Roles of the Mitogen-activated Protein Kinase Family in Macrophage Responses to Colony Stimulating Factor-1 Addition and Withdrawal*

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Colony stimulating factor-1 (CSF-1) (or macrophage CSF) is involved in the survival, proliferation, differentiation, and activation of cells of the monocyte/macrophage lineage. Because the mitogen-activated protein kinase family members extracellular signal-regulated kinases (ERKs), p38, and c-Jun N-terminal kinase are widely implicated in such cellular functions, we measured their activity in growing and growth-arrested cultures of bone marrow-derived macrophages (BMM), as well as their stimulation by saturating concentrations of CSF-1. ERK activity was approximately 2-fold higher in cycling BMM compared with growth-arrested BMM; in addition, CSF-1-stimulated BMM DNA synthesis was partially inhibited by PD98059, a specific inhibitor of MEK, suggesting a role for a mitogen-activated protein-ERK kinase (MEK)/ERK pathway in the control of DNA synthesis but surprisingly not in the control of cyclin D1 mRNA or c-myc mRNA expression. The suppression of BMM apoptosis by CSF-1, i.e. enhanced survival, was not reversed by PD98059, suggesting that a MEK/ERK pathway is not involved in this process.

Using a quantitative kinase assay, it was found that CSF-1 gave a slight increase in BMM p38 activity, supporting prior data that CSF-1 is a relatively weak stimulator of inflammatory cytokine production in monocytes/macrophages. Relatively high concentrations of the p38 inhibitor, SKB202190, suppressed CSF-1-stimulated BMM DNA synthesis. No evidence could be obtained for the involvement of p38 activity in BMM apoptosis following CSF-1 withdrawal. We were not able to show that CSF-1 enhanced BMM JNK-1 activity to a significant extent; again, no role could be found for JNK-1 activity in the BMM apoptosis occurring after CSF-1 removal.

In addition to being necessary for proliferation, colony stimulating factor-1 (CSF-1) or macrophage CSF is absolutely required for the continued survival of certain populations of cells of the monocyte/macrophage lineage (1). Several lines of evidence suggest that the maintenance of survival and the stimulation of proliferation are mediated by either different signaling pathways activated by the CSF-1 receptor or require different thresholds of activation of similar pathways (2–4). Advances have been made in describing the signal transduction molecules activated in response to CSF-1 stimulation of macrophages, although the details of the pathways leading to their activation are still in many cases unresolved (5). The role of the CSF-1 receptor in controlling passage through the G1 phase of the cell cycle has been relatively well studied, and it has been shown, for example, for cell cycle progression that continued stimulation by CSF-1 is required for the synthesis of D-type cyclins as is the up-regulation of c-myc (6–8). Relatively little, however, is known about the signal transduction pathways activated by CSF-1 involved in maintaining cell survival.

Recent research has suggested roles for different members of the MAP kinase family (ERKs, p38/(CSBP), JNK (stress-activated protein kinase)) in promoting either cell survival and/or proliferation, in response to growth factor stimulation, or apoptosis, in response to various stress stimuli. There are many examples where enhanced ERK activity has been implicated in growth factor-mediated cell survival and/or proliferation (for example, see Ref. 9 for review); significant downstream responses of ERK-dependent signal transduction pathways that have been proposed to be important for cell cycle progression are cyclin D1 and c-myc transcription, including in CSF-1-stimulated NIH-3T3 cells expressing the CSF-1 receptor (c-Fms) (Ref. 10 and references therein). The relationship between ERK activation and other responses in macrophages is, however, much more complicated than the above discussion suggests. In this cell type, activation of ERKs has not been found to correlate strongly with the level of subsequent DNA synthesis (11, 12), and there is some evidence to suggest that there may be Ras-independent control over ERK activity (5). Also, in macrophages, elevated intracellular cAMP enhances CSF-1-stimulated ERK activity while suppressing the corresponding rise in cyclin D1 mRNA, suggesting a possible disassociation (12).

The p38 and JNK cascades are primarily activated by various environmental stresses: proinflammatory cytokines (TNFα and interleukin-1), osmotic shock, ultraviolet radiation, heat shock, x-ray irradiation, hydrogen peroxide, and protein synthase. 

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‡ The abbreviations used are: CSF-1, colony stimulating factor-1; BMM, bone marrow-derived macrophages; LPS, lipopolysaccharide; TNF, tumor necrosis factor; ERK, extracellular signal-regulated kinase; p38/CSBP, cytokine suppressive anti-inflammatory drug-binding protein (also known as reactivating kinase); JNK, c-Jun N-terminal kinase; MAP kinase, mitogen-activated protein kinase; MEK, MAP-ERK kinase; MTI, 3,4-dihydroxyphenylalanine-2,5-diphenyl tetrazolium bromide; FBS, fetal bovine serum; TBST, Tris-buffered saline containing 0.5% Triton X-100; TUNEL, terminal deoxynucleotidyltranserase-mediated diUTP nick end labeling; 8BrcAMP, 8-bromo-cAMP; GST, glutathione S-transferase.
thesis inhibitors (for review, see Ref. 13). They have also been proposed to be involved in the regulation of apoptosis, because, for example, overexpression of kinases that activate them resulted in the induction of apoptosis (14, 15), withdrawal of a growth factor (nerve growth factor) from PC-12 cultures can result in a transient activation of the p38 and JNK families (14), and inhibition of p38 MAP kinase activity can suppress apoptosis (16). Contrary to its role in cellular stress responses, it has recently been proposed that CSF-1 activates p38 activity in a myeloid cell line, and a role for this enzyme in hemopoietic cell proliferation was postulated (17). p38 also has a well-established role in cytokine production by lipopolysaccharide (LPS)-activated macrophages (18). However, there has been controversy as to whether CSF-1 is a strong stimulator of cytokine production in macrophages relative to stimuli such as LPS (for review, see Ref. 19). Reports from our laboratory and those of others have shown that CSF-1 treatment of monocytes and macrophages does not lead to high levels of secreted proinflammatory cytokine production (19, 20). It is therefore of interest to know whether CSF-1 is a potent stimulator of p38 activity in cells of the macrophage lineage and what the significance of such stimulation may be.

From the above background information, it is clear that the role of the MAP kinase family in cell survival and/or proliferation is cell type- and/or stimulus-specific. It has been maintained before (5) that the relevance of signal transduction pathways to the cell biology of a growth factor such as CSF-1 must be analyzed in each individual case and is best studied in cells where the receptor (c-fms) is normally expressed and preferably in primary cultures of such cells rather than in immortalized cell lines, where proliferation and survival pathways are, ipso facto, altered. We therefore have examined the roles of the MAP kinase family members, including potentially dependent pathways, in CSF-1-treated murine bone marrow-derived macrophages (BMM); because BMM depend upon CSF-1 for survival (1), we also examined the activities of the MAP kinase family members upon CSF-1 withdrawal. We report evidence for some involvement of ERK activity in CSF-1-stimulated BMM DNA synthesis but, interestingly, and in contrast to reports in fibroblasts ectopically expressing c-fms, no evidence for its involvement in the control of cyclin D1 or c-myc mRNA expression. p38 activity was weakly induced by CSF-1. No data were obtained to support a role for p38 or JNK-1 in BMM apoptosis resulting from CSF-1 withdrawal.

EXPERIMENTAL PROCEDURES

Cell Culture—Bone marrow-derived macrophages (BMM) were obtained by culturing bone marrow cells from femurs of CBA mice in the presence of CSF-1 (21). Cells were cultured in RPMI, 15% fetal bovine serum (FBS), 30% L-cell-conditioned medium (a source of CSF-1), routinely growth-arrested by washing three times in phosphate-buffered saline, and then cultured in RPMI, 10% FBS for 18 h or for times indicated.

Immunoprecipitations and Kinase Assays—Cells were cultured and stimulated as described in the text, then lysates were prepared using a Triton-based lysis buffer supplemented with protease and phosphatase inhibitors (11). Extracts were preclarified with 20 μl of protein A-Sepharose, and samples containing equal protein were immunoprecipitated at 4 °C overnight with 0.8 μg/ml anti-ERK-1 or anti-JNK-1 antibody or with 17 μg/ml anti-p38 antibody. Immune complexes were collected using 20 μl of protein A-Sepharose and washed with 4 × 1 ml of Triton-based lysis buffer (plus phosphatase inhibitors) and 1 × 1 ml of kinase assay buffer. ERK-1 kinase assays were performed as described elsewhere (11). p38 kinase assays were performed in 10 μl of 25 mM Hepes, pH 7.4, 10 mM MgCl₂, 1 mM vanadate, 20 mM NaF, 0.5 mg/ml myelin basic protein, [γ-³²P]ATP (adjusted to a specific activity of 3.7 × 10⁶ MBq/mmol). JNK-1 assays were performed after the addition of 10 μl of assay mix (without myelin basic protein) and 10 μl of c-Jun (1–169)-GST-agarose conjugate. Reactions were for 30 min at 30 °C and terminated by the addition of SDS sample buffer. Reaction mixtures were resolved by SDS-polyacrylamide gel electrophoresis, and the relative extent of substrate phosphorylation was determined using a Fujix BAS1000 phosphorimagerr.

The above antibodies were tested for specificity in our system by carrying out immunoprecipitations using BMM lysates and immunoblotting the other MAP kinase antibodies. By this criterion, none of the antibodies cross-reacted with members of the other MAP kinase families studied. The measured ERK-1 and p38 activities were proportional to lysate protein concentration and linear over 30 min. The JNK-1 activity immunoprecipitated under the conditions used was dependent on, but not strictly proportional to, lysate concentration and was completely abolished by 0.4 μg/ml peptide used as epitope to raise the antibody.

Western Analysis—Cells extracts in Triton-based lysis buffer containing equal amounts of protein were resolved by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose in 25 mm Tris, 192 mM glycin, 20% (v/v) methanol, and membranes were blocked for 1 h with 5% nonfat milk in Tris-buffered saline containing 0.5% Triton X-100 (TBST). Membranes were probed with relevant antibodies at 1:1000 in 0.5% nonfat milk, TBST, washed extensively with TBST, and probed with swine-anti-rabbit horseradish peroxidase (1:10000) in 0.5% milk, TBST. Bound secondary antibody was detected by enhanced chemiluminescence.

Protein Determinations—Protein content of cell extracts was determined by the method of Lowry (for review, see Ref. 19). The molecular weight of DNA was extracted (22) and analyzed by 2% agarose gel electrophoresis. Any apoptosis indicated by DNA laddering was confirmed by the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay and characteristic apoptotic morphology as described previously (23).

DNA Synthesis and Cell Viability—Tritiated thymidine ([³H]Tdr) incorporation was used as a measure of DNA synthesis as before (24). To measure cell viability, BMM were cultured in 24-well plates in RPMI, 10% FBS or RPMI, 15% FBS, 30% L-cell-conditioned medium. Inhibitors were added at indicated concentrations using Me₂SO as a vehicle at a final concentration of 0.5% (v/v). No effect on survival or DNA synthesis was noted with Me₂SO at this concentration. Viable cell numbers were then determined by incubation of cultures for 2 h in the presence of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.8 mg/ml). Reduced MTT dye was solubilized over night by the addition of 0.5 volumes of acidified 15% Triton X-100, and the amount was assessed at 595 nm using a Bio-Rad model 450 microplate reader.

mRNA Levels—Approximately 1 × 10⁶ cells/extraction were used. Cells were lysed in 600 μl of RNA extraction buffer (5 μM guanidine thiocyanate, 10 mM Tris pH 7.6, 10 mM EDTA), and RNA was extracted and probed as described before (24). cDNA probes to either c-fos or cyclin D1 were radiolabeled with [α-³²P]dATP (Brea) using nick translation and used to probe the blots. Control hybridization with a β2-microglobulin probe was used to determine RNA loading and quality.

Cytokines and Other Reagents—Human recombinant CSF-1 was a gift from Chiron Corp. (Emeryville, CA). Other reagents used were from the following commercial sources: LPS, purified from Escherichia coli strain 0111:B4, Difco; myelin basic protein, Sigma; [methyl-³H]thymidine (3Mq/mmol), Hybond C nitrocellulose, Enhanced Chemiluminescence (ECL) Western blotting detection reagents and Hyperfilm-ECL, Amersham Pharmacia Biotech; stabilized [γ-³²P]ATP (148 TBq/mmol), Bresatec (Adelaide, Australia); protase inhibitors, Roche Molecular Biochemicals; c-Jun-GST fusion protein was expressed in E. coli, strain MC1061, and purified by glutathione agarose chromatography. cDNA probes were as before (24, 25).

Antibodies and Inhibitors—The following antibodies were used for immunoprecipitations and in the kinase assays: anti-ERK-1 (C-16, Santa Cruz Biotechnology), anti-p38/CSBP (obtained from J. C. Lee; recognizes both CSBP1 and CSBP2). The following antibodies were used for Western blotting: anti-ERK-2 (K23, Santa Cruz), which recognizes both ERK-1 and -2), anti-JNK-1 (C17; Santa Cruz), and anti-phosphorylated ERK (New England Biolabs, which recognizes both pERK-1 and pERK-2). SB203580 (lot AJK-19080–74) and SKB202190 (lot 10148–25A) were gifts from Smith Kline Beecham (King of Prussia, PA) and Amgen Inc. (Boulder, CO), respectively. Secondary antibody-herosperoxidase conjugates were from DAKO (Glostrup, Den...
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TABLE I
Relative ERK-1 activities in BMM

Lysates were prepared from exponentially growing BMM cultures (Cycling), from cultures that were growth-arrested by 18 h of incubation in RPMI, 10% FBS (growth-arrested), and from the growth-arrested population stimulated with CSF-1 (5000 units/ml) or LPS (100 ng/ml) for 5 min and 15 min, respectively. 50 μg of precleared lysates were immunoprecipitated and assayed for ERK-1 activity ("Experimental Procedures"). Kinase activities are expressed relative to that found in cycling cells, and the data are mean values (±S.E.) for triplicate determinations.

| Treatment     | ERK-1 activity |
|---------------|----------------|
| Cycling       | 1 (0.2)        |
| Growth-arrested| 0.5 (0.2)      |
| CSF-1         | 13.8 (0.6)     |
| LPS           | 7.4 (0.7)      |

mark). PD98059 was from New England Biolabs Inc, Beverly, MA).

LPS Contamination of Culture Media and Reagents—All practical precautions were taken for minimizing endotoxin contamination. Media and cytokines were made using pyrogen-free water (Delta West Pty Ltd., Bentley, Western Australia), and endotoxin levels in media and cytokines were routinely monitored using the Limulus lysate assay (Commonwealth Serum Laboratories, Parkville, Australia).

RESULTS

ERK Activity in Cycling and Noncycling BMM—We have previously shown that addition of CSF-1 to noncycling BMM causes an increase in ERK-1 and ERK-2 activities with a peak activity at around 5 min (11). We now show in Table I that cycling BMM, i.e. BMM maintained in the presence of saturating concentrations of CSF-1, have an approximately 2-fold higher ERK-1 activity than factor-deprived BMM, suggesting that the large and transient increase measured when growth-arrested cells are stimulated by CSF-1 (Ref. 11 and Table I) is paralleled by an elevated level of activity in cycling versus growth-arrested BMM. LPS also stimulates ERK-1 activity in BMM via a kinetically distinguishable pathway (11). The activity of ERK-1 15 min after stimulation of growth-arrested cells with LPS is also shown for comparison. The observation that CSF-1-deprived BMM have a lower ERK-1 activity than cycling cells was verified by measuring the kinetics of loss of ERK-1 activity after CSF-1 withdrawal (Fig. 1a). In other experiments, we found that the level of ERK activity per unit cell protein was maintained between 24 and 36 h after withdrawal of CSF-1 from the culture medium (data not shown). It should be noted that in these experiments the ERK-1 activities were measured in lysate samples containing equal amounts of protein; furthermore, we verified that the ERK-1 activity was not affected by cell density in these experiments because a similar activity was measured in cycling cultures harvested at the beginning and at the end of the 18-h period (data not shown).

It is generally assumed that removal of growth factor from hemopoietic cells leads to cell death via apoptosis (26). We found that removal of CSF-1 from cycling BMM led to cell death accompanied by the DNA laddering that is often characteristic of apoptosis (Fig. 1b). We also observed apoptotic macrophages within phagolysosomes of neighboring cells, which is to be expected given the known phagocytic activity of macrophages toward cells undergoing apoptosis. This removal makes quantification of apoptotic BMM in such cultures difficult; however, quantification of the morphologic and histological characteristics of late stage apoptosis both by bisbenzimide or TUNEL staining (“Experimental Procedures”) indicated that there were approximately 0.3% apoptotic BMM in cycling BMM cultures with this proportion increasing to 8–10% after 24 h of growth factor removal (data not shown). These data indicate that at least a proportion of the CSF-1-deprived BMM are dying via an apoptotic pathway.

It has previously been shown in nerve growth factor-treated PC-12 cells, for example, that growth factor withdrawal results in loss of ERK activity and cell death by apoptosis, leading to the suggestion that ERK activity is important for growth factor-mediated cell survival (14, 27). A specific inhibitor of MEK activation, PD98059, is widely used to determine the relevance of a MEK/ERK pathway to cellular function (28); we have found previously that a 30-min preincubation of BMM with this compound before stimulation with CSF-1 inhibited the activation of both ERK-1 and ERK-2 with an IC50 of 5–10 μM (Ref. 12 and data not shown). We therefore determined whether PD98059 would reverse the ability of CSF-1 to suppress apoptosis in BMM. However, it can be seen in Fig. 2 that the inhibitor of MEK activation did not do this, suggesting that ERK activity per se is not critical for the maintenance of BMM viability by CSF-1. In support of this we also found that incubation of BMM with 50 μM PD98059 did not accelerate the loss of viability (measured using MTT dye reduction) following CSF-1 with-
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Fig. 2. Effect of PD98059 and 8BrcAMP on the reversal of BMM apoptosis by CSF-1. Low molecular weight DNA was isolated from growth-arrested BMM restimulated for 24 h with CSF-1 (5000 units/ml) in the absence or presence of PD98059 (50 μM), 8BrcAMP (1 mM), or their combination, as indicated. DNA was separated by electrophoresis on a 2% agarose gel then visualized by UV light and photographed. The figure is a representative of four separate experiments.

We also examined the effect of 8BrcAMP, an agent that can suppress a number of CSF-1-induced responses in BMM while activating the ERK activity (12, 24). We see in Fig. 2 that 8BrcAMP by itself cannot prevent the reversal of apoptosis by CSF-1 but interestingly, when added with PD98059, can do so. This finding suggests that multiple pathways may be governing the maintenance of BMM viability by CSF-1 and that at least one of these pathways is ERK-dependent (see below).

We have found previously that expression of ERK activity in BMM in response to a particular stimulus did not always correlate with the subsequent appearance of a DNA synthesis response (11). This observation does not necessarily mean that ERK activity is not required for DNA synthesis to occur in BMM, especially because we have shown above (Table I; Fig. 1a) that ERK-1 activity in cycling BMM was higher than that in CSF-1-deprived cells. To test this possibility directly we examined the effect of PD98059 on DNA synthesis in CSF-1-treated BMM. We see in Table II that PD98059 inhibited CSF-1-stimulated DNA synthesis in BMM measured 20 h after the addition of both CSF-1 and the inhibitor to growth-arrested BMM, suggesting that a MEK/ERK pathway may play a role in DNA synthesis control in this system.

MEK/ERK-dependent Pathways in CSF-1-treated BMM—Previous studies in CSF-1-treated macrophages have examined the pathways governing CSF-1-dependent activation of ERK activity (29, 30); pathways downstream of the enhanced ERK activity that may be involved in, for example, the control of BMM DNA synthesis by CSF-1 have not been widely examined. Much evidence has implicated both cyclin D1 and c-Myc as important regulators of the mammalian cell cycle (31), including in CSF-1-treated macrophages (25, 32–34); cyclin D1 and c-myc transcription have been shown to be dependent upon upstream ERK activity in a number of cell systems, including CSF-1-stimulated NIH3T3 cells ectopically expressing c-Fms (see Ref. 10 and references therein). We therefore examined whether PD98059 suppresses cyclin D1 and c-myc mRNA expression as part of its inhibitory action in controlling CSF-1-stimulated BMM DNA synthesis. Our data, however, show that PD98059, even at concentrations as high as 100 μM, failed to inhibit the increase in cyclin D1 and c-myc mRNA expression resulting from stimulation of BMM with CSF-1 (Fig. 3a); in contrast, 8BrcAMP did, as we and others have previously reported (33–35). These results contrast with our earlier findings that CSF-1-stimulated c-fos mRNA expression was inhibited by PD98059 (Fig. 3b (12)). These data suggest that a MEK/ERK pathway does not govern CSF-1 stimulated cyclin D1 mRNA and c-myc mRNA expression in macrophages and that regulation of the levels of these mRNA species is different from that found in CSF-1-stimulated NIH 3T3 cells (10) (see “Discussion”). The above data indicate that, unlike 8BrcAMP, PD98059 does not suppress cyclin D1 or c-myc mRNA expression as part of its mechanism in inhibiting DNA synthesis in CSF-1 treated BMM.

Table II

| Additions | [³H]TdR incorporation (cpm/well × 10⁻⁴) |
|-----------|----------------------------------------|
| Control   | 0.25 (0.09)                            |
| CSF-1     | 22.0 (2.6)                             |
| CSF-1 + PD 98059 | 9.6 (1.9)               |

Fig. 3. Effect of inhibitors of ERK and p38 pathways on CSF-1-stimulated mRNA expression. a, total RNA was isolated from growth-arrested BMM or from BMM restimulated for 6 h with CSF-1 (5000 units/ml) in the absence or presence of PD98059 (100 μM) or 8BrcAMP (1 mM), as indicated. The three autoradiograms represent the same Northern blot hybridized successively with radiolabeled cDNA fragments of cyclin D1, c-myc, and (as a control for RNA loading) β2-microglobulin. b, total RNA was isolated from CSF-1-deprived BMM or from BMM restimulated for 30 min with CSF-1 (5000 units/ml) in the absence or presence of PD98059 (100 μM) or SB202190 (10 μM), as indicated. The two autoradiograms represent the same Northern blot hybridized successively with radiolabeled cDNA fragments of c-fos and β2-microglobulin, the latter as a control for RNA loading.

p38 Activity in Cycling and Noncycling BMM—p38 activity has been implicated in the control of cytokine biosynthesis in LPS-stimulated monocytes/macrophages (18). Activation of p38 by CSF-1, measured by its tyrosine phosphorylation status on a Western blot, has recently been reported in a murine myeloid cell line (17). Given the evidence that CSF-1 is a poor stimulator of monocyte/macrophage inflammatory cytokine production when compared with LPS (Ref. 20 and references cited in Ref. 19), we decided to compare p38 activity directly in BMM stim-
In PC-12 cells, a 2–3-fold elevation in p38 activity preceded the onset of apoptosis after nerve growth factor withdrawal (14). We therefore followed p38 activity in macrophages deprived of CSF-1 over an 18-h period, a time frame in which, given the time of onset of apoptosis in BMM (Fig. 1b), we might expect by analogy (14) to see increases in p38 activity. We show, however, that there is no significant elevation of p38 activity following CSF-1 withdrawal from cycling BMM (Fig. 4b). Similar results were obtained using the CSF-1-dependent macrophage cell line, BAC1.2FS (data not shown). In BMM, no elevation in the p38 activity was noted if its activity was monitored between 30 min and 3 h after CSF-1 removal (data not shown). What we found instead is a loss of p38 activity compared with the value for the cycling cells. It should be noted that the p38 activities are expressed relative to cell protein in each lysate and that they were not affected by cell density in these experiments because similar activities were measured in cycling cultures harvested at the beginning and at the end of the 18-h period (data not shown). Therefore, for both primary cultures of macrophages and a CSF-1-dependent macrophage cell line, withdrawal of CSF-1 results in a decrease in p38 activity with no indication of the involvement of p38 activation in the cell death that occurs when the CSF-1 is removed.

To further test the above conclusion, we examined the effect of specific inhibitors of this kinase on BMM survival. The phenolic triaryl imidazole derivatives SB203580 and SKB202190 have been shown to inhibit cytokine production in human monocytes by binding selectively to and inhibiting the enzymatic activity of p38 (18, 36); they have been widely used in the investigation of the biological functions of p38 (28). As a prelude to studying their effects on macrophage survival, we tested the inhibitory activity and selectivity of these compounds against p38 versus ERK-1 isolated from murine macrophages. SKB202190 strongly inhibited p38 activity immunoprecipitated from BMM with an IC_{50} of about 45 nM (data not shown). By contrast, there was no effect on ERK-1 activity at concentrations as high as 0.5 μM. Although the IC_{50} for the inhibition of murine p38 was slightly higher than the 10 nM reported for human p38, there is still a strong selectivity with respect to the murine p38 member(s) of the MAP kinase family. This result also confirms the specificity of the kinase assays used above (“Experimental Procedures”). The effect of SKB202190 on BMM survival was assessed using an MTT dye reduction assay. No significant effect on loss of viability induced by CSF-1 withdrawal, assessed by MTT dye reduction, was observed when the macrophages were cultured for up to 2 days in the presence of 10 μM SKB202190 (Fig. 4c). Similar results were obtained using SB203580 where we measured in vitro an IC_{50} for murine p38 of about 20 nM (data not shown). These data reinforce the conclusion arrived at above that p38 activity is not required for macrophage cell death under conditions of growth factor deprivation. When the BMM were cultured in the presence of SKB202190, administered under the same conditions as the viability experiments, the cells became highly vacuolated after 24 h, consistent with a role for p38 in oarsoregulation and indicating that, although viability was not significantly affected, the drug is still active throughout the time frame of the experiment.

Because cycling BMM have approximately 2-fold higher p38 enzymatic activity per unit cell protein than growth-arrested BMM (Fig. 4a), we measured the effect of SKB202190 on BMM DNA synthesis. The data presented in Fig. 5 show that there was an inhibitory effect of this compound on the incorporation of [3H]TdR into DNA. This effect was consistently observed at relatively high concentrations, and in five independent experiments, inhibition of [3H]TdR incorporation occurred with an IC_{50} of 4–5 μM. This high IC_{50} is comparable with what has been reported for DNA synthesis inhibition in other murine cell lines.
The actions of PD98059 differ from those of another inhibitor of CSF-1-stimulated BMM DNA synthesis, albeit a more potent one, namely 8BrCAMP (12). This cAMP analogue blocked the CSF-1-stimulated cyclin D1 and c-myc mRNA expression (Refs. 25 and 34; Fig. 3a) while raising c-fos mRNA expression and ERK activity (12, 24). However, 8BrCAMP, like PD98059, could not prevent the reversal of BMM apoptosis by CSF-1 (Fig. 2); interestingly, the combination of the two agents did so, suggesting perhaps they control complementary pathways emanating from the stimulated CSF-1 receptor, which are each important for cell survival and possibly for other cellular responses. We have found before that 8BrCAMP inhibited CSF-1-stimulated Ras activity (12), raising once again the possibility of the presence of a Ras-independent pathway controlling ERK activity in macrophages (5); perhaps enhanced Ras activity but not ERK activity is relevant for subsequent cyclin D1 mRNA or c-myc mRNA expression in CSF-1-stimulated macrophages.

p38 MAP kinase has been shown in a variety of cell systems to be strongly activated by stress signals such as osmotic shock, UV light, LPS, and inflammatory cytokines but to be activated only weakly by growth factors (see Refs. 13 and 27 for reviews). p38 is known to be involved in the control of cytokine production (e.g. TNFa in LPS-activated macrophages (18)). Some reports but not others have found that CSF-1 is a relatively poor stimulus of inflammatory cytokine synthesis (e.g. TNFa, interleukin-1) in monocytes/macrophages in vitro (19, 20, 38–40). However, it has been reported that CSF-1 causes an increase in tyrosine phosphorylation in p38 immunoprecipitates in the myeloid cell line, FD-MACII cells (17). This type of analysis does not measure enzymatic activation nor give any indication as to the degree of activation of this enzyme. We therefore decided to measure p38 activity by a specific and quantitative immunoprecipitation kinase assay in cultures of primary macrophages and to compare directly the activity induced by CSF-1 treatment to that resulting from the action of LPS. Our data show that in BMM the stimulation by CSF-1 of p38 enzymatic activity is very small compared with that stimulated by LPS (Fig. 4c) and is kinetically distinguishable from the latter (data not shown). These findings are entirely consistent with the reports from our laboratory and those of others that show that CSF-1 is a weak stimulator of cytokine biosynthesis in monocytes and macrophages compared with LPS and offer an explanation for these findings. The kinetics of p38 activation by CSF-1 and LPS are similar to those that we, and others, have previously observed for ERK-1 activation in the same cells and by the same stimuli (11), although there are
clear differences in the extent of activation. Thus CSF-1 activates both enzymes maximally at 5 min in growth-arrested cells, whereas LPS activates them maximally at 15 min; furthermore, LPS is a strong activator of both MAP kinase family members, whereas CSF-1 is a weak activator of the p38 enzyme relative to its effects on the ERK isozymes. Whether the activation of these enzymes by CSF-1 and LPS represents activation of the same intracellular pools is not clear, but in this context it is of interest that a recent report has shown that LPS preferentially activates a minor microtubule-bound subpopulation of MAP kinase (41).

The p38 pathway has been implicated to play a critical role in apoptosis, because many apoptotic signals are able to stimulate p38 activity and p38 activation is correlated with the induction of apoptosis in several cell types (14–16); however, others have found no correlation between its activation and apoptosis in other cell types and even found some activation by growth factors (see for example Refs. 17, 42). Inhibition of p38 activity has been shown to suppress apoptosis in some cases (see for example Ref. 16) but not in others (43) and to even induce apoptosis (44). In BMM (Fig. 4b) and the macrophage cell line BAC.1.2F5 (data not shown), we found a gradual decrease in p38 activity following CSF-1 withdrawal; furthermore, SKB202190 failed to inhibit the decrease in BMM viability (Fig. 4c). Our data, therefore, do not support a role for p38 family members, at least those that are susceptible to pyridinium imidazole inhibition, in macrophage cell death resulting from growth factor deprivation and indicate that CSF-1 does not maintain cell viability by suppressing p38 activity. The significance of the elevated p38 activity in cycling BMM awaits clarification. p38 activity may play a permissive role in CSF-1-driven macrophage proliferation because SKB202190 blocked CSF-1-stimulated BMM DNA synthesis, and our preliminary data suggest that it inhibits the induction of c-myc mRNA but not cyclin D1 mRNA; however, it should be noted that the concentrations required were much higher than those needed to inhibit the in vitro BMM p38 activity, suggesting that the drug might have alternative targets in BMM at the higher concentrations.

JNK activity in various cell types can be enhanced by a similar range of stress signals as p38 activity and be weakly activated by growth factors (13). We could find no evidence for an activation of JNK-1 activity in CSF-1-treated BMM above the very low levels found in the untreated population. In contrast, LPS and TNFα both gave significant increases; LPS has been shown previously to stimulate JNK activity in macrophages (45). In BMM we found no detectable increase in JNK activity as they proceeded to the apoptotic state and, therefore, no support for its role in the control of apoptosis in these cells.

In summary, the lack of association of MAP kinase family members with apoptosis and/or survival in the macrophages again highlights the need to explore the relevance of a signaling pathway to cellular function for each particular cell type; also, the atypical evidence presented above that ERK activity may not be controlling cyclin D1 mRNA and c-myc mRNA expression in CSF-1-stimulated BMM but does so in CSF-1-treated NIH 3T3 cells (cf. present paper and Ref. 10) again indicates that CSF-1 biology and associated signal transduction pathways are best studied in myeloid cells (5). The relatively weak stimulation of p38 activity by CSF-1 compared with that resulting from LPS action contrasts some actions of CSF-1 (a survival and growth factor) with those of LPS (a stress stimulus) and is consistent with some reports (19, 20), but not others (38–40), showing that CSF-1 is a relatively poor stimulator of monocyte/macrophage inflammatory mediator production.