Differences in the kinetics of activation of protein kinases and extracellular signal-related protein kinase 1 in colony-stimulating factor 1-stimulated and lipopolysaccharide-stimulated macrophages

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To determine the relevance of mitogen-activated protein kinase activity to macrophage proliferation, we measured the stimulation of myelin basic protein (MBP) kinase and extracellular signal-related protein kinase (ERK) activity in a macrophage cell line (BAC1.2F5), bone marrow-derived macrophages (BMM) and resident peritoneal macrophages (RPM). By using an 'in-gel' MBP kinase assay the activities of renaturable MBP kinases were detected, including several with molecular masses similar to those of ERK-1 and ERK-2. These represented a minor fraction of total activity and were not activated to an appreciable extent

by colony-stimulating factor 1 (CSF-1). By using a sensitive and specific immune-complex kinase assay, activation of ERK-1 by CSF-1 and lipopolysaccharide (LPS) was demonstrated. Two kinetically distinct pathways of ERK-1 activation by CSF-1 were resolved, with peak activations occurring at 5 and 15 min. The kinetics and degree of activation were similar in BMM, BAC1.2F5 cells and RPM. LPS activated ERK-1 with a single peak at 10–15 min, corresponding to the later peak of activation by CSF-1. Thus there was no strict correlation between ERK activation and macrophage proliferation.

INTRODUCTION

The mitogen-activated protein (MAP) kinase family members extracellular signal-related protein kinase (ERK)-1 and ERK-2 are activated in response to mitogenic stimulation in many cell types. Their specific role has been linked to the time course of their activation. Rapid activation of the Ras/ERK pathway is associated with proliferation in granulocyte colony-stimulating factor (CSF)-responsive cell lines [1]. In PC12 cells, prolonged activation induced by nerve growth factor is correlated with cellular differentiation, whereas rapid and transient activation after epidermal growth factor treatment is correlated with cellular proliferation [2]. Studies in fibroblast cell lines show that proliferation is correlated with prolonged activation [3,4]. It is therefore clearly important to study the time course of that activation in detail to understand the implications of MAP kinase activation for a given cell.

Macrophage colony-stimulating factor (M-CSF or CSF-1) stimulates the survival, proliferation and differentiation of cells of the monocyte/macrophage lineage via its high-affinity receptor encoded by the *c-fms* proto-oncogene [5]. To progress completely through the cell cycle, macrophages need stimulation with CSF-1 throughout most of the G₁ phase [6]. CSF-1 stimulation results in a rapid increase in the expression of immediate early genes, such as *fos* and *jun* (reviewed in [7]), which encode transcription factors that are assumed to activate other genes necessary for cell cycle progression. Although there have been several studies showing that MAP kinases are activated in macrophages or macrophage-derived cell lines by a variety of stimuli [8–14], there is still doubt as to the pathways that result in this activation. Xu et al. [14] have reported that CSF-1 activates protein kinase C- δ in the BAC1.2F5 cell line, which in turn activates the Raf-1/MEK/ERK pathway (where MEK is MAP kinase or ERK-

activating kinase). However, using the same cell line Reimann et al. [15] and Büscher et al. [10] showed that CSF-1 induces this pathway in a protein kinase C-independent manner, and other workers [16] have reported that protein kinase C- δ is not activated by CSF-1. Again, it has been reported that CSF-1 can activate phosphatidylcholine-specific phospholipase C in BAC1.2F5 cells [16] and that activation of this enzyme accompanies MAP kinase activation. However, Büscher et al. [10] reported that inhibition of phosphatidylcholine-specific phospholipase C had no effect on CSF-1-mediated MAP kinase activation. Experiments with genetically manipulated macrophage cell lines have also been inconclusive: v-Raf-transfected BAC1.2F5 cells proliferate in a CSF-1-independent manner with constitutive expression of immediate early genes, but MAP kinase is not activated [17]. Expression of dominant negative Ras in the same cell line does not affect CSF-1-dependent Raf-1 activation, but partly affects CSF-1-dependent MEK1 and MAP kinase activation [10]. Why Raf-1 activity is unaffected by dominant-negative Ras but MAP kinase activity is decreased is not clear.

The above studies have in general used myelin basic protein (MBP) kinase activity in gel-denaturation assays and/or measured the degree of tyrosine phosphorylation as a measure of ERK activity. Our aim is to determine what kinase(s), including ERKs, might be relevant in macrophages for growth control and other functions. After initially using total MBP kinase and 'in-gel' kinase assays to study the activation of protein kinases including the ERKs, we examined the time course of activation of ERK-1 in detail with a specific immune precipitate kinase assay. This assay allowed us to measure the activation of ERK-1 unambiguously, and in a detailed and quantitative manner, which is important given the correlations mentioned above for the kinetics of ERK activation and subsequent physiological

Abbreviations used: BMM, bone marrow-derived macrophages; CSBP, cytokine suppressive anti-inflammatory drug (CSAID)-binding protein ('p38'); CSF, colony-stimulating factor; ERK, extracellular signal-related protein kinase; GM-CSF, granulocyte/macrophage colony-stimulating factor; LPS, lipopolysaccharide; MAP, mitogen-activated protein; MBP, myelin basic protein; MEK, MAP kinase or ERK-activating kinase; RPM, resident peritoneal macrophages.

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response. We report that ERK-1 is activated markedly by CSF-1 in both cycling and poorly cycling macrophages by two kinetically distinct pathways; activation by lipopolysaccharide (LPS), albeit delayed, also dissociates ERK-1 activation from that of DNA synthesis.

MATERIALS AND METHODS

Cell culture

Bone marrow cells were obtained from femurs of CBA mice by aspiration. Bone marrow-derived macrophages (BMM) were prepared essentially as described [18]. Resident peritoneal macrophages (RPM) were collected by lavage of the peritoneal cavity with sterile Ca^{2+} /Mg $^{2+}$ -free PBS. BAC 1.2F5 cells, obtained from C. Sherr, were passaged in Dulbecco's Modified Eagle's Medium containing 15% (v/v) fetal calf serum, 20% (v/v) L-cell conditioned medium, 50 i.u./ml penicillin and 50 µg/ml streptomycin. BMM and BAC1.2 F5 cells were rendered quiescent before each experiment by culturing for 18–22 h in their respective media without L-cell conditioned medium [18].

Cytokines and other reagents

Human recombinant CSF-1 used throughout this study was a gift from Chiron (Emeryville, CA, U.S.A.). Recombinant mouse granulocyte/macrophage colony-stimulating factor (GM-CSF) was provided by Dr A. Burgess (Ludwig Institute for Cancer Research, Parkville, Australia). Recombinant mouse interferon- γ was provided by Hoffmann LaRoche (Tokyo, Japan). Other reagents were from the following commercial sources: LPS purified from *Escherichia coli* strain 0111:B4, Difco (Detroit, MI, U.S.A.); phorbol 12-myristate 13-acetate and MBP, Sigma (St. Louis, MO, U.S.A.); [γ - ^{32}P]ATP, Hybond C nitrocellulose, enhanced chemiluminescence (ECL) Western blotting detection reagents and Hyperfilm-ECL, Amersham (Sydney, Australia); ST-1 peptide, Protein A-Sepharose, Pharmacia LKB Biotechnology (Uppsala, Sweden); Sartobind Minisart anion-exchange filters, Sartorius (Göttingen, Germany).

Antibodies

Anti-phosphotyrosine monoclonal antibody, PY20-horseradish peroxidase conjugate, was obtained from ICN Biomedicals (Costa Mesa, CA, U.S.A.). Antibodies directed against rat-ERK-1 residues 305–327 (K-23), used for Western blotting, and against rat ERK-1 residues 352–367 (C-16), used for ERK-1-selective immunoprecipitations, were obtained from Santa Cruz Biotech (Santa Cruz, CA, U.S.A.). Rabbit antibodies directed against the C-terminal 12 amino acid residues of mouse Raf-1 was the gift of Norman Polio (Ludwig Institute for Cancer Research, Parkville, Australia).

Preparation of extracts for MBP kinase assays

Cultures of quiescent BMM were treated as indicated then rinsed twice with ice-cold PBS and scraped into ice-cold extraction buffer [50 mM Hepes (pH 7.4)/10 mM MgCl_2 /2 mM EDTA/10 mM NaF/1 mM dithiothreitol/50 mM sodium glycerophosphate/1 mM PMSF/1 mM benzamidine/0.1 mM sodium vanadate/10 µg/ml aprotinin/0.5 µg/ml leupeptin/0.7 µg/ml pepstatin A]. Cell suspensions were lysed by repeated passage through a 25-gauge hypodermic needle; then the extracts were centrifuged at 13000 *g* for 5 min. Supernatants were assayed for MBP kinase activity as described by Sturgill et al. [19].

Immunoprecipitation of tyrosine-phosphorylated proteins and immunodetection of MAP kinases

Quiescent BMM were treated as described in the text, then lysed in Triton lysis buffer [25 mM Hepes (pH 7.4)/137 mM NaCl/1% (v/v) Triton X-100/10% (v/v) glycerol/1 mM EDTA/0.1 mM sodium pervanadate/1 mM PMSF/0.5 µg/ml leupeptin/0.7 µg/ml pepstatin A/10 µg/ml aprotinin]. Samples of lysate containing equal amounts of protein were then incubated overnight with PY20 (1:10000) and then 20 µl of Protein A-Sepharose. The Sepharose beads were washed five times in lysis buffer, then boiled in 50 µl of SDS sample buffer. Proteins were resolved by SDS/PAGE with 10% (w/v) polyacrylamide resolving gels, transferred to nitrocellulose, and then probed with PY20-horseradish peroxidase conjugate (1:10000). The filter was then stripped and reprobed with anti-ERK antibody (1:10000).

'In gel' kinase assay

Cell extracts prepared as described for immunoprecipitations were resolved by SDS/PAGE with 10% (w/v) polyacrylamide gels co-polymerized with 0.5 mg/ml MBP and assayed for MBP kinase activity essentially as described [20].

MBP kinase activity of anti-ERK immunoprecipitates

Cells, cultured in six-well plates (Nunc, Roskilde, Denmark), were rendered quiescent, then treated in triplicate as described in the text. Extracts were prepared in 0.1 ml of Triton lysis buffer, then precleared with 20 µl of Protein A-Sepharose. Precleared extracts were immunoprecipitated overnight at 4 °C with 2 µg/ml anti-ERK-1. The immune complexes were collected by using 20 µl of Protein A-Sepharose, washed three times in lysis buffer, then assayed for MBP kinase activity for 30 min at 30 °C after the addition of 20 µl of MBP (final concentration 0.4 mg/ml) and 10 µl of kinase assay buffer [30 mM Hepes (pH 7.4)/15 µM ATP/25 µCi/ml [γ - ^{32}P]ATP/15 mM MgCl_2 /2 µM protein kinase A inhibitor/10 µM calmidazolium]. Phosphorylation of MBP was assessed after SDS/PAGE either by phosphorimaging or autoradiography and laser densitometry.

Ion-exchange chromatography of MBP kinases

Extracts were prepared from control BMM cultures and from cells that had been stimulated with 5000 i.u./ml CSF-1 for 4 min. Samples (1 ml) containing 2 mg of protein were applied on a Sartobind Minisart anion-exchange filter equilibrated in 50 mM β -glycerophosphate (pH 7.3)/1 mM EGTA/0.1 mM sodium vanadate/0.5 mM NaF/1 mM dithiothreitol (buffer A). The flow through was collected; the filter was washed with 2 ml buffer A and then eluted with a stepwise gradient of 0–1 M NaCl in buffer A. Samples of each fraction were resolved by SDS/PAGE and analysed by Western blotting with anti-ERK antibody. Filters were stripped and reprobed with anti-phosphotyrosine antibody.

RESULTS

MBP kinase activation is not closely correlated with macrophage proliferation

We initially measured the kinetics of MBP kinase activity in BMM stimulated with CSF-1, a cell population that proliferates

Figure 1 Stimulation of MBP kinase activity of BMM by CSF-1 and LPS

Quiescent BMM were treated with CSF-1 (5000 i.u./ml, ■) or LPS (100 ng/ml, ○) for the indicated times, then assayed for MBP kinase activity. Each point represents the mean $\pm$ S.E.M.

Table 1 MBP kinase activity is stimulated by CSF-1 in both BMM and RPM

BMM and RPM (approx. 5×10^6 cells) cultured in 10 cm tissue culture dishes were stimulated with CSF-1 (5000 i.u./ml) for 0 or 5 min, then lysed. Samples of lysate containing equal amounts of protein were analysed in triplicate for MBP kinase activity. Results are normalized to the extent of phosphorylation found in control cultures. The results from two sets of independent cultures are presented as means $\pm$ S.E.M. The level of CSF-stimulated MBP kinase activity differed between BMM and RPM by less than 20% in both experiments.

	MBP kinase activity (normalized to control)	
	RPM	BMM
Experiment 1		
Control	1 $\pm$ 0.44	1 $\pm$ 0.26
CSF-1	3.9 $\pm$ 0.091	2.5 $\pm$ 0.038
Experiment 2		
Control	1 $\pm$ 0.080	1 $\pm$ 0.037
CSF-1	1.78 $\pm$ 0.17	2.06 $\pm$ 0.066

well on addition of this growth factor. CSF-1 rapidly activated MBP kinase activity approx. 3-fold (Figure 1, ■). The dose-response curve, measured at 5 min, was similar to that for CSF-1-induced DNA synthesis [21] and other CSF-1-stimulated early events such as activation of Na^+/K^+ ATPase [18]. To analyse the possible relation between this activity and macrophage proliferation, we examined their correlation under a variety of circumstances. BMM were treated with several non-mitogenic agents known to have potent effects on macrophages. Of these, LPS (100 ng/ml) gave rise to a later peak of activity (at approx. 10–30 min) than that obtained after CSF-1 stimulation (see Figure 1, ○). No activation at any time up to 30 min was observed with the following treatments: 8-Br-cAMP (1 mM), interferon α (100 i.u./ml), interferon γ (1000 i.u./ml) or phorbol 12-myristate 13-acetate (100 nM). CSF-1 stimulated MBP kinase activity to a similar extent in a poorly proliferating mouse macrophage population, i.e. RPM [22], as in BMM (Table 1). Taken together these results show that activation of MBP kinase is not a general phenomenon but occurs in response to specific macrophage stimuli, with differing kinetics between stimuli, and

Figure 2 Analysis of ERK activation by measurement of tyrosine phosphorylation and changes in electrophoretic mobility

(a) Lysates were prepared from quiescent BMM stimulated with CSF-1 (5000 i.u./ml) for 4 min. Extracts were resolved with a Sartobind Minisart anion-exchange filter as described in the Materials and methods section. Samples from the indicated fractions were either assayed for MBP kinase activity or resolved by SDS/PAGE, transferred to nitrocellulose and immunoblotted sequentially with anti-phosphotyrosine antibody and anti-ERK antibody. (b) Quiescent cultures of BMM were treated with CSF-1 (5000 i.u./ml) for 0 or 4 min. Extracts containing equal amounts of protein were immunoprecipitated with anti-phosphotyrosine antibody. Immunoprecipitated proteins were resolved by SDS/PAGE [10% (w/v) gels], transferred to nitrocellulose, then analysed by immunoblotting with anti-phosphotyrosine antibody (left panel; lanes labelled 0 and 4' refer to 0 and 4 min of treatment with CSF-1 respectively). The filter was stripped and then reprobed with an anti-(MAP kinase) antibody that recognizes both ERK-1 and ERK-2 (right panel).

that there is no correlation between this activation and DNA synthesis in macrophages.

Analysis of MBP kinases in macrophages by anion-exchange chromatography and an 'in-gel' kinase assay

We used anion-exchange chromatography to resolve the enzymes responsible for the MBP kinase activity in BMM extracts. Two broad peaks of MBP kinase activity were resolved (Figure 2a) that co-chromatographed with both ERK-1 and ERK-2. On stimulation, the ERK-1 isoform migrated as a single band on SDS/PAGE, whereas ERK-2 migrated as a closely spaced doublet. The ERK-1 isoform and the upper band of the ERK-2 doublet co-migrated with bands visible in the anti-phosphotyrosine blot (results not shown). The presence of unphosphorylated ERK-2 shows that this particular isoform is not completely activated after CSF-1 treatment of BMM. Because we did not resolve tyrosine-phosphorylated ERK-1 from its unphosphorylated form, we cannot make any conclusions about the extent of activation of this isoform from these results. An

Figure 3 Analysis of renaturable MBP kinases by 'in-gel' assays

Extracts (20 μ g) prepared from quiescent BAC1.2F5 cells (**a**) or RPM (**b**) stimulated with CSF-1 (5000 i.u./ml) for the indicated times were resolved by SDS/PAGE with gels co-polymerized with MBP (0.5 mg/ml), then assayed for MBP kinase activity after renaturation of proteins.

increase in ERK-1 and ERK-2 immunoreactivity in anti-phosphotyrosine immunoprecipitates on stimulation of BMM with CSF-1 for 4 min indicates either an increase in tyrosine phosphorylation of the ERKs themselves or an increased association of ERKs with tyrosine-phosphorylated proteins (Figure 2b). Given the results of the anti-pTyr immunoblotting of the column fractions, at least part of this increase is directly attributable to tyrosine phosphorylation of the ERKs. The results in Figure 2(b) also suggest a similar extent of activation of the two isoforms by CSF-1.

An 'in-gel' MBP kinase assay [20] has been used to monitor MAP kinase activity, and bands of molecular mass 40–45 kDa revealed by this assay have been assumed in general to represent ERK activity. We used this assay in an attempt to verify that ERKs are enzymically activated. Our results show that in two macrophage populations that survive and proliferate in response to CSF-1, namely the BAC1.2F5 cell line (Figure 3a) and BMM (results not shown), a number of CSF-1-responsive MBP kinase bands appeared in the 35–45 kDa region; however, bands with a molecular mass expected for the MAP kinase family members [ERKs and cytokine suppressive anti-inflammatory drug (CSAID)-binding protein ('p38') (CSBP)] were minor components of the total MBP kinase activity in the renatured gel. Of the major bands observed with this technique, a kinase with subunit molecular mass 35 kDa was stimulated by CSF-1 treatment in BAC1.2F5 cells (Figure 3a). In RPM, bands at approx. 40–45 kDa were constitutively active, whereas the 35 kDa kinase was absent (Figure 3b). Other kinases with higher molecular masses seem to be activated by CSF-1, particularly a kinase of approx. 50 kDa that was consistently activated in these experi-

Figure 4 Analysis of ERK-1 activity in CSF-1- and LPS-stimulated macrophages

Extracts were prepared from BAC1.2F5 cells (**a**), RPM (**b, c**) or BMM (**d**) stimulated with either CSF-1 (5000 i.u./ml; **a, b, d**) or LPS (100 ng/ml; **c**) for the indicated times. Extracts were immunoprecipitated with anti-ERK-1 antibody or anti-Raf-1 antibody (**a**, inset); the immunoprecipitates were washed and then assayed for MBP kinase activity [or phosphorylation of catalytically inactive MEK–GST fusion protein (**a**, inset)]. Each point represents the mean $\pm$ S.E.M. for triplicate cultures. The results are representative of at least three experiments for each case.

ments (Figure 3b). Immunodepletion with anti-ERK antibodies at 5 μ g/ml failed to decrease the intensity of any of the bands observed (results not shown). This observation, coupled with the facts that (1) several bands of activity are present in macrophage extracts in the molecular-mass range 40–45 kDa and (2) activity in this range is a relatively small proportion of the total renaturable MBP kinase activity, prompted us to investigate other methods of measuring ERK activation.

Measurement of ERK activity with an immunoprecipitate kinase assay

Lysates prepared from CSF-1-stimulated BAC1.2 F5 cells were immunoprecipitated with a commercial antibody selective for ERK-1. The immunoprecipitates were washed, then assayed for MBP kinase activity as described in the Materials and methods section. Control experiments verified that activity was (1) dependent on the concentration of immunoprecipitating antibody, (2) time-dependent and (3) completely blocked by the addition of 15 μ M peptide to which the antibody was raised during the immunoprecipitation. In addition, under the conditions used in these experiments, the anti-ERK-1 antibody did not immunoprecipitate either ERK-2 or CSBP, as verified by immunoblotting of anti-ERK-1 immunoprecipitates. Using this assay, we measured activation of ERK-1 in detail in BAC 1.2F5, BMM and RPM cultures stimulated with CSF-1 for 0–30 min. CSF-1 stimulation of BAC1.2F5 cells (Figure 4a), RPM (Figure 4b) and BMM (results not shown) caused a broad biphasic activation of ERK-1 between 5 and 15 min, with activity returning to near-basal levels after 30 min. The degree of activation and its kinetics are consistent with those of Raf-1 (see Figure 4a, inset). It should be noted that the stimulation revealed by this assay (12–15-fold) is much higher than that achieved with the MBP kinase assay and also allowed resolution of the broad peak of MBP kinase activation in CSF-1-treated BMM (Figure 1).

As found with the MBP kinase assay (Figure 1), LPS stimulated ERK-1 activity in RPM (Figure 4c) with kinetics similar to the second peak of activation for the CSF-1 response in these cells (Figures 4a and 4b). Similar results were obtained with BMM (results not shown). The specificity of this assay compared with the 'in-gel' assay allows us to conclude unambiguously that ERKs are activated by LPS. We did not find an LPS response in the BAC1.2F5 cell line (results not shown), which differs from reports by others using the 'in-gel' kinase assay [17]. However, work in our laboratory (P. Valivelloo, personal communication) and others [23] has found a deficient response to LPS in BAC1.2F5 cells that could reflect strain differences between different BAC1.2F5 cells. The results in Figure 4(b) for RPM suggest that the constitutively active 40–45 kDa MBP kinases revealed by 'in-gel' analysis (Figure 3b) are probably not ERK-1 and ERK-2 and that the 'in-gel' assay is not sensitive enough to detect ERK activity unambiguously in lysates prepared from these cells. The specificity of this assay allows us to extend our earlier conclusions for MBP kinase activity, to show that activation of ERKs is not sufficient to induce macrophage DNA synthesis. Analysis of lysates from BMM treated with CSF-1 for longer times (0–3 h) did not reveal any other peaks of ERK-1 activation between 30 min and 3 h (Figure 4d).

DISCUSSION

We have shown, with a specific immune precipitate kinase assay, that ERK-1 activity is markedly stimulated by CSF-1 in both cycling (BMM and BAC1.2F5 cells) and poorly cycling (RPM) macrophages. Indirect evidence, namely enhanced tyrosine phosphorylation, indicated that ERK-2 is also activated. Analysis of extracts prepared under similar conditions by the commonly used 'in-gel' MBP kinase assay failed to detect any activation of kinases with subunit molecular mass 40–45 kDa by CSF-1. This apparent lack of sensitivity, coupled with the fact that members of the CSBP family, which are activated by LPS in macrophages, have similar molecular masses and phosphorylate MBP, led us to conclude that the 'in-gel' kinase assay by itself is unsuitable for monitoring 'MAP kinase' activity. We therefore used an

immune-complex kinase assay to demonstrate the activation of ERK-1.

Activation by LPS, albeit delayed, in BMM and RPM dissociates ERK activity from subsequent DNA synthesis in macrophages. Because of the quantitative and reproducible nature of the immune-complex kinase assay, we were able to resolve a broad, biphasic activation of ERK-1 in macrophages occurring within 30 min of stimulation with CSF-1, whereas with LPS a single activation occurred at 10–15 min that corresponded to the second peak observed with CSF-1. Contamination of reagents and culture media with endotoxin has been a major problem bedevilling studies on signal transduction pathways in macrophages [24]. That endotoxin contamination of the cytokines used in this study is not the cause of the second peak of activation was shown by two lines of evidence: first, routine measurement of endotoxin contamination by using the Limulus assay sets a lower limit of endotoxin in our stock solutions of CSF-1 and media at less than 100 pg/ml; secondly, both peaks of activation were observed in response to CSF-1 in the BAC1.2F5 cell line, which, in our hands, does not respond to endotoxin either with respect to growth inhibition or ERK activation. Our results suggest that ERK-1 is activated by at least two kinetically distinguishable pathways. We measured only small changes in total MBP kinase activity in response to CSF-1 and LPS, presumably due to high basal activity of other kinases revealed by the 'in-gel' kinase assay. We have shown, by using a specific immune-complex kinase assay, that LPS treatment of macrophages results in enhanced CSBP activity by about the same degree of stimulation as shown in the present study for ERK-1 (A. Jaworowski, unpublished work), whereas CSF-1 treatment at best results in only a weak enhancement of activity. In addition, it has recently been shown that LPS strongly activates Jun N-terminal kinase 1 in macrophages [25]. Thus it is likely that the LPS-enhanced MBP kinase activity is due to several members of the MAP kinase family, whereas that of CSF-1 is due predominantly to ERK activation.

Whether ERK activation is absolutely required for macrophage proliferation is not clear. It is clear, however, that ERK activation at levels attained by stimulation in some situations is not sufficient for proliferation to occur. The results presented in Figure 4 show that the extent of activation was similar in proliferating and in poorly proliferating macrophages or in response to mitogenic and to non-mitogenic stimuli. This suggests that the extent of stimulation of ERK activity at early times is not linked to the physiological outcome of the stimulation. This might differ from the situation in other myeloid cells, where it has been suggested that proliferation induced by granulocyte CSF is associated with rapid activation of ERK-1 and 2 and that this is limited to the proliferative response [1]. Differences in ERK activation accompanying stimuli that promote different physiological responses [2–4] show that it is very important to analyse carefully the kinetics of activation for any given system. ERK-1 activity dropped to near-basal levels between 30 min and 3 h in BMM lysates, but careful analysis has shown that the activity is twice as high in cycling populations of BMM as in growth-arrested BMM (A. Jaworowski, unpublished work). Thus a sustained activation at quite low levels might be required for a proliferative response. Recently, Kinoshita et al. [26] suggested a role for the Ras/Raf/MAP kinase pathway in mediating the IL-3/GM-CSF-dependent survival of haematopoietic cells. Because CSF-1 also promotes the survival of cells of the monocyte/macrophage lineage it would therefore be of interest to determine whether ERK activation is required for this process.

As discussed in the Introduction section, there is disagreement in the literature about pathways leading to ERK activation in

macrophages [10,14–16]. Perhaps the use of different assays could contribute to some of these disagreements. Part of the problem might also reside in the use of different BAC1.2F5 cells, because work in our laboratory (P. Vadiveloo, unpublished work) and others [23] has shown that the BAC1.2F5 cells do not respond well to LPS, including induction of ERK-1 activity (see above). This underscores the importance of examining these pathways in primary cultures of macrophages.

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