Colony-stimulating factor-1 (CSF-1) receptor-mediated macrophage differentiation in myeloid cells: a role for tyrosine 559-dependent protein phosphatase 2A (PP2A) activity

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M1 myeloid cells transfected with the wild-type (WT) colony-stimulating factor-1 (CSF-1) receptor (CSF-1R; M1/WT cells) undergo CSF-1-dependent macrophage differentiation. By mutation studies, we have provided prior evidence that tyrosine 559 in the CSF-1R cytoplasmic domain governs the Src-dependent differentiation pathway. Further components of this pathway were then sought. We report that the extent of CSF-1-mediated tyrosine phosphorylation of protein phosphatase 2A (PP2A), and the associated loss of its activity were reduced in M1 cells transfected with the CSF-1R with a tyrosine-to-phenylalanine mutation at position 559 (M1/559 cells), compared with the corresponding responses in CSF-1-treated M1/WT cells. This evidence for an involvement of a reduction in PP2A activity in

the differentiation process was supported by the restoration of the defect in the CSF-1-mediated differentiation of M1/559 cells by the addition of the PP2A inhibitor, okadaic acid. It was also found that the degree of activation of extracellular-signal-regulated kinase (ERK) activities by CSF-1 was reduced in M1/559 cells, suggesting their involvement in the differentiation process. These data suggest that PP2A and ERK form part of the Src-dependent signal-transduction cascade governing CSF-1-mediated macrophage differentiation in M1 cells.

Key words: c-Fms, ERK, okadaic acid, receptor tyrosine kinase, signalling.

INTRODUCTION

Macrophage-colony stimulating factor (M-CSF or CSF-1) controls the development of the macrophage lineage through its receptor (CSF-1R) that is encoded by the *c-fms* proto-oncogene [1]. Following CSF-1 binding, the receptor dimerizes, thereby leading to activation of its kinase domain and phosphorylation of specific tyrosines in the cytoplasmic domain [1]. The phosphorylated tyrosines appear to control signal transduction downstream of the activated CSF-1R by forming binding sites for Src-homology region 2 (SH2)-containing proteins. A number of proteins that either bind to these proteins or whose function depends on them have been identified [1]. Tyr-559 is one major phosphorylation site on the activated CSF-1R [1] and binding of Src kinases to Tyr-559 occurs in CSF-1-treated NIH-3T3 fibroblasts ectopically expressing the CSF-1R [2].

The majority of the studies examining the role of tyrosines in the cytoplasmic region of CSF-1R by their mutation have used either the NIH-3T3 cell system mentioned above [2] or the myeloid FDC-P1 cells [3]. M1 myeloid cells are a well-studied differentiation system in which a number of different stimuli (e.g. leukaemia inhibitory factor, interferon γ , interleukin-6, granulocyte/macrophage-CSF, oncostatin M and Wilms' tumour-suppressor gene product) all induce macrophage differentiation [4–9]. One of the differentiation markers reported to be induced in some of these studies is the CSF-1R (c-Fms) [6]. We

reasoned that if CSF-1R was transfected into M1 myeloid cells then exogenous CSF-1 would induce macrophage differentiation since it would bypass the need for endogenous expression of the CSF-1R and would provide a cell type for the study of macrophage differentiation in response to a critical physiological regulator, namely CSF-1. We reported recently that this can be achieved in M1 cells expressing wild-type (WT) CSF-1R (M1/WT cells) [10].

We also reported that when Tyr-559 in CSF-1R was mutated to Phe (M1/559 cells), the CSF-1-mediated differentiation was partially abrogated, as monitored by Mac-1 expression, morphology and a less-marked reduction in proliferation rate [10]. The Src-family tyrosine kinases, c-Src, c-Yes, c-Fyn and c-Hck, were tyrosine-phosphorylated in the M1/WT cells in response to CSF-1 and bound to the WT CSF-1R through their SH2 domains. The relative degree of tyrosine phosphorylation of the Src kinases, as well as their binding to the CSF-1R, was greatly reduced in the CSF-1-treated M1/559 cells. Treatment of M1/WT cells with the Src-family inhibitor, PP2, resulted in inhibition of CSF-1-mediated differentiation [10]. All of these data suggested that the reduced binding and activation of Src-family kinase(s) may contribute to the reduced ability of M1/559 cells to differentiate in response to CSF-1.

The signalling cascades downstream of Src kinases(s) in the above differentiating system have not been elucidated. We proposed that the reduced STAT3 (signal transducer and

Abbreviations used: CSF-1, colony-stimulating factor-1; CSF-1R, CSF-1 receptor; WT, wild type; PP2A, protein phosphatase 2A; ERK, extracellular-signal-regulated kinase; NBCS, newborn calf serum; MAPK, mitogen-activated protein kinase; STAT3, signal transducer and activator of transcription 3; HRP, horseradish peroxidase.

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activator of transcription 3) phosphorylation observed in CSF-1-treated M1/559 cells compared with that observed in the equivalently treated M1/WT cells was due to abrogation of Src binding to Tyr-559, associated with a loss of Src tyrosine phosphorylation and activation, leading to a significantly reduced level of macrophage differentiation [10]. In this paper we examine the dependence on Tyr-559 of the activities of certain signal-transduction molecules associated with Src-family activity in other systems. We report that the degrees of activation by CSF-1 of the phosphatase PP2A (protein phosphatase 2A) and extracellular-signal-regulated kinases 1 and 2 (ERK-1 and ERK-2) are reduced in M1/559 cells compared with the levels found in CSF-1-treated M1/WT cells. Furthermore, the reversibility of the differentiation defect in CSF-1-treated M1/559 cells by okadaic acid suggests the involvement of PP2A activity in CSF-1-induced macrophage differentiation in M1/WT cells.

EXPERIMENTAL

Reagents

Dulbecco's modified Eagle's medium was purchased from ICN-Flow Laboratories (Sydney, NSW, Australia), newborn calf serum (NBCS) from Commonwealth Serum Laboratories (Parkville, VIC, Australia) and okadaic acid from Sigma Aldrich. Recombinant human CSF-1 was a gift from Chiron Corporation (Emeryville, CA, U.S.A.).

The following primary antibodies were used: phosphoERK rabbit polyclonal antibody (New England Biolabs, Beverly, MA, U.S.A.), goat anti-rat ERK-1 polyclonal antibody, rabbit anti-rat ERK-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), mouse anti-human PP2A (36 kDa) monoclonal antibody (Upstate Biotechnology, Lake Placid, NY, U.S.A.) and horseradish peroxidase (HRP)-conjugated monoclonal anti-phosphotyrosine antibody (clone 4G10; Upstate Biotechnology). The following secondary antibodies were used: HRP-conjugated purified swine IgG to goat IgG and HRP-conjugated rabbit IgG to mouse IgG (Dako, Glostrup, Denmark). Protein A-conjugated HRP (Zymed Laboratories, San Francisco, CA, U.S.A.) was used to detect bound primary antibodies where indicated in the text.

Cells

The murine myeloid M1 cell line was kindly given by Dr Nicos Nicola (Walter and Eliza Hall Institute, Melbourne, Australia) and stable transfectants were maintained as described previously [10]. Pooled clones with similar levels of CSF-1R were used in these studies. Prior to experiments, M1 cells were cultured for 18 h in Dulbecco's modified Eagle's medium containing 1% NBCS, then stimulated at 37 °C with 5000 i.u./ml CSF-1. Cell lysates were prepared as described previously [11]. Protein concentration was determined using a Coomassie Brilliant Blue Bradford assay [12].

Immunoprecipitation

Immunoprecipitations were performed as follows: 200–1000 mg of appropriately treated cell lysate was precleared with 30 µl of a 50% (v/v) slurry of Protein-A-Sepharose 4B (Pharmacia, Uppsala, Sweden) in cell-lysis buffer, with rotation for 30 min at 4 °C. The samples were then centrifuged, and the cell pellets discarded. The supernatants were then incubated overnight

with 1–2 µg of appropriate primary antibody with rotation at 4 °C [11].

Western-blot analysis

Gels for analysis by Western blotting were electroblotted on to Hybond-C membranes (Amersham, Little Chalfont, Bucks., U.K.). Membranes were blocked in 3% (w/v) BSA, 1% (w/v) ovalbumin and Tris-buffered saline with 1% Tween-20 (TBST), and incubated with appropriate primary antibody (1:10000) for 2 h at room temperature. The membranes were washed twice in each of Tris-buffered saline and TBST (10 min), incubated with HRP-conjugated secondary antibodies (1:10000) and washed as described previously [10]. Western blots were developed by enhanced chemiluminescence (ECL; Amersham). In some cases, the blots were stripped of antibodies by extensive washing in stripping buffer containing 62.5 mM Tris/HCl, 100 mM β-mercaptoethanol and 2% SDS, then reblocked as described above for 30 min at room temperature and reprobed with different antibodies.

Phosphatase assays

PP2A was measured in triplicate in 96-well flat-bottomed microtitre plates (Disposable Products, Park Way Technology, SA, Australia). 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 /5 mg/ml BSA solution. Then, 20 µl of 50 mM Tris/HCl/0.1 mM CaCl_2 , pH 7.0, assay buffer, with or without 50 nM okadaic acid, was added to each well prior to the addition of 120 µl of *p*-nitrophenyl phosphate (Sigma; 3.0 mg/ml in 50 mM Tris/HCl, pH 7.0) and the plate incubated at 37 °C for a further 45 min. Okadaic acid was used to measure PP2A activity; a concentration of 50 nM has been shown to inhibit PP2A activity by up to 80% [13,14]. The hydrolysis of *p*-nitrophenyl phosphate was measured at 405 nm and PP2A activity was calculated as total phosphatase activity minus okadaic acid-resistant activity in pmol/min per µg of protein, as described previously [15].

Differentiation

The extent of differentiation was monitored as in [10] by morphology and Mac-1 expression. For morphological analysis, M1/WT and M1/559 cells were cultivated in 10% NBCS, 5000 i.u./ml CSF-1, 50 nM okadaic acid or CSF-1 together with okadaic acid for 24 h. Cells were cytopun and subjected to May-Grünwald and Giemsa staining or stained for Mac-1 expression as described previously [10]. Data were acquired and analysed using a FACScalibur apparatus and Cell Quest software (Becton-Dickinson, San Jose, CA, U.S.A.).

RESULTS

CSF-1-mediated tyrosine phosphorylation of PP2A is reduced in M1/559 cells

We showed previously that CSF-1 stimulates the tyrosine phosphorylation of a number of proteins in M1/WT cells [10]; however, the extent of tyrosine phosphorylation of a number of proteins, including that of the CSF-1R and Src-family kinases, was relatively reduced in CSF-1-treated M1/559 cells. Using this information we sought to examine further the molecular

Figure 1 Suppressed CSF-1-mediated tyrosine phosphorylation of PP2A in M1/559 cells

M1/WT and M1/559 cells were left unstimulated or incubated with CSF-1 for 2 min at 37 °C (see the Experimental procedures section). Nonidet P-40 lysates (200 µg) were immunoprecipitated (i.p.) with either anti-PP2A or anti-phosphotyrosine (PY) antibody and Western blotted (w.b.) with the converse antibodies. Lysates immunoprecipitated with anti-PP2A antibodies were Western blotted with the anti-PP2A antibody as a loading control.

mechanisms responsible for the reduction in the degree of differentiation induced by CSF-1 in the M1/559 cell line.

As it has been demonstrated previously that the 36 kDa catalytic subunit of PP2A is phosphorylated and inactivated *in vitro* by tyrosine kinases such as p60^{src} [16,17], we compared the extent of tyrosine phosphorylation of PP2A in CSF-1-treated M1/WT and M1/559 cells. Anti-PP2A immunoprecipitates of lysates from untreated and CSF-1-treated M1/WT and M1/559 cells were probed with anti-phosphotyrosine antibodies. Tyrosine phosphorylation of the 36 kDa catalytic subunit of PP2A was observed to be up-regulated upon CSF-1 stimulation in M1/WT cells but not in CSF-1-treated M1/559 cells (Figure 1). The apparent difference in tyrosine phosphorylation of PP2A was not due to differences in the level of 36 kDa catalytic subunit of PP2A in the PP2A immunoprecipitates (Figure 1). These observations were confirmed by the reciprocal immunoblotting of anti-phosphotyrosine immunoprecipitates with anti-PP2A antibody (Figure 1). These findings demonstrate that tyrosine phosphorylation of the catalytic subunit of PP2A following CSF-1 addition is impaired by the Tyr-559-to-Phe mutation.

Tyrosine phosphorylation of the PP2A 36 kDa catalytic subunit is associated with inactivation of PP2A phosphatase activity [16]. We examined whether this occurred following loss of PP2A phosphorylation in the CSF-1-treated M1/559 cells. PP2A activity was measured by okadaic acid sensitivity, which has been shown to be a highly specific inhibitor of PP2A at the nanomolar level [18]. This okadaic acid-sensitive activity was observed to decrease from 1 ± 0.1 pmol/min per µg in unstimulated M1/WT cells to 0.1 ± 0.05 pmol/min per µg after CSF-1 stimulation for 2 min in M1/WT cells (Figure 2, open bars), indicating that this activity, probably attributable to that of PP2A, is inactivated upon CSF-1 stimulation. However, this okadaic acid-sensitive activity in M1/559 cells was 0.9 ± 0.01 pmol/min per µg in unstimulated cells and remained at 0.7 ± 0.09 pmol/min per µg after 2 min of CSF-1 stimulation (Figure 2, closed bars), demonstrating that this phosphatase activity is not significantly reduced in M1/559 cells upon CSF-1 stimulation. This maintenance of okadaic acid-sensitive activity parallels the diminished relative PP2A tyrosine phosphorylation noted in M1/559 cells in response to CSF-1 (Figure 1), suggesting further that the okadaic acid-sensitive activity is indeed PP2A.

Figure 2 CSF-1-mediated okadaic acid-sensitive phosphatase inactivation is suppressed in M1/559 cells

M1/WT and M1/559 cell lysates were untreated (Unst) or treated with CSF-1 for 2 min at 37 °C. The okadaic acid-sensitive activity (putative PP2A) was measured in 200 µg of lysates by subtracting okadaic acid-resistant phosphatase activity from the total phosphatase activity (see the Experimental section). The values are means $\pm$ S.D. from triplicate assays in a representative experiment that was repeated at least three times. #, Not significant ($P > 0.05$) compared with untreated M1/559 cells; *, significant ($P < 0.05$) compared with untreated M1/WT cells.

Inhibition of okadaic acid-sensitive phosphatase activity restores CSF-1-mediated differentiation in M1/559 cells

Given the correlation of loss of okadaic acid-sensitive activity with the degree of CSF-1-induced differentiation, we next determined whether inhibition of this activity would restore macrophage differentiation in CSF-1-treated M1/559 cells, i.e. whether reduction of okadaic acid-sensitive activity was actually relevant to the differentiation process in this system. For this purpose okadaic acid was again used and cell morphology and Mac-1 expression (flow cytometry) again monitored as a measure of differentiation [10,19].

M1/WT cells treated with CSF-1 adopt a macrophage-like morphology, defined by decreased nuclear-to-cytoplasmic ratio, membrane ruffling and blebbing [10,20] in contrast to the blast-like cells evident when cultured in NBCS alone (Figure 3A). This apparent differentiation is abrogated dramatically in M1/559 cells [10; Figure 3A). Similarly, as reported previously [10], we observed an increase in the number of Mac-1-positive cells upon CSF-1 stimulation in M1/WT cells compared with NBCS controls within 24 h (Figure 3B). In contrast, M1/559 cells exhibited a reduced increase in the proportion of Mac-1-positive cells after CSF-1 treatment relative to M1/WT cells (Figure 3B). The combination of CSF-1 and okadaic acid restored the extent of differentiation in CSF-1-treated M1/559 cells to that observed in CSF-1-treated M1/WT cells, both in terms of morphology (Figure 3A) and Mac-1 expression levels (Figure 3B). These data are consistent with the hypothesis that the CSF-1-mediated down-regulation of PP2A activity, as inferred from its dephosphorylation and the elevated okadaic acid-sensitive activity in M1/559 relative to M1/WT cells (Figures 1 and 2), is important for differentiation in M1 cells. This defect in M1/559 cells can be bypassed by treatment with okadaic acid in conjunction with the CSF-1 addition. We observed no significant effect on the proportion of Mac-1-positive cells after okadaic acid treatment alone in either M1/WT or M1/559 cells (Figure 3B). The short time period of 24 h was used in this experiment in an attempt to avoid any non-specific toxic effects of okadaic acid. We have shown previously that 50 nM okadaic acid in-

Figure 3 Okadaic acid restores macrophage differentiation in CSF-1-treated M1/559 cells

M1/WT and M1/559 cells were cultured for 24 h in NB-CS alone (—), NB-CS and CSF-1 (5000 units/ml), NB-CS and okadaic acid (OA; 50 nM) and NB-CS, CSF-1 (5000 units/ml) and OA (50 nM). (A) Cells were cytospun and stained with May–Grünwald and Geimsa (see the Experimental section). (B) Cells were stained with either Mac-1 reactive or isotype control antibodies and assessed for Mac-1 expression by flow cytometry. The values are means $\pm$ S.D. of triplicate assays from a representative of at least three independent experiments. For CSF-1-treated M1/559 cells, the effect of okadaic acid was statistically significant (* $P < 0.05$) in this experiment and all repeat experiments carried out.

hibited PP2A activity in murine bone-marrow-derived macrophages [19].

CSF-1-mediated stimulation of ERK activity is reduced in M1/559 cells

Both c-Src and PP2A activities have been implicated in the regulation of the mitogen-activated protein kinase (MAPK) pathway in other cell systems [21–24]; in addition, ERK family members have been implicated in the pathways governing differentiation in a number of cell systems [25–27]. It is therefore possible that CSF-1-stimulated ERK activation may also be impaired in M1/559 cells. We examined this question using an anti-phosphoERK antibody that specifically identifies active forms of ERK [27]. CSF-1 was observed to activate ERK in M1/WT cells (Figure 4). The kinetics of activation were as follows; maximal at 4 min, in decline at 10 min (Figure 4) and basal levels at 30 min after CSF-1 stimulation (results not shown).

Figure 4 Reduced CSF-1-mediated ERK activation in M1/559 cells

M1/WT and M1/559 cells were untreated or treated with CSF-1 for 4 and 10 min at 37 °C in the absence or presence of okadaic acid (50 nM; 20 min pretreatment). Extracts were resolved by SDS/PAGE and Western blotted with anti-phosphoERK (pERK) antibody or anti-ERK1/2 antibody as a loading control (see the Experimental section). These results are representative of three independent experiments.

Conversely, there was far less stimulation of ERK activity in CSF-1-treated M1/559 cells at all time points (Figure 4). These findings suggest that, in this cell system, maximal ERK activation is dependent on the tyrosine at position 559 of the CSF-1R.

Since PP2A, as mentioned, has been shown to regulate MAPK activity in different cell systems [22,23], we tested whether okadaic acid addition might enhance the stimulated ERK activities. There was a partial enhancement of the ERK activity following co-treatment of M1/WT and M1/559 cells with okadaic acid and CSF-1 (Figure 4). Whether this partial increase in ERK activity in the latter cell population by okadaic acid is related to its ability to reverse the differentiation defect is unknown. Okadaic acid alone had no effect on ERK activation in either cell population, suggesting that okadaic acid acts to prevent PP2A-mediated ERK dephosphorylation, and therefore activation, rather than mediate ERK activation as such. The lower panel of Figure 4 represents a loading control probed with anti-ERK1/2 antibodies, which demonstrates the significance of the CSF-1- and okadaic acid-mediated increases in ERK activity.

DISCUSSION

The goals of this study were to continue to examine the molecular basis for the observed impairment in macrophage differentiation in M1/559 cells [10]. It is proposed in this report that there is a signalling cascade(s) downstream of Tyr-559 of the CSF-1R in CSF-1-treated M1/WT cells involving Src-family kinase phosphorylation [10], leading to PP2A tyrosine phosphorylation and loss of its phosphatase activity, and ultimately resulting in macrophage differentiation. Activation of ERK by CSF-1 also appears to be dependent upon Tyr-559. It is interesting to note that consistent with the observation that ERK activation correlates with differentiation in this system is the sensitivity of these processes to the ERK kinase (MEK) inhibitor PD98059 (N. J. Wilson, J. A. Hamilton, V. Kanagasundaram, S. T. Moss, G. Whitty and X. F. Csar, unpublished work). The above data would support the contention that the loss of maximal differentiation in these cells is due to a reduction in the extent of CSF-1-induced ERK activity, dependent upon Src kinase [10] and PP2A activity. Using CSF-1R-transfected NIH-3T3 cells, Src kinases have been found to be involved in the control of CSF-1-dependent proliferation in a pathway involving ERK activity and ultimately leading to c-Myc and cyclin D1 expression [2]. It would appear once again that the nature of the cell type can determine the final outcome of the activation of a particular CSF-1R-dependent signalling cascade [2].

PP2A is a key regulator of intracellular signalling events that lead to such diverse processes as cell-cycle regulation and proliferation [28], apoptosis [29,30] and differentiation [31–34].

Examination of PP2A tyrosine phosphorylation and phosphatase activity revealed that PP2A is tyrosine-phosphorylated only in CSF-1-treated M1/WT cells. The tyrosine phosphorylation of PP2A in CSF-1-treated M1/WT cells correlated with a down-regulation of PP2A phosphatase activity. In contrast, PP2A was not tyrosine-phosphorylated in CSF-1-treated M1/559 cells, and its phosphatase activity remained elevated upon CSF-1 stimulation. PP2A has been demonstrated to be tyrosine-phosphorylated after activation by a number of growth factors in different cell systems [35–37], and during differentiation in non-leukaemic cells [38]. Also, it was demonstrated recently that PP2A activity may be required for the interaction of middle T antigen (MT) with both PP2A and Src-family kinases, suggesting that PP2A is a necessary component of the MT–Src complex [39]. Our data suggest that PP2A may lie downstream of the Src-family kinases in a pathway leading to macrophage differentiation in CSF-1-treated M1/WT cells. *In vitro*, several receptor and non-receptor tyrosine kinases, including p60*v-src*, p56 *lck*, the epidermal growth factor receptor and the insulin receptor, are capable of phosphorylating the catalytic subunit of PP2A on Tyr-307 [16]. We suggest that it may be the Src-family kinases that are responsible in a similar manner for phosphorylating the catalytic subunit of PP2A in CSF-1-treated M1/WT cells. Whether PP2A activity can in turn be regulating Src-family kinase activity in this cell system is unknown.

Okadaic acid, presumably through inhibition of PP2A phosphatase activity, could restore macrophage-like differentiation in CSF-1-treated M1/559 cells. These data are consistent with the idea that loss of inhibition of PP2A activity in CSF-1-treated M1/559 cells is responsible for the suppressed differentiation in this cell type. By analogy, in other cells, okadaic acid has been demonstrated to augment the differentiation of HL-60 cells induced by retinoic acid [31–34]. Recently we examined the role of PP2A activity in CSF-1 signalling in another murine cell type, namely murine bone-marrow-derived macrophages [19], which proliferate in response to this ligand. In contrast to the studies in bone-marrow-derived macrophages [19] reported here, CSF-1 stimulated PP2A activity and expression, and the conclusion was that the phosphatase was important for cell-cycle progression in these cells [18]. Addition of okadaic acid to the two cell systems supported these conclusions [18].

There are a number of reports that support the view that ERK activation can be Src-dependent [23,24]. Our data comparing the degree of activation of ERK-1 and ERK-2 in CSF-1-treated M1/WT versus CSF-1-treated M1/559 cells are suggestive of a similar dependence in this system; our correlation with the extent of macrophage differentiation suggests a role for ERK activity in this process, as has been noted in other differentiating systems [25–27]. Many observations suggest that PP2A may function in the down-regulation of the ERK/MAPK pathway (see, for example, [21,22]). There was an inverse correlation between the degree of loss of PP2A activity and the degree of stimulation of ERK activity following CSF-1-treatment of M1/WT compared with M1/559 cells. This observation is consistent with an involvement of PP2A in the down-regulation of ERK activity also in this system. Moreover, addition of okadaic acid to CSF-1-treated M1/559 cells partially restored ERK activation (but not to levels observed in CSF-1-treated M1/WT cells). From our prior murine bone-marrow-derived macrophage studies discussed above, no evidence could be obtained for PP2A-mediated control of ERK activity [19].

In summary, PP2A and ERK can be added to STAT3 in the list of signal-transduction molecules in M1 cells whose activation is dependent upon the presence of Tyr-559 of the CSF-1R and possibly also dependent on Src kinase activity. How the stimu-

lation of ERK activity might control CSF-1-mediated differentiation in this system awaits clarification.

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