Protein phosphatase 2A is expressed in response to colony-stimulating factor 1 in macrophages and is required for cell cycle progression independently of extracellular signal-regulated protein kinase activity

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Colony-stimulating factor 1 (CSF-1) is required for the development of monocytes/macrophages from progenitor cells and for the survival and activation of mature macrophages. The receptor for CSF-1 is the product of the c-*fms* proto-oncogene, which, on binding ligand, can stimulate a mitogenic response in the appropriate cells. To investigate which genes are regulated in response to CSF-1-stimulation in murine bone-marrow-derived macrophages (BMM), we employed mRNA differential display reverse transcriptase-mediated PCR to identify cDNA species induced by CSF-1. Both Northern and Western blot analyses confirmed the increased expression of one of the cDNA species identified as coding for the catalytic subunit of protein phosphatase 2A (PP2A), an observation not previously reported during the response to a growth factor. To determine the significance of the increased expression of PP2A in response to CSF-1, the PP2A inhibitor okadaic acid (OA) was added to CSF-1-treated BMM and found to inhibit DNA synthesis in a dose-dependent manner. Further analysis with flow cytometry in the presence of OA led to the novel conclusion that PP2A activity is critical for CSF-1-driven BMM cell cycle progression in both early G_1 and S phases. Surprisingly, in the light of previous studies with other cells, the PP2A-dependent proliferation could be dissociated from activation by extracellular signal-regulated protein kinase (ERK) in macrophages because OA did not affect either the basal or CSF-1-induced ERK activity in BMM. Two-dimensional SDS/PAGE analysis of lysates of ^{32}P-labelled BMM, which had been treated with CSF-1 in the presence or absence of OA, identified candidate substrates for PP2A.

Key words: differential display, okadaic acid, phosphatase 2.

INTRODUCTION

Reversible protein phosphorylation, mediated by kinases and phosphatases, is a common mechanism for the regulation of basic cellular processes in eukaryotes. Protein phosphatase 2A (PP2A) is one of the four major classes of protein serine/threonine phosphatases (reviewed in [1,2]). PP2A has been shown to act at different points in the cell cycle in various systems [3–6] and has been implicated as both a positive and a negative stimulus for cell proliferation [3,6]. Stimulation of cells by growth factors can lead to an inhibitory phosphorylation of PP2A on Tyr-307 with a peak at 5–15 min. This inhibitory phosphorylation can be mediated directly, for example by the EGF receptor, or indirectly by p56^{lck} and p60^{v-src} [7,8]. Stimulation by growth factors often leads to the activation of extracellular signal-regulated protein kinase (ERK), which has been shown to be a substrate for PP2A [9] and whose activity can be inversely correlated with that of PP2A [10]. Further evidence for PP2A regulating ERK comes from the use of the tumour promoter okadaic acid (OA) and the SV40 small T antigen. At low concentrations OA is a specific inhibitor of PP2A [2] and SV40 small T antigen binds to the catalytic subunit of PP2A, preventing activation [3], in both cases leading to the activation of ERK [3,4]. In addition, the inhibition of PP2A by OA can induce the AP-1 transcription factor [11]. Fos and Jun dimerize to form the AP-1 transcription factor (reviewed in [12]) and are downstream targets of ERK activation, their transcription being immediate early responses to mitogenic stimulation. However, OA has been reported to be both an agonist and an antagonist of proliferation [4,13]; ERK activation by OA is not always correlated with proliferation, suggesting that there might be additional downstream pathways under PP2A control that are important for cell proliferation.

Colony-stimulating factor 1 (CSF-1, also called M-CSF), is a growth factor required for the survival, differentiation and activation of macrophages, and activates its receptor, c-Fms, by binding to the extracellular domain [14]. As with other growth factors and cytokines, the relevance of downstream signalling cascades to the biological function of CSF-1 is best studied in cells in which its receptor is normally expressed, and preferably not in cell lines [15–17]. The binding of CSF-1 to c-Fms leads to dimerization of the receptor, autophosphorylation and the initiation of a kinase cascade that leads to the activation of transcription factors, resulting in the expression of various genes, including those involved in proliferation [18].

The method of 'differential display' identifies subsets of total cellular mRNA between two or more cell populations by using reverse transcriptase-mediated PCR (RT–PCR) [19]; it has been used to investigate gene induction in a number of systems, including fibroblasts stimulated with fibroblast growth factor 1 [20,21], and mink lung epithelial cells treated with transforming growth factor β [22]. Using RT–PCR differential display we show here that the stimulation of murine bone-marrow-derived macrophages (BMM) with CSF-1 leads to the differential expression of several genes, including the gene encoding the catalytic subunit of PP2A. By adding OA, to inhibit PP2A activity, we provide evidence that PP2A does not effect ERK activity but is

Abbreviations used: BMM, bone-marrow-derived macrophages; CSF-1, colony-stimulating factor 1; 2D, two-dimensional; ERK, extracellular signal-regulated protein kinase; ^{3}H-TdR, [*methyl*-^{3}H]thymidine; MAPK, mitogen-activated protein kinase; OA, okadaic acid; PP2A, protein phosphatase 2A; RT–PCR, reverse transcriptase-mediated PCR.

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required for cell cycle progression in both the G_1 and S phases in CSF-1-stimulated BMM. We also identified possible targets for PP2A in these cells by using two-dimensional (2D) SDS/PAGE.

EXPERIMENTAL

Cells and media

BMM were obtained from precursor cells in murine bone marrow as described previously [23] and maintained in RPMI/10 % (v/v) fetal bovine serum plus 30 % (v/v) L-cell-conditioned medium as a source of CSF-1. The cells were growth-arrested by incubation in the absence of growth factor for 18–20 h. The granulocyte colony-stimulating factor (G-CSF)/interleukin 3-dependent NFS-60 cell line [24] was a gift from Dr. J. Layton (Ludwig Institute for Cancer Research, Parkville, Australia) and was maintained in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal bovine serum, 2 mM glutamine and 1 ng/ml G-CSF. All cells were incubated at 37 °C in air/CO_2 (19:1).

mRNA differential display

Total RNA, isolated as described [25], was treated with DNase for 4 h at 37 °C and extracted with phenol/chloroform (5:1) before precipitation with ethanol and resuspension in diethyl pyrocarbonate-treated water. In a 500 μl Eppendorf tube 0.5 μg of RNA was reverse transcribed with 2.5 μM T11MG primer, 1 × RT buffer (Promega), 125 μM dNTPs, 3.3 mM dithiothreitol, 100 units of AMV reverse transcriptase (Promega; 1 unit catalyses the incorporation of 1 nmol of dTTP into acid-insoluble form in 10 min at 37 °C) and diethyl pyrocarbonate-treated water to a final volume of 30 μl. The reaction was performed at 42 °C for 1 h and terminated by heat inactivation at 100 °C for 10 min. Differential display PCR was performed in 500 μl Eppendorf tubes as follows: 1 μl of total RT reaction was added to 29 μl of PCR cocktail (0.6 μl of 100 mM dNTPs, 3.0 μl of 10 × *Taq* buffer, 3.0 μl of 25 mM T11MG oligonucleotide, 0.6 μl of 25 mM decamer, 1500 Ci/mmol adenosine 5′-[α-[^{32}S]thio]triphosphate, 0.3 μl of *Taq* polymerase and 20 μl of water) and heated to 95 °C for 30 s, 42 °C for 2 min and 72 °C for 30 s for 40 cycles. PCR reactions were resolved on a 6 % (w/v) sequencing gel at 1250 V. Gels were dried on Whatman 3MM paper without fixing and then exposed to Kodak BioMax-MR film at room temperature. Differentially expressed bands were excised from the gel and rehydrated in 100 μl of water for 10 min at room temperature, then at 100 °C for 15 min before precipitation with 10 μl of a mixture of 3 M sodium acetate, pH 5.2, 2.5 μl of 20 mg/ml glycogen and 300 μl of ethanol. The precipitated cDNA species were resuspended in 10 μl of water; 2 μl of this was used for reamplification as before, except that 35 cycles were used. The potentially regulated cDNA species were cloned with the TA cloning kit (InVitrogen) and subjected to sequence analysis with a T7 sequencing kit (Pharmacia).

Library screening

A NFS-60 cell-derived cDNA library was plated on 90 mm Luria–Bertani agar plates at 10^5 plaques per plate and transferred to Hybond N^+ with the use of standard techniques [26]. The filters were then hybridized with ^{32}P-labelled G71B as a probe and subjected to autoradiography with Kodak BioMax film at −70 °C.

Northern analysis

Northern blot analysis was performed as described previously [27]; in brief, RNA was fractionated on a 1.2 % (w/v) agarose gel containing 1.8 % (v/v) formaldehyde and transferred to Hybond N^+ filters (Amersham). Cloned re-amplification products were radiolabelled with [α-^{32}P]dATP (Bresatec) by using nick-translation and used to probe the blots. Control hybridization with a β_2-microglobulin probe was used to determine RNA loading and quality.

Western blot analysis

BMM were harvested at 10^7 cells in 300 μl of lysis buffer [25 mM Hepes (pH 7.4)/5 mM EDTA/100 mM NaCl/1 % (v/v) Triton X-100/10 % (v/v) glycerol containing 70 i.u./ml aprotinin, 10 μg/ml leupeptin, 100 mM NaF, 0.1 mM Pefabloc and 200 μM sodium orthovanadate] and left on ice for 10 min before centrifugation at 5000 ***g*** for 5 min. The cytosolic supernatants were transferred to a new Microfuge tube and the pellets were discarded. Lysates were frozen at −20 °C until needed. For a 10-well mini-gel (Bio-Rad Laboratories, Richmond, CA, U.S.A.), 20 μl of lysate was added to 4 μl of 5 × reducing sample buffer and boiled for 3 min before loading. Proteins were size-separated by SDS/PAGE [10 % (w/v) gel]. After electrophoresis at 20 mA per gel for 2.5–3 h, the gels were transferred to Hybond C (Amersham); the membranes were blocked overnight at 4 °C in blocking buffer [4 % (w/v) BSA/1 % (w/v) ovalbumin]. An antibody against the catalytic subunit of PP2A (PP2Ac) (sc-6111; Santa Cruz Biotechnology) was added to fresh blocking buffer at the manufacturer's recommended concentration for 3 h at room temperature; membranes were washed three times in TBST [20 mM Tris/HCl (pH 7.6)/150 mM NaCl/0.1 % (v/v) Tween 20]. Fresh blocking buffer containing HRP-conjugated secondary antibody at 1:10000 dilution (rabbit anti-rat IgG; Dako) was added and membranes were incubated for 1 h at room temperature before being washed as before. Membranes were rinsed in TBS [20 mM Tris/HCl (pH 7.6)/150 mM NaCl] and subjected to enhanced chemiluminescence (Amersham ECL® reagents and Hyperfilm).

DNA synthesis analysis

BMM DNA synthesis was measured as the incorporation of [*methyl*-^{3}H]thymidine (^{3}H-TdR), as described previously [23]. Quiescent (i.e. growth-arrested) BMM (10^5 cells per well in 24-well plates) were incubated in CSF-1 (5000 i.u./ml) in the presence or absence of various concentrations of OA with ^{3}H-TdR (2.5 μCi/ml) added at 0 h. After 24 h, cells were harvested by removal of the incubation medium and the addition of 500 μl 0.2 M NaOH, followed by 500 μl of 20 % ice-cold trichloroacetic acid and incubation at 4 °C for 20 h. The lysates were then transferred to glass filters with the use of an Inotech harvester, and radioactive counts were determined by liquid-scintillation counting with a Beckman β-counter (model no. LS 3801).

Flow cytometry

BMM grown in 10 cm dishes were harvested by gentle scraping, then centrifuged and resuspended in PBS. To a 200 μl aliquot containing 2×10^5 cells was added 50 μl of stock staining solution comprising 250 μg/ml propidium iodide (Sigma Chemical Company, St. Louis, MO, U.S.A.), 5 mg/ml RNase (EC 3.1.27.5; Sigma) and 1 % (v/v) Triton X-100 in distilled water. After being stained with propidium iodide the cells were incubated in the dark at 4 °C for a minimum of 3 h, after which propidium iodide fluorescence was measured with a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.). Cell cycle analysis was performed on gated singlet populations with the use of ModFit LT Cell Cycle Analysis Software (Verity Software House, Topsham, ME, U.S.A.). Acquisition was restricted to 20000 events for each sample.

Phosphatase activity assay

BMM grown in 10 cm dishes were harvested in 200 μl of RIPA buffer [20 mM Tris/HCl (pH 7.5)/150 mM NaCl/1 % (v/v) Triton X-100/1 % (w/v) sodium deoxycholate/0.1 % SDS/2 mM EDTA]. PP2A activity was measured *in vitro* in 96-well flat-bottomed microtitre plates (Disposable Products, Park Way Technology Park, South Australia, Australia). Assays were performed in triplicate. In each well, either lysis buffer or 20 μg of cell extract was diluted to a volume of 60 μl in 20 mM $NiCl_2$ containing 2.5 mg/ml BSA (Sigma); 20 μl of 50 mM Tris/HCl (pH 7.0)/0.1 mM $CaCl_2$, either with or without OA (1 ng/ml), was added to each well before the addition of 120 μl of *p*-nitrophenyl phosphate (Sigma) (1.5 mg/ml in 50 mM Tris/HCl, pH 7.0). The reaction proceeded for 30 min at 37 °C. The hydrolysis of *p*-nitrophenyl phosphate was measured spectrophotometrically at 410 and 695 nm, and PTPase activity was calculated in nmol/min per μg as described previously [28].

Mitogen-activated protein kinase (MAPK) assays

Cytosolic cell lysates were prepared as follows: 10^7 cells were scraped in lysis buffer and left on ice for 10 min before centrifugation at 5000 ***g*** for 5 min. The pellets were discarded and kinase assays were performed as described previously [29,30]. In brief, immunoprecipitations were performed by incubating cell lysates (50 μg) overnight with 1 μg of anti-p44^{MAPK} [anti-(ERK-1)] antibody (catalogue no. sc-93; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) at 4 °C. A 20 μl slurry (50 %, v/v) of Protein A–Sepharose 4B (Pharmacia, Uppsala, Sweden) was added to the lysates and incubated for 30 min at 4 °C. The beads were pelleted and washed three times with lysis buffer. Pellets were incubated with 20 μg of myelin basic protein in kinase assay buffer [20 mM Hepes (pH 7.4)/10 mM $MgCl_2$/0.2 mM ATP/2 mM Wiptide/10 mCi of [γ-^{32}P]ATP] for 20 min at 25 °C. SDS/PAGE sample buffer was added to stop the reactions; the samples were then boiled for 5 min and subjected to SDS/PAGE [12.5 % (w/v) gel] before being stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories). The gels were dried and exposed to Kodak-IBI XAR film.

2D gel electrophoresis

Cell lysates (100 μg of protein) were subjected to isoelectric focusing at 1810 V for 15.5 h at 18 °C with pH 3–10 ampholytes (Bio-Rad Laboratories) with the use of the Bio-Rad Protean 2 electrophoresis system. The procedure was modified from that described by O'Farrell [31] by the use of isoelectric focusing tubes with an internal diameter of 3 mm as described previously [32]. After isoelectric focusing, the gel tubes were extruded with double-distilled water and equilibrated in SDS/PAGE sample buffer containing 10 % (v/v) glycerol, 5 % (v/v) 2-mercaptoethanol, 2.3 % (w/v) SDS and 62.5 mM Tris/HCl, pH 6, for 2 h at room temperature. Separation in the second dimension was performed with SDS/12.5 % (w/v) polyacrylamide gels of 0.75 mm thickness at a constant current of 25 mA per gel; the electrophoresis chamber was maintained at 4 °C. SDS/PAGE gels were stained overnight with 0.1 % Coomassie Brilliant Blue R-250 in 25 % (v/v) methanol with 10 % (v/v) acetic acid, destained in 25 % (v/v) methanol with 10 % (v/v) acetic acid, dried and subjected to autoradiography. Wide-range protein standards were used to determine molecular masses in the second dimension (Novex, San Diego, CA, U.S.A.). Isoelectric points were determined by using 2D SDS/PAGE standards, pH 4.5–8.5 (Bio-Rad Laboratories), and the Carbamylyte Calibration Kit (Pharmacia, Uppsala, Sweden). Proteins were ascribed molecular masses and isoelectric points with the use of these standards and were labelled accordingly (i.e. a protein with an apparent molecular mass of 66 kDa and a pI of 4.7 would be referred to as 66/4.7).

Quantitative determination of phosphorylation

All 2D SDS/PAGE gels were of cytoplasmic lysates containing 100 μg of protein. Gel autoradiographs were scanned with a Computerized Laser Densitometer (Molecular Dynamics, Sunnyvale, CA, U.S.A.) and the images analysed with the use of Image Quant software (Molecular Dynamics).

RESULTS

PP2A is expressed in BMM in response to CSF-1

Differential display was employed to identify genes modulated by CSF-1. After stimulation of growth-arrested BMM with CSF-1, total RNA was extracted from cells in the late G_1 phase of the cell cycle and used to generate a differential display of expressed mRNA. Highly reproducible banding patterns were observed on the differential displays and, from the particular primer combination studied, three bands seemed to be differentially expressed. These putative regulated transcripts, termed G71, G72 and G73, were excised from the gel, reamplified and cloned. A sequence analysis of individual clones of these expressed bands showed that multiple sequences were present in the same band.

The sequence identification of differential display clones and the library-derived clones are summarized in Table 1. From the three bands (G71, G72 and G73), a total of six different clones were obtained. Initial comparison with the GenBank database showed that three of the clones represented known genes: clone G72B was identified as the gene encoding murine elongation factor 1α protein, G72C encoded a rat 60 S ribosomal subunit protein, and clone G71A showed significant similarity to the human B4-2 protein [33]. Three clones, G71B, G72A and G73A, showed no significant similarities to sequences in the database. Further characterization of these clones was undertaken by using them as probes to screen an NFS-60 cell library. From the screen three clones from the G72A probe were found to be sequences for a mitochondrial protein, whereas clones from G73A revealed no similarity to known genes; these clones were not studied further. The G71B probe yielded a 460 bp cDNA clone, which sequencing analysis showed to code for the catalytic subunit of PP2A.

The 460 bp cDNA fragment (PP2A) was subsequently used as a probe for Northern blotting; the results show that PP2A

Table 1 Sequence similarities of differentially expressed clones to known genes

Growth-arrested BMM were stimulated with CSF-1 and subjected to differential display as described in the Experimental section. Individual bands isolated from the gels were numbered as shown, then cloned. Individual clones were sequenced. The sequences were compared with known sequences in the GenBank database; the similarity to known genes is shown. Clones derived from bands containing more than one cDNA species were numbered as shown.

Band	Clone	Similarity
G71	G71A	50.7% B4-2 protein
	G71B	99% PP2A catalytic subunit
G72	G72A	98% mitochondrial protein
	G72B	90.7% elongation factor 1α
	G72C	83% rat 60 S ribosomal subunit
G73	G73A	None

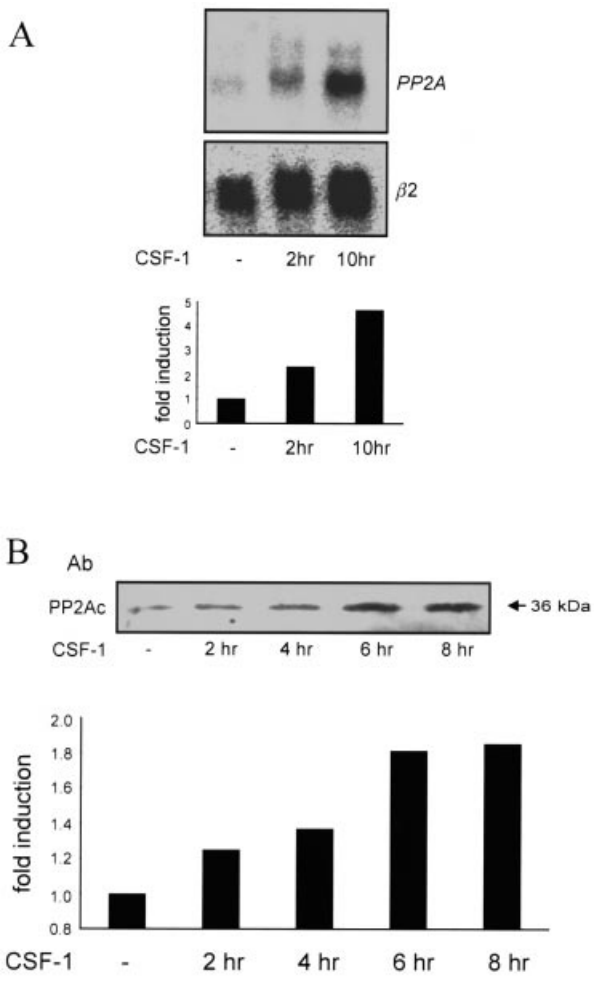

Figure 1 CSF-1 increases PP2A mRNA expression and protein levels in BMM

(**A**) Total RNA was isolated from growth-arrested BMM and, after restimulation with CSF-1 (5000 i.u./ml) at the times indicated, RNA (15 μg per lane) was subjected to electrophoresis through 1.2% (w/v) agarose gel containing 1.8% (v/v) formaldehyde, transferred to Hybond N^+ membranes then hybridized with a radiolabelled cDNA fragment of the PP2A clone obtained from differential display and library screening. β_2-Microglobulin was included as a control for RNA loading. The histogram with the β_2-microglobulin bands as reference represents fold induction as determined by densitometry. (**B**) Lysates, equalized for protein concentration, from growth-arrested BMM and from BMM after restimulation with CSF-1 for the durations indicated were immunoblotted with anti-PP2Ac antibody (Ab). The histogram represents fold induction of PP2A determined by densitometry with Coomassie Blue-stained total protein as a reference for loading. Each experiment was repeated three times; representative experiments are shown.

mRNA expression is elevated after stimulation with CSF-1 (Figure 1A). Western blotting with an antibody against the catalytic subunit of PP2A (anti-PP2Ac) showed that the elevation in mRNA expression in response to CSF-1 translated into an increase in PP2A protein (Figure 1B). Others have shown a decrease in PP2A catalytic subunit levels during differentiation [34]; however, to our knowledge this is the first report showing that the PP2A catalytic subunit is increased in response to a growth factor.

PP2A is required for cell cycle progression through both G_1 and S phases in CSF-1-treated BMM

Because we had found that PP2A mRNA and protein levels increased in response to CSF-1, we wished to explore the possible

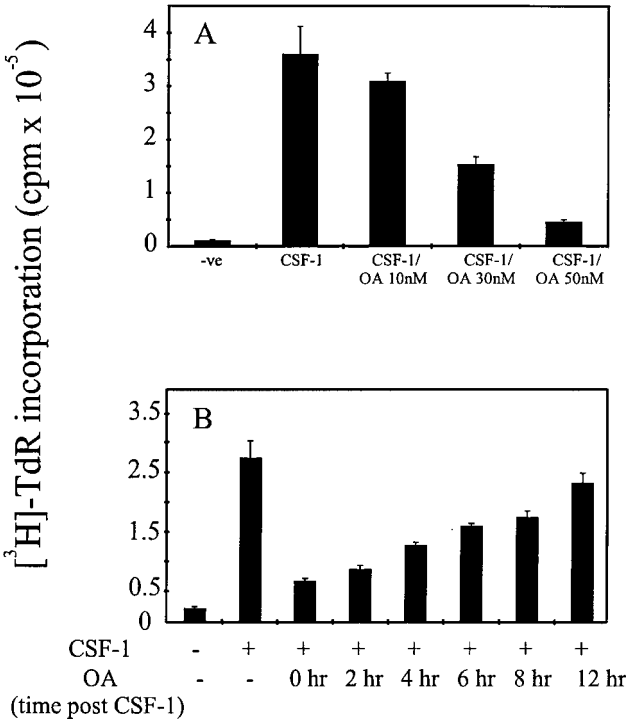

Figure 2 OA inhibits CSF-1-induced DNA synthesis in BMM

Growth-arrested BMM were cultured in 24-well plates in the presence of ^{3}H-TdR and restimulated with CSF-1 (5000 i.u./ml) in the absence or presence of OA at the concentrations indicated (**A**) or CSF-1 (5000 i.u./ml) in the absence or presence of OA (50 nM) added after stimulation with CSF-1 at the times indicated (**B**). All experiments were stopped 24 h after stimulation and DNA synthesis was measured as the incorporation of ^{3}H-TdR. The histograms are representative of three experiments, where each point is an average of triplicate wells; error bars indicate S.E.M.

role of PP2A in the CSF-1-induced proliferation of BMM. To determine the effect of PP2A on cell cycle progression, OA, a specific inhibitor of PP2A [35], was added to CSF-1-deprived BMM, in the presence or absence of added CSF-1, and DNA synthesis was quantified by ^{3}H-TdR incorporation. OA inhibited CSF-1-induced DNA synthesis in a concentration-dependent manner, with near-complete inhibition at 50 nM OA (Figure 2A), suggesting that PP2A provides a positive signal for DNA synthesis in BMM. The addition of OA to quiescent BMM in the absence of growth factor induced a slight (approx. 2-fold at 50 nM OA) concentration-dependent increase in basal DNA synthesis (results not shown). To ascertain at what point(s) in G_1 phase PP2A activity is critical for the progression into S phase, DNA synthesis was measured as above, with OA (50 nM) added at different times after stimulation with CSF-1. Growth-arrested BMM take approx. 10–12 h to enter S phase after stimulation with CSF-1 [36]. We see in Figure 2(B) that, for maximal inhibition of DNA synthesis to occur, the OA must be added within 2 h of CSF-1 because its effect is gradually diminished if added late in G_1.

To examine in more detail the effect of OA on the BMM cell cycle, DNA content was measured by flow cytometry. Four independent studies were performed; mean values for the four experiments, together with data from a representative experiment, are given in Figure 3. In Figure 3(A), accumulation of CSF-1-starved (growth-arrested) BMM in G_0/G_1 (2*N* DNA content) can be seen, confirming previous findings [37]. Incubation with CSF-1 for 24 h resulted in approx. 16% of BMM being in S phase at this time point (Figure 3B). If OA was added

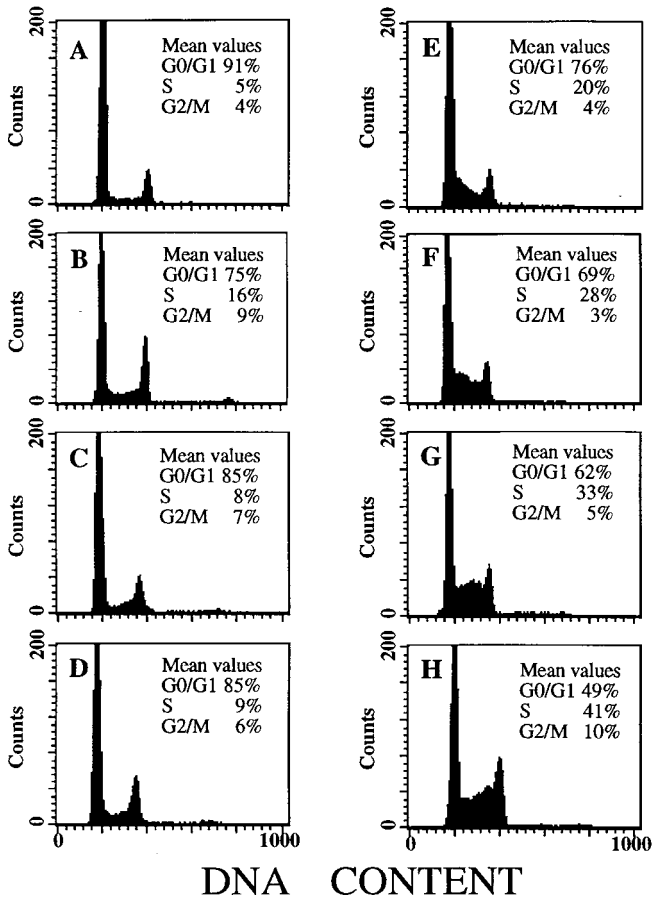

Figure 3 OA arrests BMM in both the G_1 and the S phase of the cell cycle

CSF-1-deprived BMM (**A**) were incubated for 24 h in medium containing CSF-1 (5000 i.u./ml) alone (**B**), or together with OA (50 nM) at 0 h (**C**), 2 h (**D**), 4 h (**E**), 6 h (**F**), 8 h (**G**) and 12 h (**H**) after the addition of CSF-1. At the completion of the 24 h incubation, cells were harvested, stained with propidium iodide and analysed for DNA content with a flow cytometer. The proportions of cells at the various stages of the cell cycle are plotted from a representative experiment, but mean values from four experiments are provided.

concomitantly with or 2 h after stimulation with CSF-1, the cells remain arrested in G_0/G_1, unable to progress into S phase (Figures 3C and 3D respectively) and with a cell cycle profile similar to that of the quiescent BMM (Figure 3A). Delaying the time at which OA was added to the cultures after stimulation with CSF-1 to 4, 6, 8 and 12 h increased the proportion of cells able to progress into S phase (Figures 3E, 3F, 3G and 3H respectively), confirming the results in Figure 2(B). However, flow cytometric analysis was able to show that the later addition of OA (Figures 3E–3H) resulted in an accumulation of cells in S phase to levels even higher than those observed in cycling cells (Figure 3B). This blockade of BMM in S phase prevented entry into mitosis and resumption of the 2*N* state, and was reflected in the accompanying decrease in the percentage of cells in G_0/G_1 (Figures 3G–3H). The DNA content analysis thus indicates that OA acts at two distinct stages in the CSF-1-stimulated BMM cell cycle, one preventing cells from entering S phase by a G_1-phase block and the other preventing exit from S phase and entry into G_2/M. This inhibition of S-phase entry and exit by OA presumably reflects a requirement for PP2A at these two stages.

PP2A activity is stimulated within 2 min of treatment with CSF-1 and is inhibited by the addition of OA *in vivo*

The above cell cycle analysis of CSF-1-stimulated BMM treated with OA suggests a block in early G_1 phase. To determine whether PP2A activity is acutely activated in response to CSF-1, CSF-1-deprived BMM were stimulated with CSF-1 for 2 and 10 min, in the presence or absence of OA, and phosphatase activity was measured with *p*-nitrophenyl phosphate as substrate. Total phosphatase activity increased from 0.38 nmol/min per μg to 1.65 nmol/min per μg within 2 min of CSF-1 treatment; this declined to 0.92 nmol/min per μg within 10 min (Figure 4A). Treatment of CSF-1-stimulated BMM lysates *in vitro* with OA (50 nM) showed that approx. 50% of total phosphatase activity was lost (Figure 4A) and was therefore attributable to an OA-sensitive phosphatase, presumably PP2A. When BMM were pretreated *in vivo* with OA (50 nM) before stimulation with CSF-1 (Figure 4B), the phosphatase activity mirrored that seen for OA treatment of CSF-1-stimulated lysates *in vitro*; moreover, OA added *in vitro* could not further decrease OA-pretreated, CSF-1-stimulated phosphatase activity. These results demonstrate that OA is as effective when added *in vivo* as it is *in vitro* in inhibiting phosphatase activity in CSF-1-treated BMM.

Inhibition of DNA synthesis by OA is not mediated by effects on ERK activity or c-*fos* mRNA expression in BMM

The above results show that total phosphatase activity is increased within 2 min after CSF-1-stimulation and that approx.

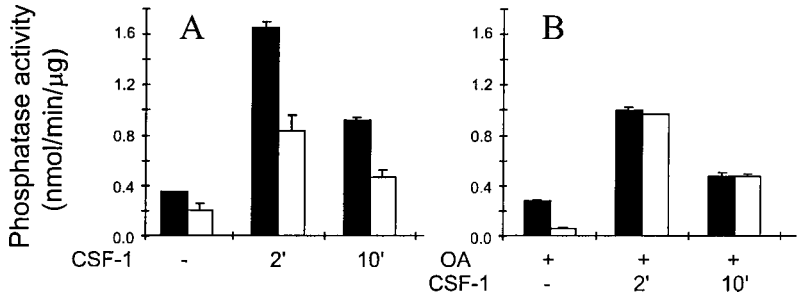

Figure 4 Inhibition of CSF-1-induced phosphatase activity in BMM by the addition of OA *in vivo*

Growth-arrested BMM were cultured in 10 cm dishes and stimulated with CSF-1 (5000 i.u./ml) for either 2 or 10 min in the absence (**A**) or presence (**B**) of OA (50 nM) pretreatment, then lysed in RIPA buffer. In both panels, total phosphatase activity in lysates is shown in the absence (black bars) or presence (open bars) of OA (50 nM) added *in vitro*. The histograms are representative of two independent experiments, where each point is an average from triplicate assays; error bars indicate S.D.

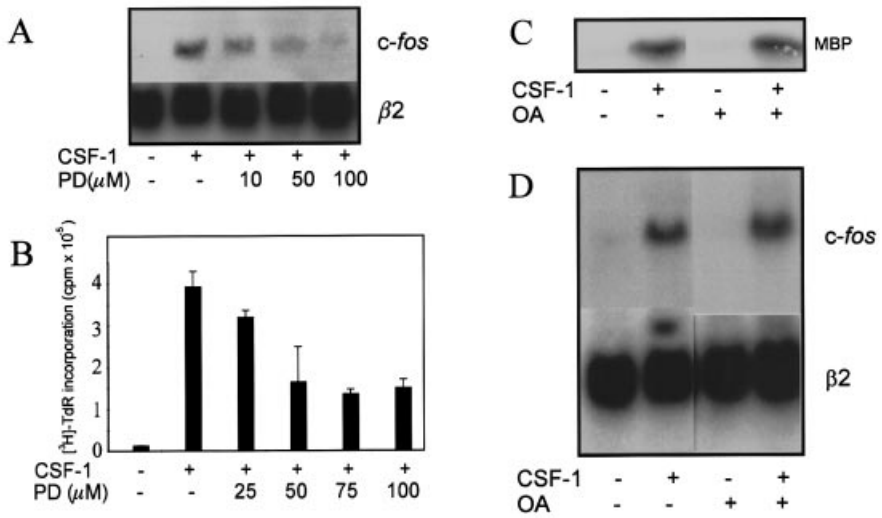

Figure 5 OA does not modulate ERK activity or the expression of c-*fos* mRNA in BMM

(**A**) Total RNA was isolated from CSF-1-deprived BMM or from BMM restimulated for 30 min with CSF-1 (5000 i.u./ml), in the absence or presence of PD98059 (PD) at the doses indicated. RNA (15 μg per lane) was subjected to electrophoresis through 1.2% (w/v) agarose gel containing 1.8% (v/v) formaldehyde, transferred to Hybond N^+ membranes and hybridized with a radiolabelled cDNA fragment of c-*fos*; β_2-microglobulin probing is included as a control for RNA loading. (**B**) Growth-arrested BMM were cultured in 24-well plates in the presence of ^{3}H-TdR and then restimulated with CSF-1 (5000 i.u./ml), in the absence or presence of PD98059 at the concentrations indicated. Experiments were stopped 24 h after stimulation and DNA synthesis was measured as incorporation of ^{3}H-TdR. The histogram is representative of three experiments, in which each value is an average for triplicate wells; error bars indicate S.E.M. (**C**) CSF-1-deprived BMM were untreated or treated with the following: CSF-1 (5000 i.u./ml) for 4 min, OA (50 nM) for 30 min or pretreated with OA for 30 min then CSF-1 for 4 min. ERK-1 was immunoprecipitated from the lysate and activity was measured as the incorporation of ^{32}P into myelin basic protein (MBP). MBP was resolved by SDS/PAGE and subjected to autoradiography. The figure is representative of three independent experiments. (**D**) Total RNA was isolated from CSF-1-deprived BMM and from BMM stimulated for 30 min with CSF-1 (5000 i.u./ml), OA (50 nM) or their combination. RNA (15 μg per lane) was subjected to electrophoresis through 1.2% (w/v) agarose gel containing 1.8% (v/v) formaldehyde, transferred to Hybond N^+ membranes and hybridized with a radiolabelled cDNA fragment of c-*fos*; β_2-microglobulin probing is included as a control for RNA loading.

50% of this activity is OA-sensitive, suggesting that PP2A activity is also stimulated by CSF-1 within this period. Importantly, this activation is abrogated by treatment with OA *in vivo*, suggesting that the early G_1-phase block caused by OA (Figures 2B and 3) might be a consequence of inhibiting the ability of PP2A to affect early signal transduction events that lead to cell proliferation. An obvious target for cell cycle regulation by PP2A is the ERK family. Members of this family have been implicated in promoting proliferation in many cell types and can be a substrate for PP2A [3]. A transcriptional target of ERK activation is the c-*fos* gene [12]. To explore the possible relationship between PP2A and ERK activities in BMM we first needed to explore the role of ERKs in the induction of c-*fos* mRNA expression and DNA synthesis. We have previously shown that the widely used specific inhibitor of MAP kinase kinase activation, PD98059 [38], inhibits CSF-1-stimulated ERK activities with maximal inhibition between 50 and 100 μM [29]. We see in Figure 5(A) that the addition of PD98059 suppressed the induction of c-*fos* mRNA in a dose-dependent manner in CSF-1-treated BMM, suggesting that a MAP kinase kinase/ERK pathway does indeed control c-*fos* mRNA expression in this system. We also show in Figure 5(B) that PD98059 leads to a dose-dependent inhibition of CSF-1-stimulated BMM DNA synthesis; however, maximal inhibition at concentrations of at least 50 μM resulted in only an approx. 60% loss of DNA synthesis. These results suggest that a proportion of the CSF-1-induced DNA synthesis in BMM is ERK-dependent.

Given the indication that ERK activity does in part control DNA synthesis in BMM, the effect of OA on ERK activity in CSF-1-stimulated BMM was examined. OA was added to BMM either alone or before stimulation with CSF-1, and ERK activity was measured. OA at 50 nM did not stimulate ERK-1 activity; neither did it potentiate or inhibit the increased ERK-1 activity in response to CSF-1 (Figure 5C); similar results were obtained for ERK-2 (results not shown). The results for ERK activity were mirrored in the effect of OA on c-*fos* mRNA expression. Figure 5(D) shows that OA did not increase c-*fos* mRNA levels when added alone, nor did it influence the CSF-1-stimulated increase in c-*fos* mRNA. These results suggest that the action of PP2A in CSF-1-stimulated DNA synthesis does not involve the regulation of ERK activity or of c-*fos* mRNA expression. In addition, the results suggest that the inhibition of ERK activity leads to only partial inhibition of CSF-1-stimulated BMM DNA synthesis, a finding different from the near-complete inhibition shown by treatment with OA (Figure 3A).

Examination of potential downstream targets of PP2A by 2D SDS/PAGE

To identify potential substrates for PP2A in CSF-1-treated BMM, which might be involved in cell cycle progression, an analysis of the effects of OA on ^{32}P incorporation into protein was performed by 2D SDS/PAGE. CSF-1 treatment of BMM for 4 min resulted in a marked elevation of protein phosphorylation compared with basal levels (compare Figures 6B and 6A). Interestingly, the addition of OA and CSF-1 together resulted in the elevated phosphorylation of only a few proteins (Figure 6C). Five such changes were clearly discernible. Two proteins whose phosphorylation was induced by CSF-1 treatment, namely 66/4.7 and 62/6.0 (Figure 6B), were hyperphosphorylated by pretreatment with OA before stimulation with CSF-1 (Figure 6C). Furthermore, three proteins not detect-

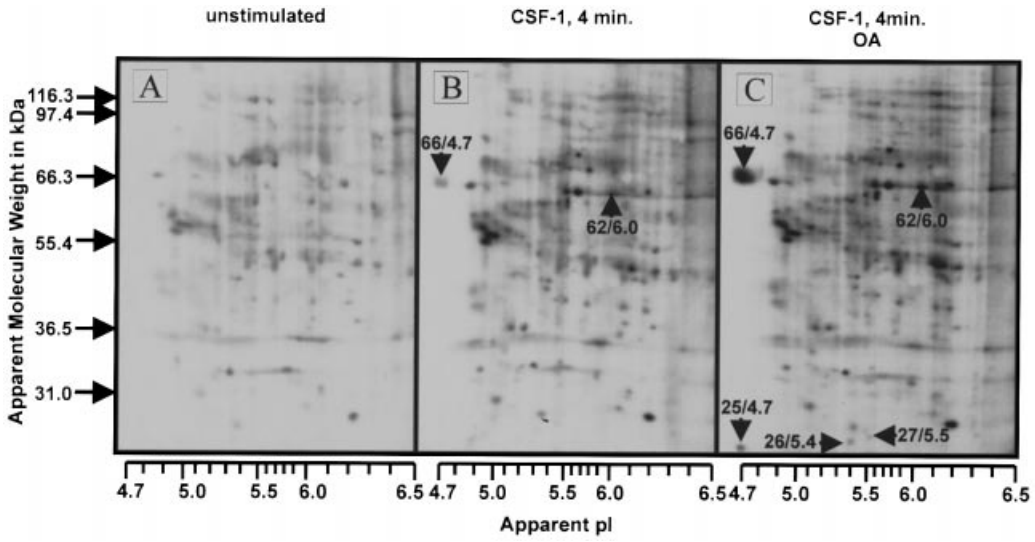

Figure 6 Identification by 2D SDS/PAGE of phosphoproteins sensitive to OA treatment

BMM were prelabelled with [^{32}P]P_i (1 mCi per 10^7 cells). Lysates were prepared from BMM that had been either CSF-1-deprived (**A**) or stimulated with CSF-1 (5000 i.u./ml) for 4 min without (**B**) or with (**C**) 30 min of OA (50 nM) pretreatment. Lysates were subjected to 2D SDS/PAGE, dried and autoradiographed.

ably phosphorylated by treatment with CSF-1, namely 25/4.7, 26/5.4 and 27/5.5, were evident after treatment with OA and CSF-1 together (Figure 6C). The effect of OA on the phosphorylation state of these five proteins is in contrast with the overwhelming conservation of the CSF-1-induced phosphorylation of a myriad of other proteins (Figures 6A, 6B and 6C).

DISCUSSION

To identify genes regulated by CSF-1 in murine BMM we have used the technique of differential display to obtain three potentially up-regulated bands on display gels. As is often observed [39,40], sequence analysis of cloned reamplification products showed that these bands contained more than one cDNA, with a total of six different clones isolated (Table 1). However, only two clones, G72A and G71B, were confirmed by Northern analysis to be up-regulated. These findings are similar to the high rates of false positives with this technique reported in other systems [39,40].

The two gene fragments expressed differentially that we isolated were used to screen an NFS-60 myeloid cell library. The G72A-derived clone showed significant similarity to a mitochondrial cDNA and was not studied further, whereas the G71B-derived clone was found to encode for the catalytic subunit of PP2A (Table 1). We confirmed by Northern blotting that there was an increased expression of the mRNA for the catalytic subunit of PP2A in response to CSF-1 in BMM, and this increase was paralleled by an increase in protein levels. To our knowledge this is the first report showing that PP2A levels are elevated in response to a growth factor. Others have shown that the catalytic subunit of PP2A is down-regulated during retinoic acid-induced differentiation of HL-60 cells [34]. In regenerating liver a 30-fold increase in PP2A mRNA expression was reported with no concomitant enhancement of protein levels [41], probably owing to the recently demonstrated tight regulation of the translation of the PP2A catalytic subunit [42].

To determine whether the CSF-1-stimulated increase in PP2A mRNA and protein levels in BMM were indicative of a requirement for PP2A activity in CSF-1-induced cell cycle progression, OA was added in various concentrations to CSF-1-stimulated BMM. This compound has been used widely in other cell types to delineate PP2A function and has been shown to potentiate or suppress cell cycle progression depending on the system studied [3,6]. We determined that the treatment of BMM with OA (50 nM) *in vivo* abrogates basal PP2A activity as well as the ability of CSF-1 to activate PP2A. In CSF-1-stimulated BMM, OA inhibited DNA synthesis with maximal inhibition at 50 nM, a concentration shown to be specific for PP2A [35], suggesting that PP2A activity is required for progression through the G_1 phase of the cell cycle in BMM. With the use of a staggered addition of OA subsequent to CSF-1 treatment in conjunction with DNA content analysis, our results indicate for the first time that OA can induce an early G_1-phase or an S-phase block at the same low concentration (50 nM); these results suggest that PP2A activity can be required at two points in the cell cycle. Others have shown that low OA concentrations can induce a G_2/M block in human myeloid leukaemic cells; in the same cells but only at high doses (500 nM) could OA cause a block at G_1/S phase [43].

Our results also demonstrate that CSF-1 induces an early phosphatase activity, a proportion of which is likely to be due to PP2A, given its sensitivity to OA. It could be that this early PP2A activity is required for the induction of cell cycle progression and its inhibition by OA might lead to the early G_1-phase block observed (Figures 2B and 3). It is unlikely that this early PP2A activity is associated with S-phase progression because OA added to BMM later than 2 h after stimulation with CSF-1 allows progression through G_1 phase but results in the accumulation of cells in S phase. It could be that the PP2A whose levels were found to be induced by CSF-1 action after 2 h (Figures 1A and 1B) is in fact the PP2A pool responsible for S-phase progression and whose inhibition by OA leads to accumulation of BMM in S phase. Obviously, further detailed monitoring of PP2A activity throughout the BMM cell cycle will be needed to clarify these issues.

In other systems, the inhibition of PP2A by OA can lead to cell proliferation [3,13,44]; our results suggest that BMM are different. PP2A inhibition has been shown to activate many key

proteins controlling cell proliferation, including signal transduction molecules such as ERKs [3,4] and the transcription factor AP-1 [11]; PP2A activity can modulate cell cycle components such as cdc2 [5]. Given that PP2A activity is increased rapidly on stimulation with CSF-1 with similar kinetics to the induction of ERK activity and c-*fos* mRNA expression [29], it might be expected that PP2A activity would be involved in their modulation in CSF-1-treated BMM. However, the lack of effect of OA on both ERK and c-*fos* modulation by CSF-1 (Figures 5C and 5D respectively) does not support such an involvement of PP2A in these cells. In addition, the different degrees of inhibition of CSF-1-stimulated DNA synthesis in BMM by OA and PD98059 (compare Figure 2A with Figure 5B) support the dissociation of PP2A and ERK activities in contrast with what has been been reported uniformly for other systems. It has been reported that differential expression of the regulatory subunit and/or of the variable subunit of PP2A can modulate the substrate specificity of the enzyme, for example by altering its intracellular localization [45]; perhaps such a mechanism might direct PP2A to substrates other than ERK in BMM. Although these results are different from those published in other reports showing that PP2A negatively regulates cell cycle progression by dephosphorylating ERKs [3,4,10,13], they are consistent with recent papers suggesting that ERK activation does not necessarily correlate with proliferation [29,46–48]. The use of PD98059 on CSF-1-stimulated BMM suggests that significant DNA synthesis can occur in these cells in the absence of significant activation of ERK and expression of c-*fos* mRNA.

Because our results for BMM suggest that PP2A influences cell cycle progression independently of ERKs, we used 2D SDS/PAGE to identify other possible targets of PP2A action. Such analysis of CSF-1-treated, ^{32}P-labelled BMM showed a widespread increase in protein phosphorylation compared with CSF-1-deprived BMM. This increase in response to CSF-1 in BMM was relatively unaffected by pretreatment with OA, with the exception of a few proteins. Three of these proteins (25/4.7, 26/5.4 and 27/5.5) were not phosphorylated in response to CSF-1 alone but were phosphorylated in response to pretreatment with OA before stimulation with CSF-1, suggesting that they are not normally regulated in response to CSF-1 within 4 min, but are downstream of PP2A activity. Two other proteins (66/4.7 and 62/6.0) were phosphorylated in response to stimulation with CSF-1 but their phosphorylation state was greatly enhanced by pretreatment with OA, suggesting they are phosphorylated by CSF-1-regulated kinases and dephosphorylated by PP2A. The OA-induced phosphorylation of the proteins not phosphorylated in response to CSF-1 and/or the hyperphosphorylation of the CSF-1-sensitive proteins in response to OA pretreatment might contribute to the early G_1-phase block observed in OA-treated CSF-1-stimulated BMM. Characterization of these proteins might reveal critical G_1-phase regulators and/or new PP2A substrates.

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