Regulation of urokinase plasminogen activator gene transcription in the RAW264 murine macrophage cell line by macrophage colony-stimulating factor (CSF-1) is dependent upon the level of cell-surface receptor

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Macrophage colony-stimulating factor (CSF-1) binds to a receptor (CSF-1R) encoded by the *c-fms* proto-oncogene and activates transcription of the urokinase plasminogen activator (uPA) gene in murine bone-marrow-derived macrophages. This article demonstrates that the murine macrophage cell line RAW264 responds to CSF-1 with inducible phosphorylation of cytoplasmic proteins on tyrosine residues but fails to induce transcription of uPA. The defect was correlated with a selective failure to maintain CSF-1Rs on the cell surface, whereas all RAW264 cells contained abundant CSF-1Rs within the presumptive Golgi/endoplasmic reticulum compartment. Transfection with a CSF-1R expression plasmid permitted CSF-1-dependent activation of the signalling pathway targeting an Ets/API (activator protein 1) element in the uPA promoter that

has been shown previously to be a target of oncogenic *ras* and protein kinase C pathways. Mutation of the expressed CSF-1R at either Y807 or Y559, sites of receptor tyrosine phosphorylation implicated in signal transduction, reduced but did not abolish uPA promoter activation by CSF-1. Activation by mutant CSF-1R plasmids was additive; there was no evidence of mutual complementation. The results indicate that maintenance of elevated uPA transcription by CSF-1 requires new receptors emerging continuously on the cell surface. Parallel, partly redundant, signalling pathways arising from phosphorylated tyrosines on the CSF-1R activate multiple *cis*-acting elements on the complex uPA promoter.

Key words: c-Fms, Ets, MAPK, signal transduction, trafficking.

INTRODUCTION

Macrophage colony-stimulating factor (CSF-1) controls the proliferation and differentiation of cells of the mononuclear phagocyte cell lineage [1]. The actions of CSF-1 are mediated through an integral membrane receptor tyrosine kinase, the product of the *c-fms* proto-oncogene. CSF-1/c-Fms action regulates the expression of specific genes in mature macrophages; for example, the urokinase plasminogen activator gene (uPA). uPA encodes an extracellular protease involved in cellular migration in many cell types including metastatic tumour cells. Expression of uPA enzymic activity in macrophages requires continued exposure to CSF-1 [2]. Both transcriptional and post-transcriptional mechanisms contribute to uPA mRNA regulation in macrophages. Upon CSF-1 removal, uPA mRNA decays rapidly, a process that is blocked by inhibitors of RNA or protein synthesis [3]. The expression of uPA is also induced by CSF-1/c-Fms expressed ectopically in NIH3T3 fibroblasts [4]. The induction of this and related genes may contribute to increased anchorage-independent growth and invasiveness in CSF-1 autocrine tumour cell lines (see [5]). The uPA promoter is conserved across species up to 8.2-kb upstream of the transcription start site [6]. Within this extended region of homology, two conserved critical growth factor-responsive elements at –2.4 and –6.9 kb [6] have been identified that correspond to the

Ets/API (activator protein 1) compound element common to genes that are inducible by oncogenic *ras* [7].

On binding of its ligand, the CSF-1 receptor, CSF-1R, dimerizes and becomes phosphorylated on a number of tyrosine residues. After initiating a signalling cascade, the receptor is covalently cross-linked, and both ligand and receptor are internalized and degraded. Continued binding of CSF-1 therefore requires the continuous replenishment of the cell-surface receptor pool. There appear to be distinct functions of particular phosphorylated tyrosines in propagating the CSF-1 signal as selected mutants have been shown to affect different subsets of the response to CSF-1 [8–10]. These previous structure/function studies on the CSF-1R have expressed the receptor ectopically in cells such as NIH3T3 fibroblasts or immature myeloid cell lines. A significant problem with these systems is that many of the components of a multimeric signalling complex assembled by the activated CSF-1R, including c-Cbl, protein tyrosine phosphatase (PTP)-1C, p150^{SHIP}, a novel adaptor protein (Mona) and a major actin-binding protein (MAYP), are expressed predominantly in haematopoietic cells or more specifically in macrophages [11–20]. Ideally, CSF-1 action would be studied in primary macrophages, but these cannot be transfected with any efficiency due to DNA-induced apoptosis under conditions of transfection [21]. We showed previously [22] that the murine macrophage cell line, RAW264, expresses CSF-1Rs but fails to induce uPA tran-

Abbreviations used: CSF-1, macrophage colony-stimulating factor; CSF-1R, CSF-1 receptor; BMM, bone-marrow-derived macrophage; uPA, urokinase plasminogen activator; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; AP1, activator protein 1; PBA, PBS containing 0.1% BSA and 0.1% sodium azide.

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scription in response to CSF-1 unless a c-Fms expression plasmid is co-transfected. The deficiency in RAW264 cells could be due to a structural defect or mutation in the CSF-1R or to a quantitative deficiency in the level of expression of the receptor. The former possibility is unlikely, since RAW264 cells do respond to CSF-1 with increased spreading on the substratum, increased uPA mRNA regulated solely at a post-transcriptional level, diminished *c-fms* transcription and a small increment in proliferation [4,23]. The latter is favoured by the observation that RAW264 cells exhibit levels of c-Fms mRNA and radiolabelled CSF-1-binding activity comparable with bone-marrow-derived macrophages (BMMs) that have been down-modulated with CSF-1 (i.e. around 4-fold lower than CSF-1-starved cells [23]). In this study we aimed to characterize the nature of the deficiency in CSF-1 signalling in RAW264 cells and how this is compensated by exogenous expression of the CSF-1R, leading to reconstitution of uPA transcriptional activation.

MATERIALS AND METHODS

Cell culture

BMMs were derived from cells obtained from the femurs of Balb/c mice, which were cultured in CSF-1 for 7 days as described previously [4]. RAW264, a macrophage cell line derived from an Abelson leukaemia virus-infected mouse [24], was obtained from the A.T.C.C. and was routinely cultured on bacteriological Petri dishes with RPMI 1640 medium supplemented with 5–10% heat-inactivated foetal calf serum, 20 units/ml penicillin and 20 µg/ml streptomycin. To avoid phenotypic drift during prolonged culture, the original aliquot obtained from the A.T.C.C. was expanded without subculture and frozen. Cells were maintained in culture for no more than one month before reinitiating culture from a fresh vial. When required, cells were cultured in the presence of 10⁴ units/ml CSF-1 (1 unit = 0.01 ng). This recombinant human CSF-1 was provided by Chiron Corporation, Emeryville, CA, U.S.A.

FACS analysis

Cell-surface expression of the CSF-1R was determined by immunolabelling followed by flow cytometry. All washes and dilutions were carried out with PBA (PBS containing 0.1% BSA and 0.1% sodium azide). To prevent receptor internalization, the cells and solutions were kept on ice at all times and 0.1% sodium azide was included in the wash/diluent solution. Briefly, 1 × 10⁶ cells were harvested and washed in PBA and resuspended in a 50-µl volume. To block non-specific Ig binding, the cells were incubated with 0.1% heat-inactivated goat serum for at least 15 min. After washing in PBA, the cells were incubated for 30–60 min with culture supernatant from the AFS98 hybridoma, which produces a monoclonal antibody specific to the CSF-1R [25]. Cells were washed as before and the cells incubated for 30–60 min with a goat anti-rat IgG F(ab')₂ fragment labelled with FITC (Serotec). Samples were kept in the dark as much as possible from this stage onwards as FITC is light-sensitive. Cells were washed once more and resuspended in 0.4 ml of PBA. Control cells were labelled with the secondary antibody alone. Samples were analysed on a FACSCalibur (Becton Dickinson) with 10⁴ events examined for each sample.

Immunofluorescence staining

Cells were cultured on glass coverslips for 2 days before labelling except in the case of the transiently transfected RAW264 cells, which were cultured for 20 h on the glass coverslips. Cells were

fixed in cold (−20 °C) methanol for 10 min and washed with PBS. After permeabilization by a 2-s exposure to 0.1% Triton X-100 in PBS and washing with 0.5% BSA in PBS, the samples were blocked by incubating in 0.2% heat-inactivated goat serum in PBS at room temperature for 15 min. c-Fms protein was detected by incubation with culture supernatant from the AFS98 hybridoma followed by indocarbocyanine (Cy3)-conjugated goat anti-rat antibody (Jackson ImmunoResearch Laboratories) with an intermediate washing step. These incubations were performed at room temperature for a minimum of 1 h and of 45 min respectively. After a final wash, the coverslips were mounted on glass slides with 1% *n*-propyl gallate/50% glycerol in PBS. Fluorescence microscopy was carried out with an Olympus Provis AX70 fitted for epifluorescence. For quantification of the transfected cells that expressed c-Fms at very high levels, 10 fields were selected randomly and total numbers of cells counted using optics designed for detecting FITC, as all cells emitted uniform, low-level fluorescence under these conditions.

Western analysis

Cells were lysed and homogenized in a 66 mM Tris/HCl/1 mM sodium orthovanadate (pH 7.4)/2% SDS buffer as described previously [26]. Protein concentrations of the cell lysates were determined using the Bio-Rad DC protein assay kit and 150 µg of protein electrophoresed on an SDS/polyacrylamide (7.5%) gel. After transfer to Amersham Hybond-C extra nitrocellulose membrane using a semi-dry transfer apparatus, the membranes were blocked with 2% teleostean gelatin (Sigma) and immunoblotted with the anti-phosphotyrosine monoclonal antibody, 4G10 (a gift from D. James, Centre for Molecular and Cellular Biology, University of Queensland, Australia). Detection was via a goat anti-mouse IgG (H + L) alkaline phosphatase conjugate (Bio-Rad EIA grade) with subsequent colorimetric development.

Plasmids and transient transfections

The parent plasmid for the luciferase reporter constructs was pGL2-B (Promega). pGL2uPA-6.6 has been described in [4] whereas pGL99, pGL119, pGL127 and pGL126 are the luciferase equivalents of the chloramphenicol acetyltransferase reporter constructs pPR99, pPR119, pPR127 and pPR126 used in that study. pGL99 was constructed by excising the uPA promoter sequences from pPR99 using *EcoRI*/*HindIII* and ligating into the *XhoI*/*HindIII* sites in the multiple cloning sequence of pGL2-B. pPR119, pPR127 and pPR126 were constructed in an equivalent manner. Introduction of 4.3 kb of the human β -actin promoter into the *SmaI*/*HindIII* sites of pGL2-B resulted in pGL β A. This plasmid was obtained from A. I. Cassady (Centre for Molecular and Cellular Biology, University of Queensland). The c-Fms expression vectors were based on pECE and pEF-BOS [26,27]. cfms-pECE was obtained from Changmin Chen (Centre for Molecular and Cellular Biology, University of Queensland) and was created by cloning the 3.7-kb murine *c-fms* cDNA into *XbaI*/*EcoRI* of pECE. pEF-cfms contained the wild-type murine *c-fms* cDNA cloned into the *XbaI* site of pEF-BOS. pEF-cfmsY559F and pEF-cfmsY807F each contained mutations in the *c-fms* cDNA that converted the specified tyrosine into a phenylalanine residue.

RAW264 cells were transiently transfected as described previously [4] but with a change of conditions to electroporation at 280 V and 960 µF immediately after mixing with the plasmid DNA. Luciferase assays were performed either with the Luciferase Assay System (Promega) or Luciferase Reporter Gene Assay, constant light signal kit (Boehringer Mannheim) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

RAW264 cells respond to CSF-1 with increased protein tyrosine phosphorylation

Despite being unable to induce transcription of uPA in response to treatment with CSF-1, RAW264 cells do respond to CSF-1, as shown by an increase in proliferation and the down-regulation of transcription from the *c-fms* gene itself. In principle, a comparison of BMMs and RAW264 cells might highlight early events that are absolutely required for the activation of uPA transcription in response to CSF-1. We recognize already that there is a quantitative reduction in sustained activation of extracellular signal-regulated kinase (ERK)-1/ERK-2 in RAW264 cells compared with BMMs treated with CSF-1 [22]. To determine whether this difference reflects the immediate response of BMMs and RAW264 cells to CSF-1, protein tyrosine phosphorylation was investigated by Western blotting with an anti-phosphotyrosine antibody. In both cell types, the levels of protein tyrosine phosphorylation increased rapidly on treatment with CSF-1. The most striking feature of the profiles of BMMs and RAW264 cells is their similarity to each other. This is emphasized by comparison with the profile of insulin-stimulated 3T3-L1 adipocyte cells, which appears to have almost no bands in common with RAW264 cells or BMMs except two that have been identified elsewhere as the mitogen-activated protein kinases (MAPKs), ERK-1 and ERK-2 (bands a and b on Figure 1) [28]. Side-by-side comparison of BMMs and RAW264 cells does reveal some differences in the profiles, the most noteworthy of which is a band of approx. 60 kDa (band c on Figure 1A) that is constitutively phosphorylated in BMMs while its phosphorylation is induced from a low level by CSF-1 in RAW264 cells, and the putative MAPK bands in RAW264 cells which, as expected, are not phos-

phorylated as heavily in response to CSF-1 as they are in BMMs. Overall, these data confirm that there are functional CSF-1Rs in RAW264 cells and that they are sufficient to initiate an 'immediate' response to CSF-1 similar to that seen in BMMs.

Cell-surface CSF-1R is differentially regulated in RAW264 cells compared with BMMs

The ability of a CSF-1R expression plasmid to overcome the apparent CSF-1 signalling defect in RAW264 cells [22] implies that the cells may have a deficit of CSF-1Rs on the cell surface. In an earlier study, this issue was addressed using radiolabelled CSF-1-binding assays [23]. The RAW264 cells were found to have 4-fold fewer CSF-1-binding sites than BMMs that had been maximally starved of CSF-1, and a corresponding lower level of *c-Fms* mRNA, but the binding activity was still readily detectable and substantial. The availability of a high-affinity anti-CSF-1R monoclonal antibody now makes it possible to examine this issue at the single-cell level and in terms of the numbers of available receptors rather than binding activity (which could be regulated independently). Figure 2(A) compares the binding of anti-CSF-1R antibody to RAW264 cells grown in the absence of CSF-1 and CSF-1-starved BMMs. The mean fluorescence was approx. 4-fold lower in the RAW264 cells, but the distribution of expression was skewed in both populations. In BMMs the majority of cells had high levels of cell-surface CSF-1Rs. In RAW264 cells, most cells had detectable CSF-1Rs but there was a tail, condensed by the logarithmic scale, of cells with much higher expression. In both populations addition of CSF-1 caused a rapid decrease in cell-surface CSF-1Rs; the residual receptor was similar in each case (Figure 2A).

Flow cytometry in these experiments detects only the cell-surface pool of CSF-1R, so we examined the location of CSF-1R within BMMs and RAW264 cells by immunofluorescence microscopy. Consistent with the FACS profile, in CSF-1-starved BMMs the majority of the cells had abundant CSF-1R protein on the cell surface, whereas lower-level expression was seen in the vicinity of the nucleus, presumably in the endoplasmic reticulum and Golgi apparatus (Figure 2B). On the cell surface, CSF-1R immunoreactivity was not distributed uniformly, with a concentration of immunoreactivity evident on the presumed leading edge of some cells (some examples are indicated with arrows in Figure 2B). The majority of RAW264 cells had detectable CSF-1R immunoreactivity. Whereas a small proportion of cells showed high-level plasma-membrane staining as expected from the FACS profile, in most cells intense immunoreactivity was detected in a perinuclear crescent, a pattern observed characteristically with Golgi-specific markers in these cells (D. A. Hume and J. L. Stow, unpublished work). Additionally, small vesicle-like structures were evident, which may have been endocytic or secretory. The exact location is not crucial to this study; the clear implication is that RAW264 cells have some defect in production of *c-Fms* protein, trafficking of the CSF-1R to the cell surface or its selective internalization.

As with the flow-cytometry data, the specificity of staining was evident from the disappearance of cell-surface immunoreactivity on treatment with CSF-1 (Figure 2B). The CSF-1R immunoreactivity in both CSF-1-treated BMMs, and less obviously in RAW264 cells, became concentrated in cytoplasmic vesicles. As noted above, the level of cell-surface CSF-1Rs in CSF-1-treated BMMs or RAW264 cells remains detectable (Figure 2A), presumably due to the establishment of a steady state in which internalization is balanced by reappearance of new receptors. The rate at which CSF-1R protein is replenished upon the cell

Figure 1 Western-blot analysis of protein tyrosine phosphorylation

(A) Comparison of BMMs and RAW264 cells that had been starved of CSF-1 overnight and treated with 10^4 units/ml CSF-1 for the indicated times (in min). The * indicates BMMs grown in the continued presence of CSF-1. Total cell lysates (150 μ g of protein) were electrophoresed on an SDS/polyacrylamide (7.5%) gel and the resultant blot incubated with the anti-phosphotyrosine monoclonal 4G10. (B) Cell lysates from RAW264 cells treated with 10^{-7} M PMA (R) and insulin-stimulated 3T3-L1 adipocyte cells (L1) were analysed as described in (A). Arrows a and b mark the positions of the ERK-1 and ERK-2 (MAPK p44 and p42) bands. Band c is not defined.

Figure 2 Comparison of CSF-1R expression in BMMs and RAW264 cells by immunofluorescence

(A) Cell-surface expression of CSF-1R on starved BMMs and RAW264 cells as measured by flow cytometry. BMMs (—CSF-1) were starved of CSF-1 for 16 h while RAW264 cells (—CSF-1) were grown in the absence of CSF-1. The +CSF-1 profiles refer to cells which were then treated with 10^4 units/ml CSF-1 for 1 h. Baseline fluorescence was measured on cells labelled with the secondary antibody alone. The mean value of fluorescence intensity for each profile is marked on the histogram. (B) Immunofluorescence localization of CSF-1R with anti-CSF-1R antibody. Note the delineation of the plasma membrane, with particular concentration on the presumed leading edges (some examples marked with arrows) in CSF-1-starved BMMs (panel a) and the disappearance of surface staining and appearance of intracellular vesicles in cells restimulated with CSF-1 for 1 h (panel b). In RAW264 cells, CSF-1R immunoreactivity in most cells is concentrated in crescents surrounding the nucleus in the absence of CSF-1 while some cells exhibit strong surface staining (panel c). After 1 h of CSF-1 treatment, surface staining vanished and more intracellular vesicles were visible (panel d).

surface can be assessed by examining the reappearance of receptor following CSF-1 removal from CSF-1-treated cells (Figure 3). In BMMs, increased numbers of cell-surface CSF-1Rs were detected at the earliest time point examined (30 min) and peaked by around 8 h, whereas in RAW264 cells there was a time lag of

2–4 h before any replenishment of cell-surface receptors was detectable.

The apparent deficiency in production/trafficking of CSF-1Rs in RAW264 cells clearly raises the question of whether the transfection of the cells with a c-Fms expression plasmid, which

Figure 3 Reappearence of CSF-1Rs on the cell surface of BMMs and RAW264 cells after withdrawal of CSF-1

BMMs were cultured in the continued presence of CSF-1 while RAW264 cells were grown in the presence of CSF-1 for a minimum of 18 h before removal of the growth factor. Cells were harvested 0.5, 1, 2, 4 and 8 h after withdrawal of CSF-1 and cell-surface expression of CSF-1R analysed by flow cytometry. The increase in mean fluorescence above baseline fluorescence of the cells at each of the time points is plotted.

permits a CSF-1 response at the level of uPA transcription, really does reconstitute normal surface expression. We therefore measured the level of cell-surface c-Fms expression in transfected cells. Approx. 2–3% of the cells transfected with a c-Fms expression plasmid demonstrated high levels of cell-surface CSF-1R-associated fluorescence (Figure 4A). This percentage was lower than expected and difficult to reconcile with the substantial CSF-1 response observed in such transfected cells when a uPA promoter-reporter gene is co-transfected (see below). A time course of cell-surface CSF-1R expression 4, 8, 12 and 24 h after transfection confirmed that the number of highly expressing cells did not simply peak at an earlier time point and then decay to these levels (results not shown). The explanation was revealed by immunofluorescence microscopy where the elevated level of expression of CSF-1Rs in a larger percentage of cells was visibly obvious. The majority of the cells overexpressing high levels of CSF-1R had the receptor sequestered intracellularly in the perinuclear region and additionally had numerous CSF-1R-immunoreactive cytoplasmic vesicles resembling those observed in CSF-1-treated BMMs (Figure 4B). CSF-1R-superpositive cells comprised up to approx. 16% of the total cell population, as estimated by counting cells in 10 separate randomly selected fields. We propose that the transfected RAW264 cells go through cycles of cell-surface expression and internalization into cytoplasmic vesicles followed by accumulation in the Golgi complex prior to rapid transport to the cell surface. The trigger for internalization in the absence of CSF-1 may be the point at which enough CSF-1Rs are on the cell surface for spontaneous dimerization to occur, generating signalling activity that is responsible for the increased baseline reporter expression in transfected cells (see below). According to this view, all the cells overexpressing the receptor acquire the ability to respond to CSF-1 even though only a small proportion have the receptor on the surface at any moment in time. It would appear from the pattern of CSF-1R localization that transfection/overexpression does not overcome the defect in Golgi–plasma membrane translocation in RAW264 cells, but simply increases the amount of receptor produced so that a sufficient amount gets to the surface to allow a response.

Figure 4 Expression of the CSF-1R in transfected RAW264 cells

RAW264 cells were electroporated with 10 μ g of pGL2uPA-6.6 and either 10 μ g of pEF-BOS (+ pEF-BOS) or 5 μ g of pEF-cfms plus 5 μ g of pEF-BOS (+ pEF-cfms). After 20 h in culture, the expression of the CSF-1R was analysed by both flow cytometry and immunofluorescence. (A) FACS analysis of the cell-surface expression of c-Fms. The percentage of cells falling in the high-expression region increased from 0.3% to 2.5% when pEF-cfms was included in the transfection. (B) Immunofluorescence analysis of the cells transfected with pEF-cfms plus pEF-BOS as described above. Three cells transfected with pEF-cfms demonstrate different distribution of the overexpressed c-Fms. Note the much less bright cells (arrows) which are presumed not to be expressing transfected pEF-cfms.

Multiple tyrosine residues within the CSF-1R are required for signalling in RAW264 cells

Our earlier studies implicated a signalling pathway linking the CSF-1R to Ras/Raf/MAPK activation and ultimately to phosphorylation of the transcription factor Ets-2, which activated the uPA promoter through the conserved Ets/AP1 elements [22]. Mutation of one critical tyrosine within the human CSF-1R (Y809F) abolished the activity of the receptor in this pathway in *c-fms*-transfected fibroblasts [22], extending previous studies showing that the human Y809F mutation in CSF-1R reduces the ability of the activated receptor to promote anchorage-independent growth in fibroblasts [29], differentiation of an immature myeloid leukaemia cell line [8] and binding and activation of the Src family of kinases [30]. Having established that the complementation of the defect in RAW264 cells by a c-Fms expression plasmid does involve increased expression of CSF-1R, we wished to determine whether the Y809 phosphorylation required for uPA promoter activation in fibroblasts is also required in the macrophage system. An additional question was whether this is the only tyrosine absolutely required for this pathway. Therefore, we assessed the effect of two mutations in the mouse CSF-1R: Y807F (equivalent to human Y809F) and Y559F, which has also been implicated in the binding and activation of the Src family of kinases by activated CSF-1R [9].

Co-transfection of a wild-type c-Fms expression plasmid increased the basal activity of the 6.6-kb uPA promoter in transfected RAW264 cells approx. 2-fold without added CSF-1

Figure 5 Effect of Y807F and Y559F mutants of the CSF-1R

(A) RAW264 cells were electroporated with 10 μ g of pGL2uPA-6.6 plus 10 μ g of expression/control plasmids comprised of 10 μ g of pEF-BOS (+ pEF-BOS), 5 μ g of pEF-cfms or the equivalent mutant plasmid plus 5 μ g of pEF-BOS (+fms, +Y559F or +Y807F) or 5 μ g each of both the mutant plasmids (+Y559F + Y807F). Luciferase activities were normalized to the +pEF-BOS sample. This experiment was performed three times in duplicate with the +Y559F + Y807F transfection included in only two of these experiments. The error bars represent the S.E. (B) Cells were transfected as above and cell-surface CSF-1Rs were detected by immunofluorescence and FACS analysis as described in Figure 4.

(Figure 5). Compared with the wild-type receptor, both of the CSF-1R mutants resulted in a 70–80% decrease in the CSF-1-induced activity of the uPA reporter gene, but neither completely abolished the CSF-1 response (Figure 5). Hence, both phosphorylated tyrosines are required for maximal CSF-1 signalling, but neither is absolutely sufficient to initiate the response leading to uPA promoter activation. To ensure that the loss of activity in the mutants was not due simply to reduced cell-surface expression, we carried out FACS analysis of the transfected cells. The increased level of CSF-1R expression on cells transfected with the mutant receptors was indistinguishable from that of cells transfected with the wild-type receptor (Figure 5B). As with the wild-type receptor, only a small proportion of transfected

cells expressed elevated levels of the mutant receptor on the cell surface.

Upon activation, the CSF-1R dimerizes. In principle, two mutant receptors might potentially complement each other if each provides one functional docking site for signal-transduction molecules containing SH2 domains, i.e. a Y807F/Y559F heterodimer might be more active than a homodimer of either mutant. To test this possibility, we co-transfected both mutant receptors together, but the response was essentially the sum of the two individual responses to the mutant receptors and no evidence of complementation was found (Figure 5).

Both Y807 and Y559 of CSF-1R reduced the induction of uPA promoter activity by CSF-1 substantially. Both residues have been shown to be necessary for maximal activation of the Src family of kinases [9,30], and Src kinases have been implicated in the regulation of uPA transcription [31]. The mutant receptors appear distinct in that Y559F had a selective effect on the basal activity of the uPA promoter elicited by plasmid alone and the effects of the two mutant plasmids were purely additive (but not synergistic). Each phosphotyrosine may contribute to different aspects of the CSF-1 signal, a proposal consistent with the observation that the Y807 mutation selectively reduced differentiation-inducing activities of the CSF-1R in murine myeloid cells without preventing mitogenic activation [8]. The residual CSF-1 response seen with each mutant is difficult to interpret because of the presence of endogenous receptor; it could be due to an increase in the number of wild-type endogenous receptors on the cell surface of the transfected cells accompanying the mutant receptors or possibly the formation of active heterodimers with wild-type receptor. The important point is that more than one putative docking-site phosphotyrosine in the CSF-1R is absolutely required for maximal activation of the uPA promoter by CSF-1. Given the extended sequence conservation of the uPA promoter, and the multiple CSF-1-responsive elements, we favour a mechanism in which different signals arise in parallel from the receptor and target different *cis*-acting elements.

Ets/AP1 is a target for the CSF-1 signalling pathway in transfected RAW264 cells

In a previous study of the uPA promoter responding to basic fibroblast growth factor in fibroblasts [6] the Ets/AP1 element at –2.4 kb was found to be necessary but not sufficient to mediate maximal activation of promoter activity. In the first description of the CSF-1/c-Fms signalling system in RAW264 cells, we showed that deletion of this element from the –6.6-kb promoter reduced both CSF-1-stimulated and basal activity by more than 80% [22] and we had found previously that the Ets/AP1 element alone responded to both phorbol esters and oncogenic Ras [4,7]. We therefore examined whether the Ets/AP1 element alone is sufficient to elicit a CSF-1 response. A luciferase reporter construct under the control of 90 bp of the uPA upstream sequences encompassing the –2.4-kb enhancer linked to the minimal uPA promoter (from –114 to +398, including the first intron; pGL99) was co-transfected with the c-Fms expression plasmid and cells were exposed to CSF-1. Constructs with mutations in either half of the Ets/AP1 element were also available (pGL119, pGL127), as was a construct simply under the control of the uPA minimal promoter/intron 1 alone (pGL126). As expected, pGL99 was activated by CSF-1 addition to c-fms-transfected cells (Table 1). The action of the CSF-1/c-Fms signal on the proximal promoter was not due to a general increase in transcriptional activity of the cell as luciferase expression from the plasmid controlled by the β -actin promoter was not affected by CSF-1/c-Fms. As a positive control, we

Table 1 The Ets/AP1 element at —2.4 kb of the uPA promoter responds to CSF-1 signalling in transfected RAW264 cells

RAW264 cells were transiently transfected with 10 µg of a reporter construct together with either 5 µg of a c-Fms expression plasmid, cfms-pECE, or 5 µg of the control parent plasmid, pECE. The uPA-reporter constructs were under the control of a 90-bp fragment encompassing the Ets/AP1 element linked to the uPA minimal promoter/first intron. pGL99 contained the wild-type Ets/AP1 element while pGL119 and pGL127 contained mutations in one half or the other of this element. pGL126 was under the control of the minimal promoter/first intron alone. pGLβA was a luciferase reporter construct under the control of 4.3 kb of the β-actin promoter. After transfection, the cells were divided into four subgroups, one of which was the control. To the +CSF-1 and +CSF-1/PMA cells, 10⁴ units/ml CSF-1 was added immediately after the transfection. After 6 h in culture, 10⁻⁷ M PMA was added to the +PMA and +CSF-1/PMA cells. After a total of 24–30 h in culture, the luciferase activities were determined. These values were normalized to the untreated pECE co-transfection for each reporter. This experiment was performed three times in duplicate with pGL126 and pGLβA included in two of the experiments. Values are presented with the S.E.

Plasmid	Treatment	+ pECE	+ cfms-pECE
pGL99 (wild-type)	Control	1.00 ± 0.00	2.16 ± 0.54
	+ CSF-1	0.96 ± 0.06	9.05 ± 3.29
	+ PMA	6.64 ± 1.50	14.79 ± 5.86
	+ CSF-1/PMA	5.99 ± 1.26	24.81 ± 10.32
pGL119 (Ets mutant)	Control	1.00 ± 0.00	2.31 ± 0.27
	+ CSF-1	0.96 ± 0.06	5.08 ± 0.72
	+ PMA	3.68 ± 0.41	7.37 ± 1.23
	+ CSF-1/PMA	3.55 ± 0.40	10.44 ± 1.72
pGL127 (AP1 mutant)	Control	1.00 ± 0.00	1.71 ± 0.28
	+ CSF-1	0.92 ± 0.03	3.48 ± 0.73
	+ PMA	2.98 ± 0.61	5.18 ± 1.67
	+ CSF-1/PMA	2.91 ± 0.62	7.26 ± 2.07
pGL126 (minimal promoter)	Control	1.00 ± 0.00	1.90 ± 0.38
	+ CSF-1	0.95 ± 0.06	4.58 ± 1.36
	+ PMA	3.62 ± 0.61	5.78 ± 2.14
	+ CSF-1/PMA	3.23 ± 0.63	8.15 ± 2.92
pGLβA (β-actin promoter)	Control	1.00 ± 0.00	1.42 ± 0.22
	+ CSF-1	0.92 ± 0.03	1.88 ± 0.30
	+ PMA	1.94 ± 0.02	2.79 ± 0.42
	+ CSF-1/PMA	1.93 ± 0.07	2.99 ± 0.39

included the phorbol ester, PMA, alone or in combination with CSF-1. PMA was somewhat more active than CSF-1 in activating the promoter activity of pGL99 and the two in combination caused a small additional increment. Removal of the 90-bp fragment selectively reduced CSF-1-, PMA- or CSF-1 plus PMA-stimulated activity approx. 2–3-fold, but the minimal promoter (pGL126) retained responsiveness to all three treatments. Mutations in either the Ets or the AP1 half of the Ets/AP1 element reduced CSF-1, and CSF-1 plus PMA-stimulated activity to the same extent as complete removal of the 90-bp fragment. The results indicate that CSF-1/c-fms transfection does reconstitute the signalling pathway targeting the Ets/AP1 element. The activation of this element in isolation is comparatively weak, and additional CSF-1- and PMA-responsive elements appear to be present in the proximal promoter region.

Conclusions

The maintenance of high levels of uPA mRNA in primary macrophages depends upon the continuous presence of CSF-1. When CSF-1 is removed, uPA mRNA is rapidly degraded in a manner that is dependent upon new RNA synthesis [3]. In previous studies, we demonstrated that ERK-2 (MAPK p42) activation and Ets-2 phosphorylation was sustained in the continuous presence of CSF-1, and provided several lines of evidence that this pathway was required for activated uPA transcription [4,7,22]. Because the CSF-1R is internalized and

degraded following CSF-1 binding, continuous signalling implies a requirement for the production of new cell-surface receptors. The studies in this report imply that the rate of receptor trafficking can be limiting for the response to CSF-1.

There has been considerable recent interest in the concept of 'quantitative signalling', particularly with reference to the Ras/Raf/MAPK pathway. For example, in PC12 cells the 'magnitude' of the Ras signal determines the balance between continued proliferation and progress towards neuronal differentiation [32,33]. The activation of uPA transcription by CSF-1 appears to be another example of quantitative signalling, the quantitative variable being the duration and sustained nature of the signal delivered to the cell. In this study we have presented evidence that the limiting determinant of whether the signal is sustained is whether the CSF-1R is replenished on the cell surface at a sufficient rate. Using the RAW264 model we have confirmed that CSF-1/c-Fms signalling targets the Ets/AP1 element of the uPA promoter, consistent with evidence that phosphorylation of the pointed domain of Ets-2 is a critical event in the CSF-1 signalling pathway in primary macrophages [22]. The evidence that Y807 (human residue 809) is required for this pathway in RAW264 cells validates studies with fibroblasts; the discovery that Y559 is also required offers new challenges in uncovering the interactions between signals generated from the CSF-1R.

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