A novel 110 kDa form of myosin XVIIIA (MysPDZ) is tyrosine-phosphorylated after colony-stimulating factor-1 receptor signalling

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Macrophage colony-stimulating factor (M-CSF or CSF-1) controls the development of macrophage lineage cells via activation of its tyrosine kinase receptor, c-Fms. After adding CSF-1 to M1 myeloid cells expressing CSF-1R (CSF-1 receptor), tyrosine phosphorylation of many cellular proteins occurs, which might be linked to subsequent macrophage differentiation. The biological significance and characterization of such proteins were explored by a dual strategy comprising two-dimensional SDS/PAGE analysis of cell lysates of CSF-1-treated M1 cells expressing the wild-type or a mutated receptor, together with an enrichment strategy involving a tyrosine-phosphorylated receptor construct. In the present study, we report the identification by MS of a novel, low-abundance, 110 kDa form of myosin XVIIIA (MysPDZ, myosin containing PDZ domain), which appears to be preferentially tyrosine-phosphorylated after CSF-1R activation when compared with other known isoforms. Receptor mutation studies indicate that CSF-1R-dependent tyrosine phosphorylation of p110myosin XVIIIA requires Tyr-559 in the cytoplasmic domain of the receptor and is therefore Src-family kinase-dependent. Gelsolin, Erp61 protein disulphide-isomerase and possibly non-muscle myosin IIA were also tyrosine-phosphorylated under similar conditions. Similar to the more abundant p190 isoform, p110 myosin XVIIIA lacks a PDZ domain and, in addition, it may lack motor activity. The phosphorylation of p110 myosin XVIIIA by CSF-1 may alter its cellular localization or target its association with other proteins.

Key words: colony-stimulating factor-1 receptor, macrophage differentiation, myosin XVIIIA, Src kinase, two-dimensional SDS/PAGE, tyrosine phosphorylation.

INTRODUCTION

Macrophage colony-stimulating factor (M-CSF or CSF-1) controls the development of the macrophage lineage from bone-marrow precursors [1]. Its receptor is the homodimeric tyrosine kinase, c-Fms, which dimerizes and undergoes autophosphorylation after CSF-1 (colony-stimulating factor-1) binding and has a so-called kinase insert region [2,3]. The molecular pathways governing CSF-1-driven proliferation and differentiation are beginning to be unravelled [1,4]. One approach is to assess the role of tyrosine residues in CSF-1R (CSF-1 receptor) because of their capacity to bind to signalling molecules by domains such as SH2 (Src homology 2) domain and PTB (phosphotyrosine-binding) domain [5,6]. A number of tyrosine residues in the cytoplasmic domain of CSF-1R are phosphorylated after CSF-1 action [2]; mutational studies have been performed on some of these tyrosine residues to gain an insight of the downstream pathways and functional consequences depending on the particular tyrosine in question [1]. It is becoming apparent that the nature of the cell harbouring the receptor can determine the consequences of mutating a particular tyrosine [7,8]. Tyr^{559} in the JX (juxtamembrane) domain and Tyr^{807} in the CT (C-terminal) kinase domain of the murine CSF-1R (Tyr^{561} and Tyr^{809} in the human receptor respectively) are two such residues whose functions have been assessed. The former appears to be a binding site for Src-family kinases [9–11], whereas the latter has been suggested to play a role in CSF-1-driven differentiation [1] or proliferation [12], depending on the cell type in which it is expressed [8].

Leukaemic cell lines are convenient model systems to analyse the molecular pathways governing haematopoietic differentiation and to determine how interference with these pathways can lead to the leukaemic state. The murine myeloblastic cell line, M1, was generated from a spontaneous leukaemia and can be induced to undergo macrophage-like differentiation by a number of agents including interferons [13], interleukin-6 [14], LIF (leukaemia inhibitory factor) [14] and oncostatin M [15]. Previous studies on the differentiation of M1 cells into macrophage-like cells with a number of different stimuli reported a gradual differentiation in development [13]. The CSF-1R (c-Fms) appeared during these developmental changes in response to LIF [16], transfection of ets-2 [17] or the Wilms' tumour suppressor gene [18]. We reasoned that transfection of the CSF-1R may lead to a rapid and significant induction of a macrophage-like phenotype when

Abbreviations used: BMM, bone-marrow-derived macrophage; CLB, cell lysis buffer; CSF, colony-stimulating factor; CSF-1R, CSF-1 receptor; M-CSF, macrophage CSF; CT, C-terminal; 1D, one-dimensional; ERK, extracellular-signal-regulated kinase; FBS, foetal bovine serum; GST, glutathione S-transferase; HRP, horseradish peroxidase; JX, juxtamembrane; LIF, leukaemia inhibitory factor; MysPDZ, myosin containing PDZ domain; NBCS, newborn calf serum; NP40, Nonidet P40; PY, phosphotyrosine; Shc, Src-homology collagen; SH2 domain, Src homology 2 domain; TBS, Tris-buffered saline.

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CSF-1 is added and thereby provide a convenient cellular model to explore CSF-1-driven differentiation pathways. Accordingly, it was found that, after transfection with murine CSF-1R, M1 cells differentiated rapidly into macrophage-like cells in response to CSF-1 as judged by morphological changes, Mac-1 expression and reduced proliferation rate [11]. Therefore this system provides a convenient model for CSF-1-induced differentiation, while also allowing for analysis of the effects of mutating key residues, e.g. tyrosine residues. In separate studies of this system, we had found previously that a mutation of Tyr^{559} to phenylalanine in the CSF-1R led to reduced CSF-1-induced differentiation and Src tyrosine phosphorylation [11], whereas a corresponding mutation in Tyr^{807} led to a differentiation defect which depended on Shc (Src-homology collagen) tyrosine phosphorylation [4].

Tyrosine phosphorylation of signal transduction molecules occurs after the interaction of a ligand, such as CSF-1, with its cognate receptor and is easily monitored by probing or immunoprecipitating with anti-PY antibodies (where PY stands for phosphotyrosine). Since tyrosine-phosphorylated forms of signalling proteins are probably present in low abundance after cell activation by ligands, enrichment procedures may be necessary for the mapping of signalling pathways involving the characterization of these protein forms. In the present study, we used a combination of 2D (two-dimensional) SDS/PAGE, an enrichment procedure involving binding to GST (glutathione S-transferase) fusion constructs of the CSF-1R and MS to identify the proteins that were tyrosine-phosphorylated after CSF-1 treatment by a Src (Tyr^{559})-dependent pathway. We were able to identify a novel p110 kDa form of myosin XVIIIA (MysPDZ, myosin containing PDZ domain) which is tyrosine-phosphorylated in an Src-dependent manner after CSF-1R signalling.

EXPERIMENTAL

Reagents

DMEM (Dulbecco's modified Eagle's medium), streptomycin and penicillin were purchased from ICN–Flow Laboratories (Sydney, NSW, Australia) and FBS (foetal bovine serum) and NBCS (newborn calf serum) were from Commonwealth Serum Laboratories (Parkville, Victoria, Australia). Recombinant human CSF-1 was a gift from Chiron Corp. (Emeryville, CA, U.S.A.). The following primary antibodies were used: rabbit polyclonal non-muscle myosin II heavy chain isoform A (Covance Research Products, Princeton, NJ, U.S.A.), mouse monoclonal 4G10 conjugated with HRP (horseradish peroxidase) (4G10–HRP; Upstate Biotechnology), rabbit polyclonal phospho-ERK (ERK stands for extracellular-signal-regulated kinase; New England Biolabs, Beverly, MA, U.S.A.), rabbit polyclonal anti-Src, goat polyclonal anti-ERK-2, goat polyclonal anti-gelsolin, goat polyclonal anti-Erp61 and mouse monoclonal anti-$p52^{Shc}$ antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). The following secondary antibodies were used: HRP-conjugated, affinity-purified, swine immunoglobulins to rabbit immunoglobulins (Dako, Glostrup, Denmark) and HRP-conjugated rabbit immunoglobulins to mouse immunoglobulins (Dako).

Cells

M1 murine myeloid cells were first depleted of any $CSF\text{-}1R^+$ ($c\text{-}Fms^+$) cells [11] and then transfected with wild-type c-*fms* (M1/WT) or c-*fms* with the Tyr^{559} mutated to phenylalanine (M1/559) or Tyr^{807} mutated to phenylalanine (M1/807), as described previously [4,11]. After stable transfection, clones of the M1 populations were pooled and then sorted by FACS to achieve a mixed population of cells with similar receptor number.

Murine BMMs (bone-marrow-derived macrophages) were grown and treated with CSF-1 as described in [19].

Cell culture and differentiation

M1 cell lines were derived, grown and treated with CSF-1 as described previously [4,11]. Mac-1 expression was examined as follows [11]: 2×10^5 cells were washed twice with ice-cold TBS (Tris-buffered saline) containing 1 % FBS, resuspended in 50 μl of either anti-Mac-1 hybridoma cell supernatant or isotype-matched control (rat anti-mouse $IgG2_b$) and then left on ice for 1.5 h. The cells were washed three times with ice-cold TBS containing 1 % FBS, resuspended in FITC-conjugated anti-rat IgG and left on ice for a further 30 min. Cells were then washed twice with ice-cold TBS containing 1 % FBS and resuspended in 500 μl of TBS. Fluorescence was measured using an FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.). Acquisition was restricted to 10 000 events for each sample and Mac-1-positive cells were calculated by subtracting the isotype-matched control value from the Mac-1-positive value. Results were analysed by using CellQuest version 3.0.1 (BD Biosciences Immunocytometry Systems, San Jose, CA, U.S.A.). Student's t tests were performed on the percentage of differentiated cells, and a $P \leqslant 0.05$ was considered to be statistically significant. Proliferation was assessed by counting the cells every 24 h for 3 days. Student's t tests were performed on the cell number on day 3.

CSF-1R surface expression

M1 cell lines were assessed for CSF-1R surface expression as described previously [11]. Briefly, 2×10^5 cells were washed twice with ice-cold TBS containing 1 % FBS, resuspended in 50 μl of either anti-CSF-1R hybridoma cell supernatant [20] or isotype-matched control (rat anti-mouse $IgG2_a$) and then left on ice for 1.5 h. The method used was similar to the method described above for Mac-1 surface expression.

NP40 (Nonidet P40) lysate preparation

NP40 lysates were prepared as described in [21] and contained a complete protease inhibitor cocktail (Mini; Roche, Mannheim, Germany).

2D SDS/PAGE

Immobiline dry strips (pH 3–10; linear; Amersham Biosciences, Uppsala, Sweden) were rehydrated overnight [8 M urea/0.5 % (v/v) Triton X-100/0.5 % (v/v) Pharmalytes 3–10/0.01 M dithiothreitol]. Cell lysate (100 μg), diluted 1:4 in sample solution (9 M urea/0.06 M dithiothreitol/2 % Pharmalytes 3–10/0.5 % Triton X-100), was applied to the rehydrated strips and run on a horizontal Multiphor II Electrophoresis unit (Amersham Biosciences) at 20 °C for a total of 100 kV/h. The second-dimension separation was performed as described previously [22].

Prestained standards (BenchMark) from Invitrogen (Carlsbad, CA, U.S.A.) were used for apparent molecular-mass assignments.

Western-blot analysis of SDS/polyacrylamide gels

Gels were analysed by Western blotting as described in [22]. In some cases, the blots were stripped of antibodies by extensive washing with stripping buffer containing 62.5 mM Tris/HCl, 0.1 M 2-mercaptoethanol and 2 % (w/v) SDS, then blocked with a 3 % (w/v) BSA and 1 % (w/v) ovalbumin solution for 1 h at room temperature (25 °C) and, finally, re-probed with different antibodies, as described previously [21].

Affinity enrichment and identification of CSF-1R domain-associated phosphoproteins

CSF-1R domain fusion protein constructs were prepared by standard PCR methods to amplify the JX (amino acids 540–595) and CT (amino acids 681–976) coding regions of the CSF-1R. The PCR products were subsequently cloned as *Bam*HI–*Eco*RI fragments into pGEX-2TK (Amersham Biosciences) and introduced into the *Escherichia coli* strain TKX1 (Stratagene, La Jolla, CA, U.S.A.), which contains an inducible tyrosine kinase. Tyrosine phosphorylation was confirmed by Western-blot analysis with anti-PY antibody. The GST fusion proteins were induced as described previously [23] and purified from the bacterial cell lysates by affinity-binding to glutathione–Sepharose beads (Amersham Biosciences) [24].

M1/WT cells (1×10^9 cells) were treated with CSF-1 (5000 units/ml) and NP40 extracts were prepared as described above. Extracts were incubated (4 °C, 30 min) with fusion protein (10 mg), washed with 10 vol. of CLB (cell lysis buffer) and eluted in 100 μl of CLB containing 100 mM phenylphosphate (Sigma). The eluted material was dialysed overnight in TBS (4 °C) and concentrated using a Minicon concentrator (Amicon, Beverly, MA, U.S.A.) according to the manufacturer's instructions.

Peptides were extracted from digests [25] and desalted on Zip Tip microcolumns (Millipore, Bedford, MA, U.S.A.) before being loaded on to a Voyager Elite MALDI-TOF (matrix-assisted laser-desorption ionization–time-of-flight) mass spectrometer (PerSeptive Biosystems, Framingham, MA, U.S.A.). Peptide profiles were searched against a non-redundant protein database of NCBI (National Centre for Biotechnology Information) [26].

Antiserum against myosin XVIIIA

A region of the cDNA for KIAA0216 (GenBank® accession no. D86970), encompassing the peptides determined from the p110 form of myosin XVIIIA (MysPDZ) and spanning residues 3651–4317, was subcloned into a pGEX vector, expressed and then purified as described previously [23]. An antiserum against myosin XVIIIA was raised by immunization of rabbits; the IgG fraction was purified using a Protein A–Sepharose column (Amersham Biosciences) and is referred to as anti-myosin XVIIIA.

Immunoprecipitation

Lysates were precleared by incubating 500 μg of NP40 lysates from appropriately treated cells with 50 μl of a 25 % (v/v) slurry of Protein A–Sepharose 4B (Amersham Biosciences) with rotation for 30 min at 4 °C. The samples were then centrifuged at 16 060 ***g*** for 5 min and the pellets were discarded. The samples were then incubated overnight with 1 μg of antibody with rotation at 4 °C; 50 μl of a 25 % slurry of Protein A–Sepharose 4B was added and the mixture was incubated for 1 h at 4 °C. The beads were then pelleted and washed three times with CLB. Samples were boiled for 5 min in SDS/PAGE sample buffer, subjected to 1D SDS/PAGE (10 % gel) and transferred on to a nitrocellulose membrane for Western-blot analysis.

Protein staining

SDS/polyacrylamide gels were stained overnight with 0.1 % (w/v) Coomassie Blue (PhastGel Blue R250 stain; Amersham Biosciences) in 25 % (v/v) methanol and 10 % (v/v) acetic acid, destained in 25 % methanol and 10 % acetic acid and then dried.

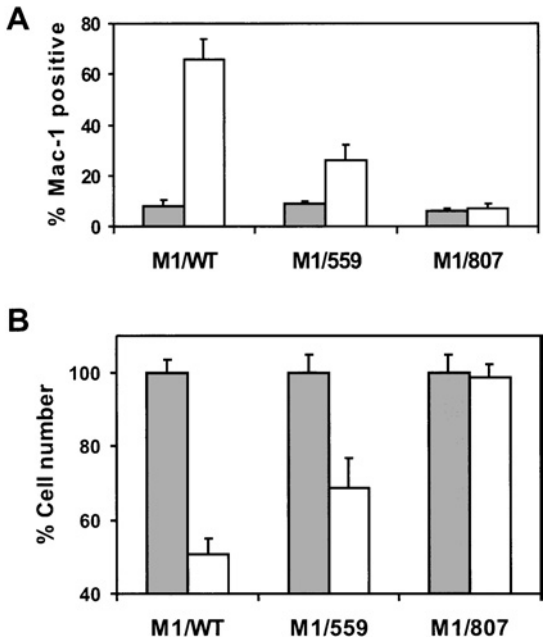

Figure 1 CSF-1-induced differentiation in M1 cells

(**A**) M1/WT, M1/559 and M1/807 cells were cultured for 72 h in 10 % (v/v) NBCS alone (untreated; shaded bars) or with CSF-1 (5000 units/ml; open bars). Cells were stained with either Mac-1-reactive or isotype-control monoclonal antibodies and assessed for Mac-1 expression by flow cytometry. Results are expressed as means ± S.D. for triplicate assays from a representative of three independent experiments. (**B**) Cells were cultured as in (**A**) and viable cell numbers were measured. Results are the means ± S.E.M. for triplicate cultures from a representative experiment that was repeated twice more. Cell numbers were standardized as a percentage of those observed in each cell line in NBCS alone.

Quantitative determination of phosphorylation

ECL® autoradiographs were scanned using a computerized laser densitometer (Molecular Dynamics, Sunnyvale, CA, U.S.A.) and the images were analysed using Image Quant software (Molecular Dynamics).

RESULTS

CSF-1-induced differentiation paradigm in M1 cells

Effect of mutations at Tyr^{559} and Tyr^{807} in CSF-1R

We engineered M1 cells to express the normal (wild-type) CSF-1R (previously, they were termed M1/WT cells to distinguish them from cells engineered to express mutant CSF-1R forms by depleting the small numbers of CSF-$1R^+$ cells from a starting M1 cell population, followed by CSF-1R transfection [11]). The M1/WT and M1 cells expressing mutated CSF-1R were subsequently used as pools of individual clones expressing similar CSF-1R levels ([11]; see below). We have shown previously that CSF-1-treated M1/WT cells, but not parental M1 cells, underwent macrophage-like differentiation, as judged by morphology, increased forward and side-scatter characteristics, increased adherence and Mac-1 expression, and reduced proliferation [11]. We have reported that M1 cells expressing the CSF-1R with a mutation at Tyr^{559} (M1/559 cells) demonstrated a partial loss in differentiation capability after CSF-1 treatment [11]; M1 cells with a mutation at Tyr^{807} (M1/807 cells) exhibited a more significant block [4]. For the various cell populations, the effects of CSF-1 in the same experiment on two criteria of differentiation, namely Mac-1 expression and cell proliferation, are shown in Figures 1(A) and 1(B).

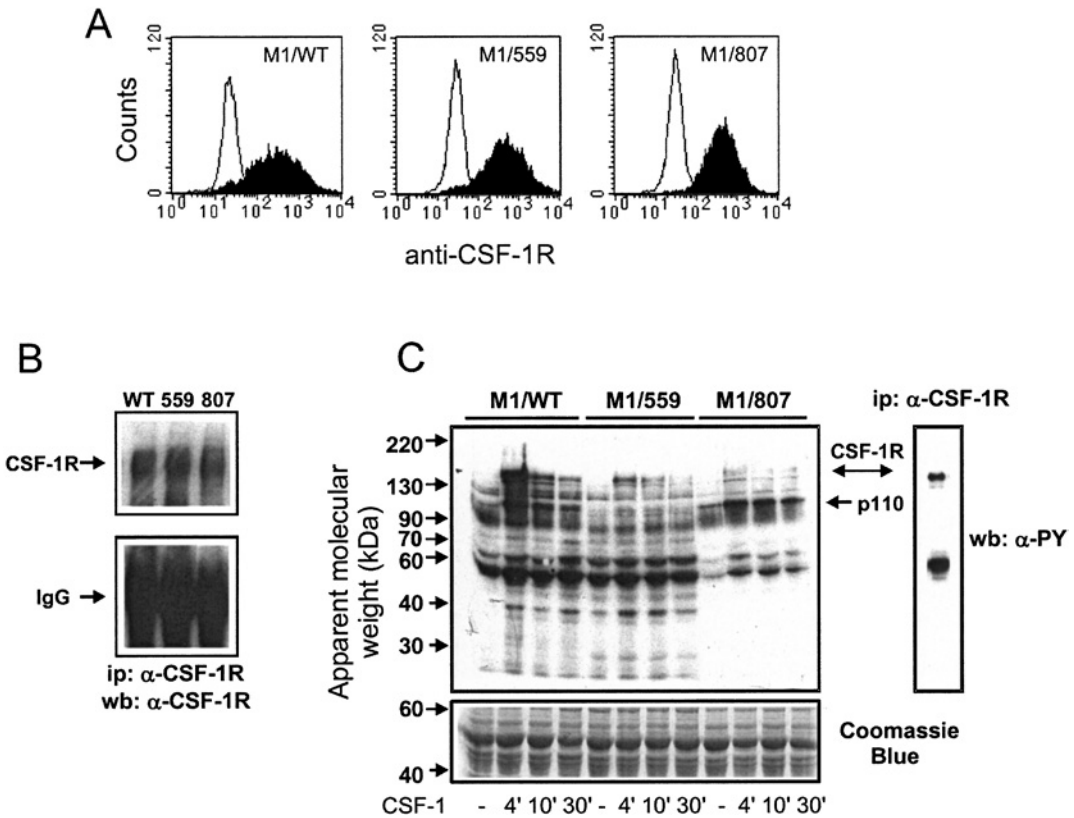

Figure 2 Phosphotyrosine analysis of CSF-1-treated M1/WT, M1/559 and M1/807 cells by 1D SDS/PAGE

(**A**) M1/WT, M1/559 and M1/807 cells were stained with either anti-CSF-1R monoclonal antibody (closed plots) or isotype control (open plots) and assessed for CSF-1R surface expression by flow cytometry (see the Experimental section). Representative histograms are shown. (**B**) M1/WT, M1/559 and M1/807 cells were cultured in 10% NBCS and protein extracts were as described in the Experimental section. The levels of CSF-1R (c-Fms) in each cell type were determined by Western-blot analysis using anti-CSF-1R antibodies. (**C**) M1/WT, M1/559 and M1/807 cells were either untreated or treated with CSF-1 (5000 units/ml) for various time periods as indicated. The kinetics of tyrosine phosphorylation was determined by probing Western blots of cell lysates with an anti-PY (α-PY) antibody (upper panel; see the Experimental section). The lower panel depicts the same lysates stained with Coomassie Blue as a loading control. The identity of CSF-1R was determined by immunoprecipitating (ip) with the anti-CSF-1R (α-CSF-1R) antibody and probing the Western blots with α-PY (right panel). The results presented are representative of three independent experiments.

CSF-1 induced tyrosine phosphorylation and M1 cell differentiation

The relative extent of CSF-1-dependent differentiation cannot be accounted for by variation in CSF-1R expression in the different M1 cell lines. The comparable levels of cell-surface CSF-1R levels (flow cytometry) and the Western blot of CSF-1R (c-Fms) levels in cell lysates of untreated cell lines are shown in Figures 2(A) and 2(B) respectively. In previous studies, we explored the signal transduction pathways that were dependent on Tyr^{559} (the Src-binding site) and Tyr^{807} (with the above M1 cells). Evidence was obtained for the involvement of Src [11] and Shc [4] in CSF-1-driven macrophage differentiation in this system. In the present study, we examined whether analysis of the tyrosine phosphorylation profiles obtained after CSF-1 treatment of various M1 populations might be a useful strategy to help define the signal transduction cascades downstream of Tyr^{559} and Tyr^{807}. These signal transduction cascades may prove critical for CSF-1-R-dependent cellular changes, particularly differentiation.

In other cells, CSF-1 induces tyrosine phosphorylation of numerous downstream proteins in whole cell extracts [1,4,9,27–29]; this also occurs in M1/WT cells within 4 min ([11]; Figure 2C) and, as seen in other cell types, is transient with the phosphorylation approaching basal levels within 30 min after stimulation. Interestingly, it can also be observed in Figure 2(C) that, viewed overall, the extent of the CSF-1-induced tyrosine phosphorylation in M1/559 and M1/807 cells mirrors the ability of these cell lines to differentiate in response to CSF-1 (Figure 1). Analysis of the respective apparent molecular-mass patterns of the tyrosine-phosphorylated proteins indicates similarities between CSF-1-treated M1/WT and M1/559 cells except for the obvious reduction in the extent of tyrosine phosphorylation of a number of proteins, particularly proteins in the range of approx. 110–180 kDa (Figure 2C). By comparison with both M1/WT and M1/559 cells, there is an even more widespread reduction in the degree of tyrosine phosphorylation of many proteins in CSF-1-treated M1/807 cells. However, there is some selective dysregulation of tyrosine phosphorylation. For example, a 110 kDa band (Figure 2C, p110) is poorly phosphorylated in CSF-1-treated M1/559 cells, but its tyrosine phosphorylation appears to be relatively normal in CSF-1-treated M1/807 cells (i.e. similar to that in M1/WT cells). Taken together, these results suggest that the decreased ability of CSF-1-treated M1/559 and M1/807 cells to differentiate is due to significant and, in some cases, specific perturbations in signal transduction cascades involving tyrosine phosphorylation.

It is reasonable to expect that the extent of tyrosine phosphorylation of the CSF-1R itself may reflect the overall relative degree of tyrosine phosphorylation found in the CSF-1-treated M1 cell lines of the present study. Tyrosine phosphorylation of a

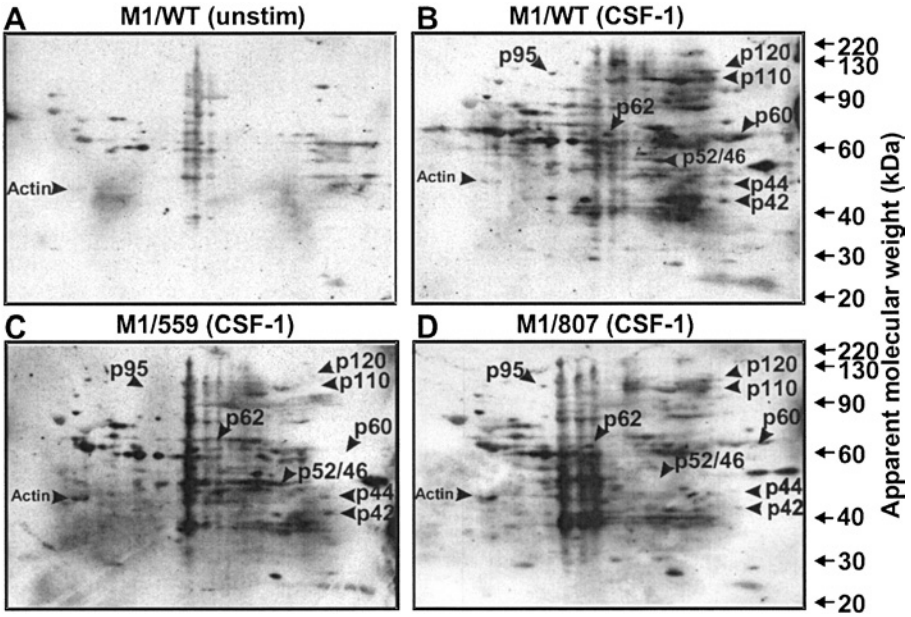

Figure 3 Phosphotyrosine analysis of CSF-1-treated M1/WT, M1/559 and M1/807 cells by 2D SDS/PAGE

M1/WT, M1/559 and M1/807 cells were untreated or treated with CSF-1 for 4 min or treated with CSF-1 (5000 units/ml). Protein extracts were resolved by 2D SDS/PAGE and patterns of tyrosine phosphorylation were determined by Western-blot analysis using α-PY. (**A**) Untreated M1/WT cells; (**B**) CSF-1-treated M1/WT cells; (**C**) CSF-1-treated M1/559 cells; (**D**) CSF-1-treated M1/807 cells. Some proteins were reproducibly observed to be specifically affected by either of the CSF-1R mutations; these proteins (indicated by arrows) include p120, p110, p95, p62, p60, p52, p46, p44 and p42 (numbers refer to mass in kDa). The results presented are representative of five independent experiments.

165 kDa protein was observed to increase after CSF-1 treatment of M1/WT cells (Figure 2C). Previous immunoprecipitation studies with these cells have demonstrated that this protein is the CSF-1R ([11]; see also Figure 2C). The relative degrees of CSF-1-dependent CSF-1R tyrosine phosphorylation were similar to those for the overall tyrosine phosphorylation in the CSF-1-treated M1/559 and M1/807 cells and paralleled the loss in their ability to differentiate in response to CSF-1 (Figures 1 and 2C). The reduced overall tyrosine phosphorylation in CSF-1-treated M1/559 cells compared with CSF-1-treated M1/WT cells is presumably related to loss of Src activation (Tyr^{559} has been reported to be the Src-binding site [9,11]), whereas that in CSF-1-treated M1/807 cells may be related to a reduction in CSF-1R autokinase activity [1].

Strategies for the identification of CSF-1R-associated phosphoproteins with a putative role in differentiation

2D SDS/PAGE analysis

We next determined whether some of the above tyrosine-phosphorylated proteins observed after 1D SDS/PAGE analysis could be identified by 2D SDS/PAGE. The increased tyrosine phosphorylation of many proteins can be seen again in CSF-1-treated M1/WT cells as was the relative degree across the various M1 cell populations (Figures 3A–3D). We also found comparable results when the three CSF-1-treated M1 populations were ^{35}S-methionine labelled to improve detection sensitivity and the lysates immunoprecipitated with anti-PY antibodies (results not shown). The corresponding Coomassie Blue-stained protein profiles demonstrated the relative invariance of sample loading, electrophoretic conditions and proteomes (results not shown). To assist the 2D SDS/PAGE analysis of the various tyrosine phosphorylation patterns, we used actin as a standard phosphoprotein, which is found to be tyrosine-phosphorylated in each of the CSF-1-treated M1 populations (Figures 3A–3D; also see below).

We have found previously that, compared with CSF-1-treated M1/WT cells, Src kinase phosphorylation and association with Tyr^{559} CSF-1R were both significantly reduced after stimulation of M1/559 cells with CSF-1 [11]. Immunoprecipitation of CSF-1-treated cell lysates with anti-PY antibody, followed by 2D SDS/PAGE and re-probing with anti-PY and anti-c-Src antibodies, demonstrated that the p60 phosphoprotein labelled in Figure 3(B) is c-Src (results not shown). A significant reduction in its level of tyrosine phosphorylation can be observed (cf. Figure 3B with Figure 3C), as well as a relative maintenance of the level in CSF-1-treated M1/807 cells (cf. Figure 3B with Figure 3D). These relative changes were also confirmed by immunoprecipitation (results not shown). This approach also identified p52/46 as Shc, confirming our previous findings [4]. The significant reduction in its tyrosine phosphorylation in CSF-1-treated M1/807 cells (cf. Figure 3B with Figure 3D) is also consistent with our previous results [4]; there is a smaller reduction in its relative degree of tyrosine phosphorylation in CSF-1-treated M1/559 cells. The p44/42 proteins (Figure 3B) were similarly shown to be ERKs, with a similar large relative reduction in tyrosine phosphorylation in CSF-1-treated M1/807 cells being consistent with the relative loss of ERK enzymic activity shown by us in a previous study [4]; the smaller relative reduction in ERK tyrosine phosphorylation in CSF-1-treated M1/559 cells was again similar to the results obtained for Shc and was reflected in the change in its kinase activity (N. Wilson and J. A. Hamilton, unpublished work).

Enrichment and identification of other CSF-1R-dependent tyrosine-phosphorylated proteins

After CSF-1R activation, a number of tyrosine residues in its various cytoplasmic domains, e.g. the JX and CT domains, are tyrosine-phosphorylated [1,7,8]. It is possible that some signalling proteins, including those that are also tyrosine-phosphorylated

downstream of CSF-1R activation, associate with phosphorylated tyrosine residues in specific domains of the activated CSF-1R, either directly or indirectly via other binding proteins. To be able to characterize these associated signalling proteins, enrichment strategies need to be developed. For this purpose, we attempted to mimic, to some extent, regions of the activated CSF-1R by expressing the JX [11] and CT domains of the cytoplasmic region of the CSF-1R as GST fusion proteins in TKX1 *E. coli*, a strain containing an inducible tyrosine kinase (see the Experimental section). We were able to confirm that tyrosine phosphorylation was induced in the constructs. These constructs allow large amounts of cell lysates to be examined and, therefore, we used them in an attempt to enrich, and then characterize, the associated tyrosine-phosphorylated proteins in lysates of CSF-1-treated M1/WT cells.

To test this strategy, we measured Shc association with the constructs because of its presence in complexes containing the activated CSF-1R [30] and because of the above results. It can be seen in Figure 4(A) that binding of Shc (p46, p52; see above) was observed only with tyrosine-phosphorylated JX (JX-PY) and not with its non-phosphorylated form; in addition, there was no detectable binding to unphosphorylated or tyrosine-phosphorylated GST. When the tyrosine-phosphorylated JX and CT constructs were applied to lysates from CSF-1-treated M1/WT cells, a number of tyrosine-phosphorylated proteins could be shown to associate in a construct-specific manner (Figure 4B). The phosphoproteins were not detected in untreated M1/WT cells (results not shown).

For JX-PY, it was observed that there were tyrosine-phosphorylated proteins (p120, p110, p95 and p62) that associated with it (Figure 4B) and these proteins could be the same as those whose tyrosine phosphorylation was Tyr^{559}-dependent in CSF-1-treated cells (see Figures 3B and 3C). We attempted to determine whether it is possible to affinity-purify them in sufficient quantities for sequence determination by MS. As shown in Figure 4(C), enrichment was obtained with this approach and a number of Coomassie Blue-stained bands could be observed. A different profile was found with the corresponding enrichment procedure using the CT-PY construct and is included for comparison. The molecular masses of many of the bands associating with both constructs are similar to what was shown above by PY blotting; therefore they could be the same proteins.

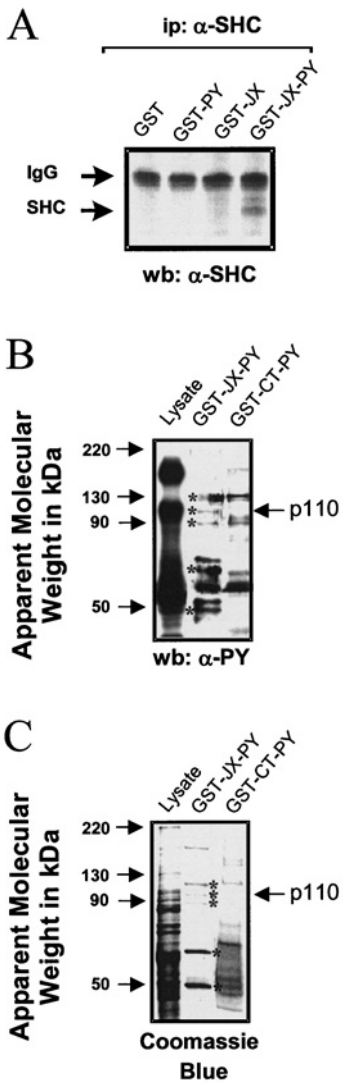

Figure 4 Affinity enrichment of GST–JX-PY-associated tyrosine-phosphorylated proteins in CSF-1-treated M1/WT cells

(**A**) M1/WT cells were treated with CSF-1 (5000 units/ml) for 4 min; NP40 lysates were extracted and incubated with unphosphorylated GST (GST), tyrosine-phosphorylated GST (GST-PY), GST–JX and GST–JX-PY respectively (see the Experimental section). After incubation, Shc was released from the GST fusion protein–glutathione resin pellet using phenylphosphate before immunoprecipitation (ip) with anti-SHC antibodies, separation of the proteins by 1D SDS/PAGE and Western blotting (wb) with anti-SHC antibodies. (**B**) The same cell lysates were incubated with either GST–JX-PY or GST–CT-PY fusion proteins. The bound fractions were separated by 1D SDS/PAGE and the blots were probed with anti-PY. The p120, p110, p95, p62 and p45 bands are labelled (asterisk). (**C**) JX-PY- and CT-PY-bound proteins were resolved by 1D SDS/PAGE and stained with Coomassie Blue. The p120, p110, p95, p62 and p45 bands, which associated with JX-PY (indicated by asterisk), were identified by MS (see Table 1). These bands appear to correlate with the bands labelled in (**B**) and might therefore represent the same proteins.

Identification of a new form of the unconventional myosin, myosin XVIIIA (MysPDZ)

The p110 protein binding to JX-PY was isolated from the 1D SDS/polyacrylamide gel (Figure 4C) and identified by MS as an isoform of myosin XVIIIA (Table 1; the human gene is Myo18A [31]). This unconventional myosin was initially named MysPDZ (myosin containing PDZ domain) and, as the name suggests, it is a PDZ-domain-containing myosin-like protein [32]. MS results suggest that this p110 isoform of myosin XVIIIA lacks the PDZ domain and the majority of the myosin head domain, given the position of the amino acids identified (see Table 1) and the apparent molecular mass of the isoform (see Figures 4C and 5B). We shall refer to this isoform as p110myosin XVIIIA (see the Discussion section). However, myosin XVIIIA has been reported to be expressed only as 230, 205 and 190–195 kDa forms in cells [32]. Very recently, Mori et al. [33] have shown by Western-blot analysis that by far the most predominant form of myosin XVIIIA in M1 cells is a 190 kDa band, coded by the so-called 'MysPDZβ' mRNA; however, the protein lacks the PDZ domain. There was a weak band at 230 kDa, coded by the so-called 'MysPDZα' mRNA; this isoform contains the PDZ domain [33]. A 110 kDa form has not been reported.

In the present study, we confirmed (Figure 5A) the presence of p110myosin XVIIIA in lysates of untreated and CSF-1-treated M1/WT cells by immunoprecipitation and Western-blot analysis with an antibody raised against a region which encompasses, but is not restricted to, the p110 isoform (see the Experimental section). Bands of 110 and 190 kDa were detected, with the 190 kDa band

Table 1 Proteins that associate with GST–JX-PY after stimulation of M1/WT cells with CSF-1

M1/WT cells were treated with CSF-1 (5000 units/ml) for 4 min; NP40 lysates were extracted and incubated with GST–JX-PY, eluted and resolved by 1D SDS/PAGE (see the Experimental section; Figure 4C). Some proteins which are bound to the GST–JX-PY (Figure 4C) were identified by MALDI–TOF-MS. Accession numbers are indicated. The positions of the peptides observed for p110myosin XVIIIA in relation to the full-length p230myosin XVIIIA molecule (or MysPDZ) are indicated. A schematic representation of the relationship between the p230 form and the p190 and p110 forms of myosin XVIIIA is presented in Figure 7.

Protein (kDa)	Sequence (position of the peptide from MysPDZ sequence [33])	Accession no. (identity %)	Identity
p110	FDVLAPHLTK (1121–1130)	BAA93660 (100)	Myosin XVIIIA (MysPDZ)
	ISELTSELTDR (1287–1297)	BAA93660 (100)	
	LHLEGQQVR (1140–1148)	BAA93660 (100)	
	NTGESASQLLDAETAER (1299–1320)	BAA93660 (100)	
	VVSLEAELQDISSQESKDEASLAK (1511–1535)	BAA93660 (100)	
p120	ADQLEQTKR	AAC36508 (100)	Non-muscle myosin heavy chain A
	AKQTLENERGELANEVK	AAC36508 (100)	
	DFSALESQLQDTQELLQEENRQK	AAC36508 (100)	
p95	SGALNSNDAFVLK	NP_666232 (100)	Gelsolin (actin-depolymerizing factor)
p62	FISDKDASVVGFFR	S41661 (100)	ERp61 (protein disulphide-isomerase); glucose-regulated protein; endoplasmic reticulum protein; phospholipase Cα
	DLLTAYYDVDYEK	S41661 (100)	
	ALEQFLQEYFDGNLKR	S41661 (87.5)	

representing $>90\%$ of antigenic material. The protein levels of the two myosin XVIIIA immunoreactive forms did not alter after a brief treatment with CSF-1 for 4 min.

Tyrosine phosphorylation of p110myosin XVIIIA by CSF-1

Preferential tyrosine phosphorylation of p110myosin XVIIIA by CSF-1

We then monitored the CSF-1-dependent tyrosine phosphorylation of the respective myosin XVIIIA forms in the immunoprecipitates by stripping and probing the same Western blots as above with anti-PY antibodies. As seen in Figure 5(A), both the p110 and the p190 forms were phosphorylated by treatment with CSF-1, with the relative anti-PY reactivity of the p110 being at least equal to that of the 190 kDa form, suggesting that the former might be preferentially tyrosine-phosphorylated. The anti-PY antibody also detected a band at 205 kDa in anti-myosin XVIIIA immunoprecipitates; a weak 230 kDa band was also detected by anti-PY antibodies (results not shown).

Src-dependent tyrosine phosphorylation of p110myosin XVIIIA

As mentioned above, we were able to show, by 2D SDS/PAGE analysis, that a p110 protein was tyrosine-phosphorylated in response to CSF-1 and the phosphorylation was dependent on the Tyr^{559} of CSF-1R (Figure 3). It is seen in Figure 5(B) that this protein co-migrates with an anti-myosin XVIIIA-reactive protein. The initial observation that the mutation of CSF-1R at Tyr^{559} impaired CSF-1-mediated differentiation and Src phosphorylation in M1 cells [11], and the fact that these changes correlated with a loss of p110myosin XVIIIA phosphorylation (Figures 3B and 5B), suggest that the latter tyrosine phosphorylation lies in a pathway dependent on Src activation. Furthermore, the purification strategy that led to the identification of the p110myosin XVIIIA in cell lysates involved its capacity to bind to the tyrosine-phosphorylated form of the JX domain of CSF-1R, which contains Tyr^{559} [9].

Tyrosine phosphorylation of p110myosin XVIIIA is increased by CSF-1 in macrophage-like cells

Having observed p110myosin XVIIIA to be rapidly tyrosine-phosphorylated in the murine leukaemic M1/WT cell line, we sought to determine if it was similarly present and phosphorylated acutely in response to CSF-1 in a primary cell type, namely BMM. After immunoprecipitation with anti-myosin XVIIIA antibodies, a faint band corresponding to the 110 kDa form of myosin XVIIIA was evident in BMM (Figure 5C, upper panel) which was tyrosine-phosphorylated by treatment with CSF-1 for 4 min.

Next, we investigated whether CSF-1-dependent myosin XVIIIA tyrosine phosphorylation was also observed in M1/WT cells that had undergone discernable macrophage differentiation in response to CSF-1 as opposed to the short-term studies above. Experiments in which M1/WT cells were cultured either in serum alone or in the presence of CSF-1 for 2 days revealed that, although p110myosin XVIIIA protein levels were not altered, it was tyrosine-phosphorylated in CSF-1-containing cultures and not in serum alone (Figure 5C, lower panel). The increase in tyrosine phosphorylation of p110myosin XVIIIA in M1/WT cells after CSF-1 treatment for 2 days correlates with CSF-1-induced differentiation into macrophage-like cells [11].

p120, p95 and p62 proteins

The other proteins identified by the enrichment strategy, namely p120, p95 and p62, were isolated from 1D SDS/polyacrylamide gels (Figure 4C) and identified by MS (see the Experimental section) as non-muscle myosin IIA, gelsolin and ERp61 protein disulphide-isomerase respectively (Table 1). The 45 kDa (p45) band (Figure 4C) was identified by MS as γ actin (results not shown).

The above results suggest that non-muscle myosin IIA, gelsolin and ERp61 might be tyrosine-phosphorylated by CSF-1 in M1/WT cells. However, to confirm this and to exclude artifacts due to co-migrating proteins, we endeavoured to immunoprecipitate the proteins in question and examine their tyrosine phosphorylation status by Western-blot analysis. By this approach, the tyrosine phosphorylation of both gelsolin and ERp61 in lysates of CSF-1-treated M1/WT cells could be confirmed (Figure 6); however, the only commercially available antibody to non-muscle myosin IIA failed to immunoprecipitate the protein (results not shown).

DISCUSSION

The present study continues those on the signalling cascades downstream of the activated CSF-1R in a CSF-1-dependent

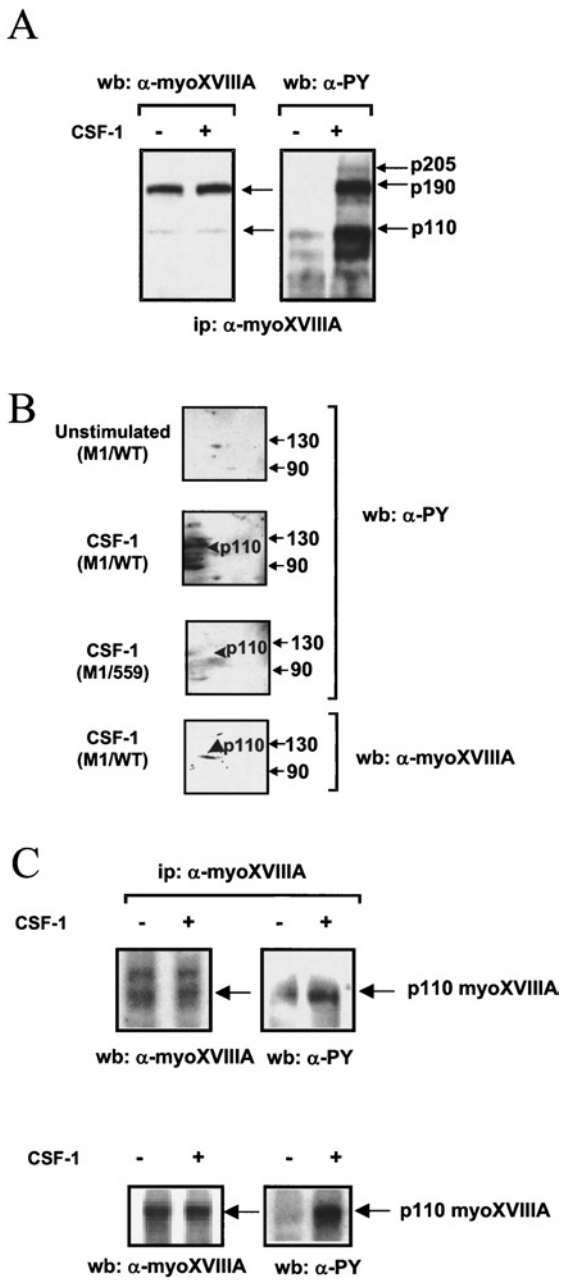

Figure 5 CSF-1-dependent tyrosine phosphorylation of p110myosin XVIIIA

(**A**) M1/WT cells (1×10^7 cells) were treated with CSF-1 (5000 units/ml) for 4 min; NP40 lysates were extracted, immunoprecipitated with anti-myosin XVIIIA antibody, resolved by 1D SDS/PAGE, transferred on to nitrocellulose and probed with either anti-myosin XVIIIA or anti-PY antibodies. The anti-myosin XVIIIA antibodies identified a minor 110 kDa protein signal and a dominant 190 kDa protein, as indicated. Subsequent re-probing with anti-PY antibodies revealed tyrosine phosphorylation in CSF-1-treated cells, which was weak at 205 kDa, but strong at 190 and 110 kDa. (**B**) M1/WT and M1/559 cells (1×10^7 cells) were either untreated or treated with CSF-1 (5000 units/ml) for 4 min. Protein extracts were resolved by 2D SDS/PAGE and patterns of tyrosine phosphorylation determined by Western-blot analysis using anti-PY antibodies as indicated. The sections are enlargements of a region from the same gels seen in Figure 3. Membranes were stripped and re-probed with anti-myoXVIIIA antibodies. The p110myosin XVIIIA protein that was identified by both anti-myoXVIIIA and anti-PY antibodies is indicated with an arrow. The apparent molecular-mass determination was the same as for Figure 3. (**C**) Upper panel: murine BMMs (1×10^7 cells) were either untreated (−) or treated (+) with CSF-1 (5000 units/ml) for 4 min. NP40 lysates were extracted, immunoprecipitated with anti-myosin XVIIIA antibody, resolved by 1D SDS/PAGE, transferred on to nitrocellulose and probed with anti-myosin XVIIIA and anti-PY antibodies. Lower panel: M1/WT cells (1×10^7 cells) were cultured in serum alone (−) or in the presence (+) of CSF-1 (5000 units/ml) for 2 days. Lysates were extracted, resolved as above and probed with anti-myosin XVIIIA and anti-PY antibodies.

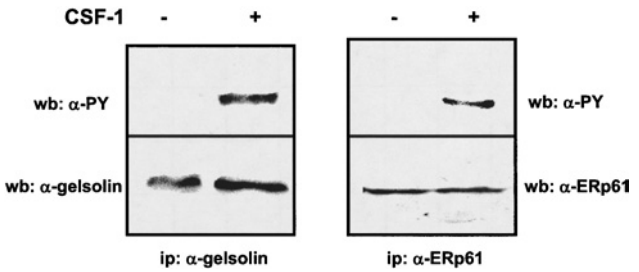

Figure 6 CSF-1-dependent tyrosine phosphorylation of gelsolin and Erp61 in M1/WT cells

M1/WT cells were either untreated (−) or treated (+) with CSF-1 (5000 units/ml) for 4 min as indicated. Protein extracts were immunoprecipitated with anti-gelsolin and anti-ERp61 antibodies respectively, and subjected to Western blotting with anti-PY antibody as well as with the immunoprecipitating antibodies.

M1-cell macrophage differentiation model [4,11]. We found previously, using 2D SDS/PAGE and Western blotting of tyrosine-phosphorylated proteins and M1/807 cells, that tyrosine phosphorylation of p46/52^{Shc} is required for CSF-1-mediated differentiation in this cell system [4]. In the present study, the same strategy was used to compare the CSF-1-dependent differentiation and tyrosine phosphorylation profiles in M1/WT, M1/807 and M1/559 cells. This approach enabled us to show that tyrosine phosphorylation of ERK, similar to that of Shc [4], was very much dependent on Tyr^{807} in the C-terminal region of the CSF-1R and that it correlated with CSF-1R kinase activity [1,34] and the extent of macrophage-like differentiation. These results on ERK phosphorylation confirmed our findings on the relative ERK activity in CSF-1-treated FD/WT and FD/807 cells [4]; in support of an actual role for ERK activation, addition of the MEK (mitogen-activated protein kinase/ERK kinase) inhibitor PD98059 blocked the CSF-1-induced differentiation in M1/WT cells (N. J. Wilson and J. A. Hamilton, unpublished work). It also allowed us to demonstrate, in contrast, that not only was Src tyrosine phosphorylation dependent on Tyr^{559} in the JX region of the CSF-1R (Tyr^{559} is the Src-binding site) [11], but also the extent of its tyrosine phosphorylation did not correlate well with the extent of the CSF-1-dependent differentiation since it was relatively intact in CSF-1-treated M1/807 cells. Unfortunately, this approach does not easily allow us to identify less obvious and perhaps unknown signalling molecules.

The above strategy was therefore extended in the present study to include an enrichment component designed to characterize eventually additional signalling proteins associated with the activated CSF-1R. This extension involved the use of tyrosine-phosphorylated GST fusion constructs of the cytoplasmic domains of the CSF-1R. An assessment of the usefulness of this approach will be published elsewhere (M. Cross and J. A. Hamilton, unpublished work). As a result, we were able to identify a novel form of an unconventional myosin and show that, along with gelsolin, ERp61 and quite probably non-muscle myosin IIA, it was tyrosine-phosphorylated by CSF-1.

We have termed this novel protein p110myosin XVIIIA. The position of each identified peptide and its relationship to the full-length myosin XVIIIA amino acid sequence are outlined in Table 1. Myosin XVIIIA is an unconventional myosin and, as the name implies, is classified as class XVIII [31]; three isoforms (230, 205 and 190 kDa) were originally reported by Furusawa et al. [32]. Recently, the genomic structure and differential expression of two of the spliced murine isoforms were described and were termed 'MysPDZα' (230 kDa) and 'MysPDZβ' (190 kDa)

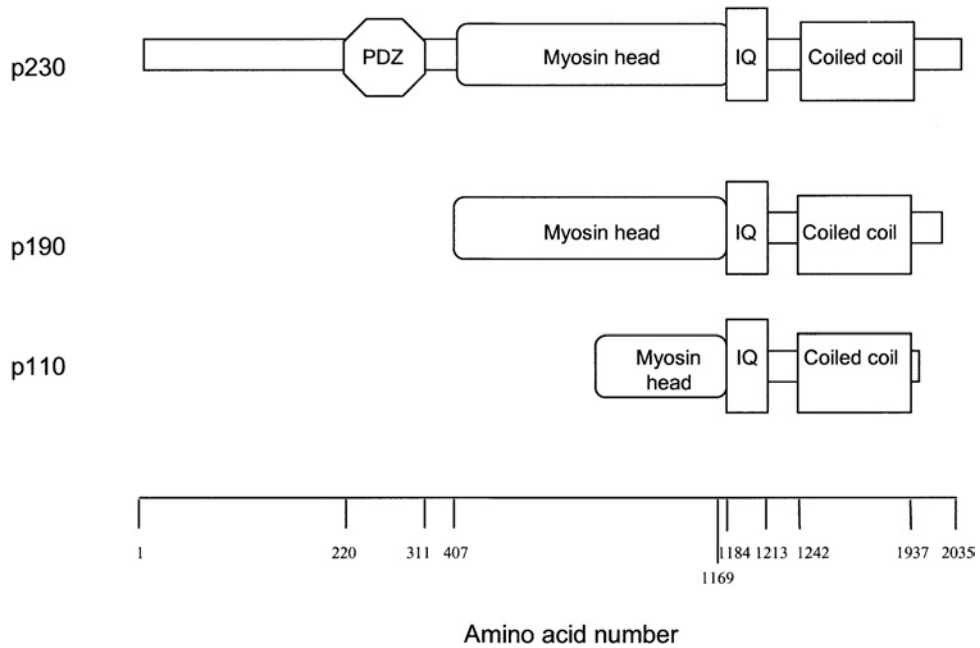

Figure 7 Relationship between p110myosinXVIIIA and larger isoforms

MS analysis has identified the p110 protein that associates with GST–JX-PY (Figure 4C) to be myosin XVIIIA (Table 1). The relationship between p110myosin XVIIIA and the previously identified p230myosin XVIIIA ('MysPDZα') and p190myosin XVIIIA ('MysPDZβ') isoforms, the latter lacking the PDZ domain [33], is represented schematically. Utilizing the MS data generated in the present study, the positions of the peptides observed for the p110myosin XVIIIA isoform, in relation to the full-length p230myosin XVIIIA molecule (MysPDZ; Table 1), enable us to propose the boundaries of p110myosin XVIIIA. The amino acid positions which define the boundaries of each domain within the sequence of myosin XVIIIA are depicted. IQ domain, ilimaquinone (IQ) calmodulin-binding region.

[33]; the latter isoform was proposed to be haematopoietic-cell-specific. With the particular antibody used, we observed that the 190 kDa protein was the one most readily detectable in M1 cells and, therefore, perhaps the most abundant (Figure 5A). Consistent with this suggestion, it has been found that the 190 kDa isoform was clearly more abundant than the 230 kDa isoform both at the mRNA and protein levels in undifferentiated M1 cells [33]. In the present study, the full-length 230 kDa myosin XVIIIA molecule was not detected in M1 cells by our antibody. However, probing antimyosin XVIIIA immunoprecipitates with anti-PY antibodies revealed a weakly reactive 230 kDa band (results not shown). Based on the published genomic structures for 'MysPDZα' and 'MysPDZβ' [33] and our MS data, schematic representations of the p230, p190 and p110 forms are depicted in Figure 7. The simplest interpretation of our results is that the p110 kDa isoform results from a post-translational cleavage of one of the higher-molecular-mass forms, possibly the 190 kDa isoform. MS analysis suggests that this proposed cleavage occurs within the myosin head domain of myosin XVIIIA, since the peptides identified are located towards the C-terminal end of the myosin head domain, in the IQ domain and in the coiled-coil region, resulting in the formation of the p110myosin XVIIIA isoform. It is also possible, however, that there is a second translational start site in the myosin XVIIIA mRNA, giving rise to our p110myosin XVIIIA. Our results suggest a preferential tyrosine phosphorylation of the less abundant 110 kDa isoform over the 190 kDa molecule; if p110myosin XVIIIA arises via a cleavage mechanism, then in our view the relative degree of tyrosine phosphorylation observed is more consistent with the tyrosine-phosphorylated p110 isoform arising from cleavage of a non-tyrosine-phosphorylated higher-molecular-mass isoform rather than of a tyrosine-phosphorylated isoform.

The function(s) of myosin XVIIIA is unknown and the identification of isoforms with and without PDZ domains is intriguing. Myosins, including the unconventional ones, are implicated in numerous cellular processes [35]. Mori et al. [33] provided evidence suggesting that the PDZ-containing 'MysPDZα' co-localizes both with the ER–Golgi complex and partially with actin, in the leading, ruffling edge of the cell, whereas 'MysPDZβ' (p190myosin XVIIIA) did not; therefore it was suggested that they may have different functions in membrane ruffling and trafficking pathways. However, Furusawa et al. [32] have shown that the 230 kDa form of myosin XVIIIA ('MysPDZα') localized in filamentous networks, which were distinct from those containing actin and/or tubulin. Similar to p190myosin XVIIIA ('MysPDZβ'), the p110myosin XVIIIA has a myosin light-chain-binding IQ motif (see Figure 7). The Most N-terminal peptide found starts with residue 1121 (Table 1), which is near the C-terminus of the motor domain. Assuming that the C-terminus is intact, it is unlikely that there is enough of the motor domain present to constitute an active motor. The long coiled-coil domain is similar to that seen in class II myosins and implies the formation of a filamentous structure predominantly through this domain, which may be the mode by which p110myosin XVIIIA and p190myosin XVIIIA interact with multiprotein structures in the cytoplasm [33]. Perhaps of relevance is the finding that the coiled-coil domain of myosin XVIIIA binds to Jak3 in a tyrosine phosphorylation-independent manner and inhibits apoptosis induced by interleukin-2 deprival of BAF-BO3β cells [36]. The PDZ-containing 'MysPDZ' isoform appears to be present in adherent macrophage populations and is induced after differentiation of M1 cells into macrophages by LIF [33].

Tyrosine phosphorylation of myosin XVIIIA after CSF-1 treatment may alter its cellular localization or target its association

with other proteins or structures within a cell. CSF-1-stimulated tyrosine phosphorylation of p110myosin XVIIIA was dependent on the Tyr^{559} within CSF-1R. Considering the evidence for Tyr^{559} to be the binding site on the activated CSF-1R for Src-family kinases [10,11], it is reasonable to hypothesize that tyrosine phosphorylation of p110myosin XVIIIA lies within an Src-dependent pathway. Tyrosine phosphorylation of p110myosin XVIIIA after CSF-1 treatment suggests a probable interaction with the SH2 domains of associated proteins [37]. The p110myosin XVIIIA molecule contains eight tyrosine residues (myosin XVIIIA; Swiss-Prot accession no. Q9JMH9) that lie within an f_N-Yxx-hydrophobic residue motif, where f_N is either a hydrophilic or hydrophobic residue for a type I or III SH2-binding site respectively [38,39].

The functional significance in our system of CSF-1-induced tyrosine phosphorylation of gelsolin, ERp61 and presumably non-muscle myosin IIA remains to be elucidated. After the addition of CSF-1, M1/WT cells and macrophages spread, and the CSF-1R is rapidly internalized ([40]; X. F. Csar and J. A. Hamilton, unpublished work). Gelsolin has been reported to form multi-component complexes with the CSF-1R, Shc and actin in CSF-1-treated macrophages [41]. Myosin II is critical for processes such as cellular shape changes, migration and the contraction processes [42]; interestingly, in terms of our proposal, myosin II has been linked with Src kinases in macrophages [43]. Its association with JX-PY is consistent with the finding that a myosin regulatory light chain is present in CSF-1R-containing multi-protein complexes [41]; myosin light chain has also been demonstrated to be involved in Ca^{2+} influx during macrophage activation [44]. The precise role of CSF-1-dependent tyrosine phosphorylation of these proteins in CSF-1-dependent cytoskeletal changes, for example, remains to be elucidated. We have not formally demonstrated that they are the p120, p95 and p62 proteins in Figure 3 whose CSF-1-dependent tyrosine phosphorylation appears to be reduced in M1/559 cells and therefore Src-dependent. Further studies are in progress to test these possibilities.

In our differentiation model, we have shown that the strategy of monitoring the dependence of the tyrosine phosphorylation status of signalling proteins on a particular tyrosine residue (namely Tyr^{807}) in the activated CSF-1R, when coupled with functional overexpression studies, was able to demonstrate a role for Shc tyrosine phosphorylation in the CSF-1-mediated differentiation [4]. Obviously, similar overexpression and also 'knock down' approaches need to be undertaken to determine the roles of p110myosin XVIIIA, gelsolin, non-muscle myosin IIA, etc. in the M1-cell macrophage differentiation system. It should be noted that additional tyrosine-phosphorylated proteins can be seen to be associated with the tyrosine-phosphorylated CSF-1R constructs in CSF-1-treated M1/WT lysates (Figure 4B). We suggest that the combination of similar biochemical and cellular strategies will allow the characterization of other putative signal-transduction molecules involved in CSF-1-mediated differentiation. Since a major effect of CSF-1 on human bone-marrow precursor cells and monocytes is differentiation into macrophages [45], our differentiation model may be relevant to such normal differentiation programmes and provide a useful representative system for their molecular analysis. Furthermore, this model may have some clinical relevance, since the major effect of CSF-1 on the blast cells of acute myeloblastic leukaemia patients is a differentiation-inducing activity [46].

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