF (1200 U/ml) for 30 min. MKP-1 expression was analyzed by Northern blotting.
PKC ε was immunoprecipitated from the Triton-soluble fraction of cells stimulated or not with M-CSF (1200 U/ml) for 10 min. The cytosolic, membrane, and cytoskeletal fractions from A and B were isolated, and 25 μg of protein from each fraction was separated by 8% SDS-PAGE. Detection of PKC isoforms was performed by Western blot using specific Abs. C, PKC ε was immunoprecipitated from the membrane fractions of macrophages stimulated with M-CSF (1200 U/ml) for different periods of time. PKC ε activity was measured as described in Materials and Methods.

FIGURE 6. M-CSF induces the activation of membrane-bound PKC ε. A. Quiescent macrophages were left untreated or were stimulated with M-CSF (1200 U/ml) for 10 min. B. As a positive control for PKC βI translocation, macrophages were left untreated or were incubated with PMA (100 ng/ml) for 10 min. The cytosolic, membrane, and cytoskeletal fractions from A and B were isolated, and 25 μg of protein from each fraction was separated by 8% SDS-PAGE. Detection of PKC isoforms was performed by Western blot using specific Abs. C, PKC ε was immunoprecipitated from the membrane fractions of macrophages stimulated with M-CSF (1200 U/ml) for different periods of time. PKC ε activity was measured as described in Materials and Methods.

Discussion

In this report we used bone marrow-derived macrophages because they constitute a homogeneous population of macrophages that can be either rendered quiescent by removing M-CSF from the medium or induced to proliferate in response to this specific growth factor. Transfection of these primary cultures is very inefficient (41). For this reason, in many experiments we need to use chemical inhibitors to assay the involvement of specific molecules in macrophage biology. In this report we have provided evidence that activation of the MEK/ERK pathway is necessary for macrophage proliferation in response to M-CSF. Blockage of ERK-1/2 activation caused a growth arrest of macrophages at the G1 phase of the cell cycle, without inducing programmed cell death. Our results indicate that ERK activity is necessary for the M-CSF-induced progression through the G1 phase or the entry into the S phase of the cell cycle. In fact, the activation of these kinases is also necessary for the proliferation of other cell types in response to specific growth factors or serum (32, 33, 65). We still do not know the exact mechanism by which ERK-1/2 control macrophage proliferation. However, it has been described that these kinases can mediate c-Myc phosphorylation on serine 62 (66, 67); the activation of this transcription factor is necessary for M-CSF-induced macrophage proliferation (68). Besides, ERK-1/2 may also act as positive regulators of cyclin D1 expression (69), which is required for progression through the G1 phase of the cell cycle in response to M-CSF (7). The fact that blockage of ERK activation did not induce macrophage apoptosis suggests that the expression of genes involved in M-CSF-induced macrophage survival is not under the control of the ERK cascade and contrasts with the observation that ERK activity is involved in the GM-CSF/IL-3-induced survival of hemopoietic cells (70). In bone marrow-derived macrophages, separate pathways regulating proliferation or survival may be activated in parallel by M-CSF.

The time course of ERK activation is a critical aspect for determining some cellular responses, including cell proliferation (35–37, 49, 50). We have determined the time course of ERK-1/2 activation in M-CSF-stimulated bone marrow macrophages by studying both the state of phosphorylation of these kinases and their ability to phosphorylate MBP in an in-gel kinase assay. Our results confirm previous observations about the transient pattern of ERK activity in M-CSF-stimulated macrophages (22). However, by using a totally distinct substrate of phosphorylation, a recombinant Ets-2 protein, it has been recently described that M-CSF induces persistent activation of ERK-1/2 in bone marrow macrophages (71). In our experiments we detected some residual ERK activity at prolonged times of M-CSF stimulation, which may be sufficient to mediate Ets-2 phosphorylation.

During the macrophage response to M-CSF, the major part of ERK inactivation correlated with the synthesis of the phosphatase MKP-1, thus suggesting that this phosphatase is involved in the negative control of ERK activity in our system. The induction of MKP-1 in bone marrow macrophages confirms a previous report that described accumulation of the mRNA coding for this phosphatase after stimulation of the macrophagic cell line BAC1.2F5.
with M-CSF (38). However, we have further explored the mechanisms involved in the negative control of ERK activity by studying the signaling pathway that mediates MKP-1 induction in M-CSF-stimulated macrophages.

In contrast to what has been described in serum-stimulated fibroblasts (51), induction of MKP-1 by M-CSF was not dependent on activation of the MEK/ERK cascade. Thus, MKP-1 is not expressed in M-CSF-stimulated macrophages as a consequence of the ERK pathway being able to induce its own attenuation in a direct negative feedback loop. Instead, M-CSF ensures the expression of this phosphatase by an alternative mechanism.

In this report we show that the induction of MKP-1 is dependent on PKC activation. In parallel studies we found that LPS also induces the expression of MKP-1 in bone marrow macrophages through a PKC-dependent pathway. In that report we also showed that, in contrast to what has been described in other systems, an increase in the intracellular levels of cAMP did not induce the expression of this phosphatase. This indicates that important differences exist regarding transcription of the MKP-1 gene in different cell types. Our observations suggest a major role for PKC-dependent events in the control of MKP-1 expression in macrophages.

The time course of ERK activity was significantly extended in macrophages in which MKP-1 expression was inhibited in response to a PKC inhibitor. Although we cannot discard the involvement of phosphatases other than MKP-1, especially since some dephosphorylation of ERK-1/2 still occurred in the absence of normal MKP-1 induction, our results allow us to conclude that PKC plays an important role in the control of the time course of ERK activity by inducing the expression of MKP-1.

There is increasing evidence that individual PKC isoforms mediate specific events in signal transduction (16). We have shown that bone marrow macrophages express PKC isoforms βI, ε, and ζ. In contrast to the observations recently reported by Pingel et al. (72), we did not detect the expression of PKCε and PKCζ in this cell type, although the experiment was conducted several times. The fact that MKP-1 induction was not altered by rottlerin, a specific inhibitor of PKCζ, further supports our results. Differences in the pattern of expression of PKC isoforms in a certain cell type may be caused by the state of maturation of the cells (73–76) or by the specific culture conditions. In fact, there are significant variations in the expression of PKC isoforms when comparing different macrophagic cell lines (21, 77–79) or even different primary monocytic/macrophagic populations (80–83), perhaps as a consequence of their specific state of differentiation/maturation. To study the response of bone marrow macrophages to M-CSF, we need to use nonconfluent cultures of macrophages that maintain a high rate of proliferation, because prolonged culture of these cells in the presence of M-CSF correlates with a loss of their capability to further proliferate in response to M-CSF or other growth factors. Besides, before the stimulation with M-CSF, we need to render the cells quiescent by incubating them in the absence of this growth factor for 16 h. The use of macrophages at a different stage of terminal differentiation may explain the discrepancies between our results and the data reported by Pingel et al. (72).

When we analyzed the selective implication of PKC isoforms, we found no evidence that PKCζ could mediate the expression of MKP-1 in response to M-CSF. First, the expression of this phosphatase was inhibited by GF109203X, which blocks the activation of conventional and novel PKCs, but does not effectively inhibit atypical isoforms at the doses used in our experiments (64). Second, the expression of MKP-1 induced by M-CSF did not decrease by pretreating the macrophages with wortmannin, a specific inhibitor of PI3-K. This enzyme mediates the generation of PI(3)P production, a second messenger that activates PKCζ (17). Taken together, these results suggest that PKCζ is not involved in the M-CSF-induced signaling pathway that leads to MKP-1 expression.

Our results indicate that of the other two PKC isoforms expressed in macrophages, the most likely candidate to mediate the induction of MKP-1 by M-CSF is PKCε. First, calphostin C has been shown to inhibit the novel isoforms of PKC, including PKCε, more efficiently than the conventional ones (63). Second, in bone marrow macrophages, prolonged treatment with PMA causes the complete depletion of PKCβI, but leads to only a slight down-regulation of PKCε. Similarly, PKCε has been also shown to be resistant to prolonged PMA treatment in three macrophagic cell lines and in several other cell systems (84–88). The fact that this treatment only reduces slightly MKP-1 induction by M-CSF supports the involvement of PKCε in this event. Third, PKCε is associated with the plasma membrane in both untreated and M-CSF-stimulated macrophages. The constitutive presence of this isoform in the plasma membrane has also been described in some other cell types and in the macrophagic cell line U937 (15, 89). In bone marrow macrophages we have detected significant activation of PKCε within 15 min of M-CSF stimulation. Our results allow us to conclude that the only presence of PKCε in the macrophage membrane fraction is not sufficient for the activation of this isoform. In our system stimulation with M-CSF is required for PKCε to become active, perhaps as a consequence of both the generation of DAG (14) and the induction of conformational or phosphorylation-based modifications of the enzyme. Moreover, DAG generated in response to M-CSF mainly derives from the hydrolysis of phosphatidylcholine mediated by a phospholipase C isoform specific for this phospholipid (90). Production of DAG without generation of inositol trisphosphate, a second messenger that triggers the mobilization of intracellular calcium, may explain the lack of translocation of PKCβI in our system. In conclusion, MKP-1 expression in response to M-CSF correlates with the activation of PKCε, and the absence of this activation in nonstimulated macrophages allows us to explain why MKP-1 is not expressed under nonstimulated conditions.

However, although PKCε is the best candidate to mediate the induction of MKP-1 by M-CSF, we cannot discard the involvement of other signaling molecules that have not been studied in this report. In fact, we did not detect a total inhibition of MKP-1 expression in macrophages treated with specific PKC inhibitors. This suggests that PKC is required for this process, but some other mechanism may participate in the induction of MKP-1 by M-CSF.

In this report we have shown that ERK-1/2 are required for macrophage proliferation, but not survival, in response to M-CSF. Our results also yield relevant insights into the mechanisms that control the duration of ERK activity in M-CSF-stimulated macrophages. M-CSF induces the activation of ERK-1/2 and the expression of MKP-1 through two distinct pathways. Induction of MKP-1 is mediated by the activation of a GF109203X/calphostin C-sensitive isoform of PKC, putatively isoform ε.

Acknowledgments

We thank Dr. Antonio García de Herreros for discussing the experiments regarding PKC and for some reagents. We also thank Drs. Rich A. Maki, Jorge Moscat, and Teresa Díaz-Meco for some reagents and for their helpful advice about signal transduction; Dr. Peter Parker for the anti-PKC Abs; Dr. Michael Weber for the anti-ERK1/2 Abs; Dr. Rodrigo Bravo for the
plasmid containing the full-length MKP-1 cDNA; Dr. José A. García Sanz for his advice about some protocols; and Jaume Comas and Rosario González, from the flow cytometry facility of the Serveis Científico-Tècnics, Universitat de Barcelona, for their helpful assistance. We also thank Martin Cullell-Young for reviewing the manuscript.

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