Molecular Biology of the Cell
Vol. 11, 3835–3848, November 2000

Colony-stimulating Factor-1 Receptor Utilizes Multiple Signaling Pathways to Induce Cyclin D2 Expression

Arunangsu Dey,*† Hongyun She,†‡ Leopold Kim,* Allan Boruch,‡ Deborah L. Guris,§ Kristen Carlberg,‖ Saïd M. Sebti,¶ David T. Woodley,‡ Akira Imamoto,§ and Wei Li*‡#

‡Department of Medicine, Division of Dermatology, University of Southern California Keck School of Medicine, Los Angeles, California 90033; *The Ben May Institute for Cancer Research, and §Center for Molecular Oncology, University of Chicago, Chicago, Illinois 60637; ‖Fred Hutchinson Cancer Research Center, Seattle, Washington 98104; and ¶H. Lee Moffitt Cancer Center and Research Institute, University of South Florida, Tampa, Florida 33612

Submitted June 15, 2000; Revised August 8, 2000; Accepted August 16, 2000
Monitoring Editor: Carl-Henrik Heldin

Colony-stimulating factor-1 (CSF-1) induces expression of immediate early gene, such as c-myc and c-fos and delayed early genes such as D-type cyclins (D1 and D2), whose products play essential roles in the G1 to S phase transition of the cell cycle. Little is known, however, about the cytoplasmic signal transduction pathways that connect the surface CSF-1 receptor to these genes in the nucleus. We have investigated the signaling mechanism of CSF-1-induced D2 expression. Analyses of CSF-1 receptor autophosphorylation mutants show that, although certain individual mutation has a partial inhibitory effect, only multiple combined mutations completely block induction of D2 in response to CSF-1. We report that at least three parallel pathways, the Src pathway, the MAPK/ERK kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway, and the c-myc pathway, are involved. Induction of D2 is partially inhibited in $Src^{-/-}$ bone marrow-derived macrophages and by Src inhibitor PP1 and is enhanced in v-Src-overexpressing cells. Activation of myc's transactivating activity selectively induces D2 but not D1. Blockade of c-myc expression partially blocks CSF-1-induced D2 expression. Complete inhibition of the MEK/ERK pathway causes 50% decrease of D2 expression. Finally, simultaneous inhibition of Src, MEK activation, and c-myc expression additively blocks CSF-1-induced D2 expression. This study indicates that multiple signaling pathways are involved in full induction of a single gene, and this finding may also apply broadly to other growth factor-inducible genes.

INTRODUCTION

Colony-stimulating factor-1 (CSF-1 or M-CSF) ensures survival and selectively stimulates proliferation of cells of the monocyte and macrophage lineage (Stanley, 1986). CSF-1 binds to and activates its cell surface protein tyrosine kinase receptor (CSF-1R/c-Fms) (Sherr and Stanley, 1990). The immediate early substrate for the CSF-1R tyrosine kinase is the receptor itself (Sengupta *et al.*, 1988; Li *et al.*, 1991). Six phosphotyrosine residues in the human and/or the mouse CSF-1 receptor (CSF-1R) (Y561/Y559, Y571/Y569, Y699/Y697, Y708/Y706, Y723/Y721, and Y809/Y807) have been identified (Rohrschneider *et al.*, 1997; Roussel, 1997; Bourette and Rohrschneider 2000). The principal function of the receptor tyrosine autophosphorylation is to recruit cytoplasmic signaling proteins that have Src-homology 2 or phosphotyrosine-binding domain (Pawson, 1995). Y561 binds Src family kinases, which have been reported to play an important role in CSF-1–stimulated DNA synthesis in the context of NIH 3T3 cells (Courtneidge *et al.*, 1993; Roche *et al.*, 1995). Y569 is required for activation and endocytosis of CSF-1 receptor (Myles *et al.*, 1994), but no binding molecule has been identified. Y697 and its carboxy-end amino acids show a $Y^{697}XNX$ motif and bind the adapter protein growth factor receptor-binding protein-2 (Grb2) (van der Geer and Hunter, 1991). Grb2 links tyrosine kinase receptors to the Ras/extra-

† These authors contributed equally to this work.
# Corresponding author. E-mail address: wli@hsc.usc.edu.
Abbreviations used: BMM, bone marrow-derived macrophages; CSF-1, colony-stimulating factor-1; CSF-1R, CSF-1 receptor; D2, cyclin D2; ERKs, extracellular signal-regulated kinases; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Grb2, growth factor receptor-binding protein-2; ODC, ornithine decarboxylase; PDGF, platelet-derived growth factor; PI-3K, phosphotidylinositol 3-kinase; SH2, Src-homology domain 2; $Src^{-/-}$, Src-deficient; v-Src, viral form of Src.

cellular signal-regulated kinase (ERK) pathway through Sos (Pawson and Schlessinger, 1993). The Y697F mutation (together with the Y721F mutation) inhibits CSF-1–stimulated DNA synthesis in Rat-2 fibroblasts (van der Geer and Hunter, 1993) but not in FDC-P1 myeloid cells (Bourette *et al.*, 1995). Y706 appears to mediate CSF-1–stimulated STAT-1 (signal transducers and activators of transcription 1) activation and STAT-1 interaction with CSF-1R (Novak *et al.*, 1996). Y721 is the binding site for both phosphotidylinositol 3- kinase (PI3-K) (Shurtleff *et al.*, 1990; Reedijk *et al.*, 1992) and phospholipase C-γ2 (Bourette *et al.*, 1997). PI3-K and phospholipase C-γ2 have been reported to cooperate in mediating CSF-1–induced macrophage differentiation (Bourette *et al.*, 1997). Mutation at Y809 of the human CSF-1R severely impairs the receptor-mediated mitogenesis in NIH 3T3 (Roussel *et al.*, 1990) and RAT-2 (Reedijk *et al.*, 1990; van der Geer and Hunter, 1991) cells. The equivalent Y807F mutant of the mouse CSF-1R, however, shows only a moderate defect in inducing c-myc gene expression in FDC-P1 cells, and CSF-1R-Y807F–expressing cells grow even faster than cells expressing the wild-type CSF-1R in the presence of CSF-1 (Bourette *et al.*, 1995). Other reported CSF-1R–binding proteins include GTPase-activating protein (Reedijk *et al.*, 1992), Shc (Lioubin *et al.*, 1994), c-Cbl (Wang *et al.*, 1996), and Ship-1 (Lioubin *et al.*, 1996), but their binding sites remain unknown. Our current understanding is that through these phosphotyrosine sites, CSF-1R engages in multiple parallel cytoplasmic signaling pathways, leading to gene expression and ultimately cell survival and proliferation.

D-type cyclin (D1, D2, and D3) expression is induced by mitogens during the G1-to-S phase transition of the cell cycle (Sherr, 1993; Pines, 1995). Induced D-type cyclins form complexes with cyclin-dependent kinases Cdk-4 and Cdk6, which in turn phosphorylate the retinoblastoma (Rb) gene product, thereby helping to cancel its growth-inhibitory effects by dissociating Rb from transcription factor E2F (Weinberg, 1995; Sherr 2000). The Rb-free E2F then initiates another wave of gene expression that drives cells through the "restriction point" of late G1 phase. After this point, the cells become irreversibly committed to complete the cycle. Consistent with the role of D-type cyclins in cell proliferation, overexpression of D1 shortens the G1 phase of the cell cycle (Quelle *et al.*, 1993) and rescues the mitogenic defect of the human CSF-1 receptor Y809F mutation (Roussel *et al.*, 1995). Moreover, microinjection of anti-D1 antibodies prevents cells at the G1 phase from entering the S phase of the cell cycle (Baldin *et al.*, 1993; Quelle *et al.*, 1993). While in cell culture D1, D2, and D3, which show tissue-specific expression, do not seem to functionally overlap (Kato and Sherr, 1993), D-type cyclins appear to compensate each other in most tissues and organs during mouse development. Individual knockout of D1 and D2 genes in mice does not affect the overall development of the animals but rather targets specialized tissues and cell lineages (Fantl *et al.*, 1995; Sicinski *et al.*, 1995; Sicinski *et al.*, 1996). Lack of D1 gives rise to mice with smaller size and developmental defects in retinas and breasts (Fantl *et al.*, 1995; Sicinski *et al.*, 1995). Mice lacking D2 show hypoplastic testes in males and sterileness due to failed proliferation of ovarian granulosa cells in response to follicle-stimulating hormone (Sicinski *et al.*, 1996). However, despite the importance of D-cyclin genes in control of cell growth and development, the signaling pathways that mediate the cell surface signals to the expression of these genes remain poorly understood.

Taken together, during the G1-to-S phase transition CSF-1 induces a cascade events of signal transduction that include CSF-1R activation, protein tyrosine phosphorylation, engaging in cytoplasmic signaling networks, activation of yet-to-be identified transcription machinery, induction of immediate early genes such as c-myc and c-fos, induction of delayed early genes such as D-cyclins and Cdk inhibitors, inactivation of Rb and activation E2F transcription machinery, induction of the third wave of genes, and "crossing" the G1 restriction point. To gain insights into the mechanisms of D-cyclin regulation, we have used various approaches to understand how CSF-1R signaling regulates D2 expression in the contexts of myeloid cells and macrophages. Results of this study indicate that Src, MAPK/ERK kinase (MEK), and c-myc pathways contribute to CSF-1–stimulated full expression of D2 gene.

MATERIALS AND METHODS

FDCP-1 cells expressing the wild type or various mutants of the mouse c-fms/CSF-1R were kindly provided by Dr. Larry Rohrschneider (Fred Hutchinson Cancer Research Center, Seattle, WA). BAC1.2F5 cells and purified mouse CSF-1 were the gifts of Dr. E. Richard Stanley (Albert Einstein College of Medicine, New York, NY). Mouse cyclin D1 and cyclin D2 cDNAs and anti-D1 (B35) and anti-D2 (mAbD2–34B4-7) antibodies were gifts of Dr. Martine Roussel (St. Jude Children Resarch Hospital, Memphis, TN). Monoclonal anti-D1 antibody (D1-72-13G) was purchased from ZYMED Laboratories (South San Francisco, CA). c-Myc, ornithine decarboxylase (ODC) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNAs were kindly provided by Dr. Nissim Hay (The University of Illinois at Chicago, Chicago, IL). Antiactive mitogen-activated protein kinase (MAPK) pAb (V6671) was purchased from Promega (Madison, WI). Antiv-H-Ras monoclonal antibody (Ab-1), which cross reacts with K-Ras and N-Ras, for immunoprecipitation was purchased from Oncogene Science (Uniondale, NY). Anti-Ras monoclonal antibody (R02120) for Western blotting was from Transduction Laboratories (Lexington, KN). ^{125}I-protein A (70–100 mCi/mg) was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). Horseradish peroxidase-conjugated anti-mouse IgG (NA931) and electrochemiluminescence reagents were purchased from Amersham Life Science (Indianapolis, IN). Farnesyltransferase inhibitor FTI-277 and geranylgeranyltransferase I inhibitor GGTI-298 were used as previously described (Lerner *et al.*, 1995, 1997). MEK inhibitor PD98059 [2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one] and PI3-K inhibitor wortmannin were gifts from Dr. Marsha Rosner (University of Chicago, Chicago, IL). Other inhibitors were obtained as indicated.

Cell Culture

FDCP-1 cells expressing various mouse fms/CSF-1R constructs were maintained and stimulated with CSF-1 as previously described (Bourette *et al.*, 1995). Approximately 80% confluence of cells were used for all experiments. Murine BAC1.2F5 macrophages were grown in alpha medium (Life Technologies-BRL, Grand Island, NY) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum, penicillin G (50 U/ml), streptomycin sulfate (50 mg/ml), and 10% (vol/vol) L cell-conditioned medium as the source of CSF-1. Cells were arrested at G1 by incubating in CSF-1–free medium for 20 h and restimulated with mouse CSF-1 (13.2 or 4.4 nM).

Table 1. Effects of inhibitors on CSF-1–induced D2 and myc[a]

Name of inhibitor	Target(s)	Decrease (D)/ increase (In) On D2	Decrease (D)/ increase (In) On c-myc
FTI-277	H-ras, N-Ras	D	—
GGTI-298	K-Ras	D	—
PP1	Src family kinases	D	—
Lithium	GSK-3β	—	—
Rottlerin	PKCδ/CaMK-3	D	D
Staurosporin	PKC/MLCK/PKA/PKG	D	D
Wortmannin	PI-3K	—	—
SB-202190/SB-203580	SAPK2a/p38 and SAPK2b/p38β	D	D
PD98059	MEK1	D	—
Rapamycin	$p70^{S6K}$, cdk2	—	—
LY294002	PI3-K	—	—
Anisomycin	SAPK2a/p38 and SAPK2b/p38b	In	In

[a] Based on results of Northern blot analysis of three independent experiments under each condition. The D and In are defined by 30% or more decrease or increase in reference to CSF-1 alone. −, No or insignificant effect.

List of Inhibitors Used and Their Pretreatment Conditions

The panel of chemical inhibitors, their targets, time for pretreatment, and concentrations used in our screening assays were as follows: FTI (Ras, 24–48 h, 10 μM), GGTI (Ras, 24–48 h, 1 μM), PP1 (Src kinases, 6–12 h, 5 μM), Li^+ (GSK-3β, 25 min, 1 mM), rottlerin (PKCδ, 60 min, 1–3 μM), HA1004 (PKA/CaMKII, 20 min, 2–10 μM), wortmannin (PI-3K, 15 min, 100–500 nM), SB202190 (p38-α/β MAPK, 30 min, 0.35 μM), PD98059 (ERKs, 15 min, 5–30 μM), rapamycin ($p70^{S6K}$, Cdk2, 30 min, 1 μM), and staurosporin (PKC/MLCK/PKA/PKG, 30 min, 1–3 nM). Their effects on CSF-1–stimulated c-myc and D2 expression are summarized in Table 1.

Establishment of v-Src and myc-ER Stable Cell Lines

Retroviral vector pMV7 containing the chick v-Src gene was used to transfect Bosc-23 cells, and a neomycin-resistant population was selected for producing replication-incompetent virus stock. The myc-ER fusion gene (from Dr. Hay, University of Illinois at Chicago, Chicago, IL) was subcloned into the retroviral vector pBabe, transfected into Bosc-23 cells, and selected for neomycin-resistant population. Conditioned media of the cultured cells were used as viral stocks. BAC1.2F5 cells (1×10^6 in 60-mm tissue culture dish) were incubated in the medium with corresponding viral stocks (2 ml/dish) for 4 h in the presence of polybrene (5 μg/ml). Media were removed and cells incubated with fresh CSF-1–containing medium for 2 d before selection under G418 (1 mg/ml). The mixed population and isolated individual clones were maintained in culture medium containing 300 μg/ml G418. Fresh medium was added every 4 d. Expression of the v-Src and the myc-ER fusion proteins were analyzed by Western immunoblot analyses with anti-Src antiserum (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-ER monoclonal antibody (from Dr. Geoffrey Greene, University of Chicago, IL). Results were visualized by binding to ^{125}I-protein A and autoradiography.

Preparation of Bone Marrow-derived Macrophages

Bone marrow-derived macrophages (BMM) from wild-type, $Src^{+/-}$, and $Src^{-/-}$ mice were extracted and cultured as described elsewhere (Tushinski *et al.*, 1982). Day 5 adherent cells were pooled and either frozen in liquid nitrogen for future use or continued in culture for additional 6 d. For experiments, day 11 BMM cells ($\sim 7 \times 10^6$ cells) were deprived of CSF-1 for 18–20 h and restimulated with murine CSF-1 (4.4 nM) for indicated times.

Northern Blot Analysis

Total RNA was isolated using RNeasy kit (Qiagen, Chatsworth, CA). Equal amounts of RNA were size-fractionated on 1% agarose gel containing 0.66 M formaldehyde, transferred to Duralon nylon membrane (Stratagene, La Jolla, CA), and cross-linked to membrane by UV and baking under vacuum at 80°C for 2 h. Membrane was prehybridized in 50% formamide, 6× SSPE, 0.5% SDS, 5× Denhart's solution, 100 μg/ml sheared and denatured salmon sperm DNA at 42°C, and then hybridized in the same buffer in the presence of radiolabeled cDNA probes. Membrane was washed two times in 2× standard saline citrate at 50°C for 20 min, followed by washing three times with 0.2× standard saline citrate, 0.1% SDS for 1 h total at 60°C. Between hybridizations with different cDNA probes, previously bound probe on the membrane was stripped off by boiling for 10 min in the solution of 0.1× SSPE and 0.5% SDS, and rinsing two times with 1× SSPE before re-prehybridization.

Immunoprecipitation and Western Blot Analyses

Triton X-100 soluble extract of the cells was immunoprecipitated with anti-Ras antibody (Ab-1, 2 μg/ml, 2 h at 4°C), followed by a rabbit anti-mouse IgG antibody (3 mg/ml, 1 h at 4°C). The immunoprecipitates were resolved in SDS gel, transferred to a nitrocellulose membrane (Micron Separations, Westboro, MA), and immunoblotted with anti-Ras monoclonal (R02021) and secondary rabbit anti-mouse IgG antibodies. The results were visualized by incubating with ^{125}I-protein A followed by autoradiography. For direct Western blot analysis, cells were solubilized in lysis buffer as previously described (Li *et al.*, 1991). After centrifugation (15,000 × *g* for 8 min at 4°C), protein concentrations of cell extracts were measured by using Bio-Rad MicroAssay and equalized. Aliquots of equal amounts of total cellular proteins were resolved in 8% SDS-PAGE (acrylamide:bis, 30:0.8) and transferred to nitrocellulose membranes. After washing once with Tris-buffered saline (TBS), twice with TBS-Triton X-100 (0.1%), and once more with TBS, the membrane was blocked in TBS containing 5% (wt/vol) bovine serum albumin (Intergen, Purchase, NY) before incubation with the corresponding antibodies. Immunoreactive bands were detected with either horsesradish peroxidase and electrochemiluminescence (Amersham Life Science) or with ^{125}I-protein A and autoradiography.

Transfection

NIH3T3-cfms cells were cultured in DMEM supplemented with 10% fetal calf serum and streptomycin and penicillin (50 U/ml). Cells in 100-mm tissue culture dishes (~40% confluence) were transfected with 25 μg/dish of the indicated DNA constructs by using SuperFect reagent (Qiagen) according to the manufacturer's instructions and incubated for 2 h. The ratio of DNA to SuperFect reagent was 1:2 (wt/vol). Media were changed to fresh DMEM with 10% fetal calf serum and incubated for additional 48 h. Cells were then incubated in low serum (0.2%) medium overnight before CSF-1 treatment.

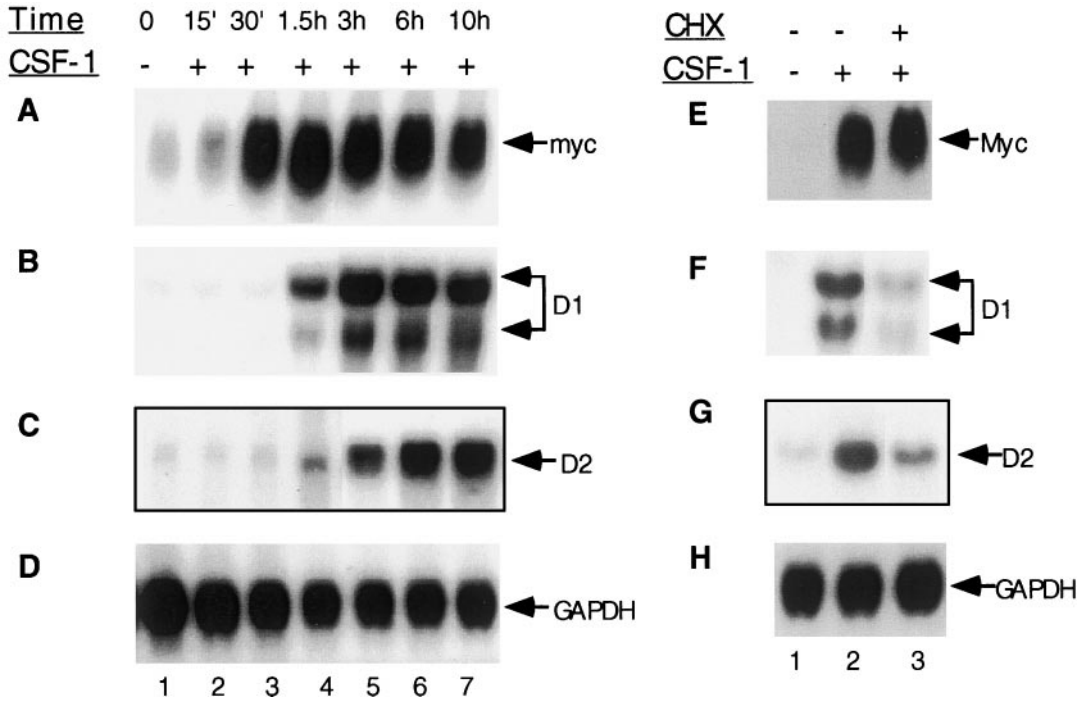

Figure 1. Time courses of CSF-1–stimulated transcription of c-myc, cyclin D1, and cyclin D2 genes in BAC1.2F5 macrophages. G1-arrested BAC1.2F5 cells were either unstimulated or stimulated with mouse CSF-1 (4.4 nM) at 37°C for the indicated times. In certain experiments (E–H), cells were preincubated with cycloheximide (10 μM) for 20 min before addition of CSF-1. Total RNA was isolated and analyzed for expression of the indicated genes by Northern blotting (30 μg RNA/lane) by using ^{32}P-labeled c-myc, D1, and D2 cDNA probes. Expression of GAPDH gene was included as control for RNA sample loading. The time of CSF-1 stimulation: E, 45 min; F and G, 5 h. Framing of C and G emphasize the subsequent focus on D2.

RESULTS

Time Courses of Cyclin D2 and D1 Expression in BAC1.2F5 Cells in Response to CSF-1

Mouse macrophage cell line BAC1.2F5 requires continued presence of CSF-1 for survival and proliferation (Morgan *et al.*, 1987). After Go/G1 arrest in CSF-1–free medium and restimulation with CSF-1, these cells reenter synchronously through G1 into the S phase of the cell cycle. Thus, BAC1.2F5 cell line represents an ideal cell culture system for studying CSF-1 signaling and cell cycle regulation. First, we set out to compare the time courses of c-myc, D1, and D2 induction in response to CSF-1. Cells were either untreated or treated with CSF-1 for different periods of time, and total RNA was isolated and analyzed for induction of c-myc, D1, and D2 by Northern blot analysis. It is shown in Figure 1 that induction of myc, an immediate early gene in response to almost all growth factors, could be detected as early as 15 min after addition of CSF-1 and reached a steady-state level by 30 min (Figure 1A), consistent with a previous study (Orlofsky and Stanley, 1987). Induction of D1 mRNA was not detectable until 90 min after CSF-1 stimulation and the level stayed constant for at least 10 h (Figure 1B), similar to the previously reported data (Matsushime *et al.*, 1991). The earliest detection of D2 induction was around 180 min (The slight induction at 90 min was not always reproducible.), and the expression reached a steady-state level by 6 h (Figure 1C). Loading of the RNA samples is indicated by GAPDH mRNA levels (Figure 1D). Thus, the temporal relationships among myc. D1 and D2 in response to CSF-1 are myc $>$ D1 $>$ D2. To test whether induction of c-myc, D1, and D2 requires de novo protein synthesis, cells were pretreated with cycloheximide (10 μM) for 30 min before CSF-1 stimulation. Cycloheximide treatment had no effect on induction of c-myc (Figure 1E, lane 3), but significantly inhibited D1 (Figure 1F, lane 3) and D2 (Figure 1G, lane 3) expression in response to CSF-1, indicating that de novo protein synthesis is required for D1 and D2 induction. Loading of the RNA samples was indicated by GAPDH control (Figure 1H).

CSF-1R Engages Multiple Signaling Pathways in Full Induction of D2

We were interested in the cytoplasmic signaling mechanism by which CSF-1R regulates expression of these genes, and decided to focus on the induction of D2 because little has been reported on it. We first studied this problem at the receptor level, by testing the effects of individual and combined mutations in the phosphotyrosine sites of the mouse CSF-1R, which are stably expressed in mouse myeloid progenitor cell line FDC-P1 (Rohrschneider and Metcalf, 1989; Myles *et al.*, 1994; Bourette *et al.*, 1995). CSF-1 stimulated a similar kinetics of the CSF-1R activation and c-myc, D1, and D2 expression in FDC-P1 to that in BAC1.2F5 cells (our unpublished results), suggesting that the intracellular signaling pathways at least for the three genes tested in these

two cell lines are compatible. The mouse CSF-1R mutants, including Y559A, Y569F, Y697F, Y706F, Y721F, Y807F, YTF (Y697F, Y706F, Y721F triple mutation), and Y5F (Y559F, Y697F, Y706F, Y721F, and Y807F), were expressed at similar levels in FDC-P1 cells (Myles *et al.*, 1994; Bourette *et al.*, 1995). Locations of the phosphotyrosine sites in the mouse CSF-1R and their known binding proteins are schematically shown in Figure 2A. Total RNA was isolated from each of these cell lines either untreated or treated with CSF-1 and analyzed for induction of D2. It is shown in Figure 2B that a similar fold induction of D2 was detected in cells expressing wild-type (lanes 1 and 2), Y569F (lanes 3 and 4), Y706F (lanes 7 and 8), and Y721F (lanes 9 and10) CSF-1R. Reduced induction of D2 was detected in cells expressing Y559A (lanes 13 and 14), Y697F (lanes 5 and 6), Y807F (lanes 11 and 12), and YTF triple (lanes 15 and 16) mutants. The Y5F mutation, however, almost completely abolished CSF-1–induced D2 expression (lanes 17 and 18). Quantitation of induced D2 expression (shown underneath Figure 2B) in fold increases was calculated based on reference to each of the corresponding GAPDH levels (Figure 2B, bottom). The relative percentage of the induction by the various mutant CSF-1Rs in reference to the wild-type CSF-1R is shown in Figure 2C. These results indicate that mutation at certain individual phosphotyrosine sites can partially block CSF-1R signaling to induce D2, and implicate that CSF-1R engages multiple cytoplasmic signaling pathways in a full induction of D2.

To further identify the possible signaling pathways that mediate CSF-1–stimulated expression of D2, we undertook a two-step approach by 1) starting out with screening a panel of inhibitors (as listed in Table 1) that are known under certain concentrations to block specific cellular signaling pathways, and 2) confirming the above-mentioned results with genetic approaches. As shown in Table 1, results from testing 12 inhibitors show that three main signaling pathways play a role in mediating CSF-1–stimulated D2 expression: the Src pathway, the c-myc pathway, and the MEK/ERK pathway. Inhibition of any one of the three pathways only partially (30–50%) inhibited the CSF-1–stimulated expression of D2 (Figure 6). The rest of the inhibitors used had either <10% inhibition or no inhibition (our unpublished results). We set out to further establish the roles of these three pathways in induction of D2 in response to CSF-1.

Src Partially Mediates CSF-1-stimulated Induction of D2

Src kinase has long been reported to play an important role in CSF-1–stimulated DNA synthesis in the context of NIH 3T3 fibroblasts (Courtneidge *et al.*, 1993; Roche *et al.*, 1995). To definitively address the role of Src in CSF-1R signaling to D2 as well as D1 and c-myc genes in macrophages, we isolated BMMs from wt, $Src^{+/-}$ and $Src^{-/-}$ mice and used these cells to test CSF-1–induced expression of D2, as well as D1 and myc. There was little visible difference between the wt and $Src^{+/-}$ mice and the BMM cells isolated from these mice. The $Src^{-/-}$ mice are, however, smaller in size than their wt and $Src^{+/-}$ littermates due to defects in bone remodeling and osteopetrosis (Soriano *et al.*, 1991). The relative number of BMM cells that were recoverable from a $Src^{-/-}$ mouse was five times less than that from its wt or $Src^{+/-}$ littermate. Nonetheless, the $Src^{-/-}$ BMM cells were able to proliferate for 11 d in culture in response to CSF-1 with a slightly slower doubling time (26 vs. 22 h), indicating that Src is not essential for mitogenic signaling by CSF-1R in macrophages. Total RNA was isolated from these cells and analyzed for CSF-1–induced gene expression. It is shown in Figure 3A that >50% of CSF-1–stimulated D2 expression was blocked in $Src^{-/-}$ (lane 6 vs. 2) and slightly inhibited in $Src^{+/-}$ (lane 4 vs. 2) BMM cells, in comparison to wt BMM cells (lane 2). In contrast, CSF-1 induced a comparable expression of D1 in wild-type, $Src^{+/-}$, and $Src^{-/-}$ BMM cells (Figure 3B, lanes 2, 4, and 6). The slight increase of D1 in $Src^{-/-}$ cells was apparently due to its higher RNA loading (Figure 3D, lane 6). Similarly, CSF-1–induced c-myc (Figure 3C) and c-fos (with an independent stimulation time of 30 min, because c-fos induction declines after 30 min; our unpublished results) expressions in these cells were unaffected. These results suggest that Src is a specific upstream activator of D2 gene in macrophages.

To confirm the above-mentioned results, we stably expressed v-Src in the mouse macrophage cell line BAC1.2F5 and tested whether it would increase D2 expression in the absence of CSF-1. We first compared proliferation of the v-Src cells with that of parental BAC1.2F5 cells. As shown Figure 4B, the 2F5-v-Src cells proliferate in a similar rate to that of parental BAC1.2F5 cells in response to CSF-1 (Figure 4, B vs. A), suggesting that expression of constitutively active v-Src did not significantly alter the cell cycle profile of the cells. Expression of the v-Src protein was indicated by anti-Src antibody immunoblotting (Figure 5A, lanes 3 and 4 vs. 1 and 2) (The 50-kDa protein has long been noticed as a hallmark of v-Src-expressing cells.) and by increased protein tyrosine phosphorylation (Figure 5B, lanes 3 and 4). Interestingly, consistent with the results of $Src^{-/-}$ BMM cells, expression of v-Src caused elevated expression of D2 even in the absence of CSF-1 (Figure 5C, lane 3 vs. 1). CSF-1 stimulation further enhanced the D2 expression to the full induction in these cells (lane 4 vs. 2). In contrast, the overexpressed v-Src had no effect on D1 (Figure 5D) or myc (Figure 5E) expression. We further tested the effect of the Src kinase inhibitor PP1. Pretreatment of the cells with PP1 up to 1 μM partially blocked D2 (Figure 5F), but not D1 (Figure 5G) or myc (Figure 5H), expression. It is noticed that the background levels (in the absence of CSF-1) of D1 and D2 varied among experiments, likely due to some difference after the 16 h CSF-1 deprivation (G1 arrest). Taken together, the above-mentioned findings demonstrate that Src kinase plays an important role in CSF-1R signaling to D2 gene, but the full induction of D2 by CSF-1 would require participation of other parallel pathway(s).

c-Myc Acts as an Upstream Activator of D2 Gene

To study the role of c-myc in CSF-1–induced D2 expression, we used myc-ER fusion gene, which is schematically shown in Figure 6A. The transactivating activity of myc in the myc-ER fusion protein can be regulated by 4-hydroxytomaxifen (4-HT) (Littlewood *et al.*, 1995). This myc-ER fusion gene construct was stably introduced into BAC1.2F5 cells. When lysates of 2F5-myc-ER clones were immunoblotted with anti-ER monoclonal antibody, a 95-kDa protein was detected in four of the five clones shown (lanes 2 to 6), but not in the parental cells (lane 1). Lysate of 293 cells transiently transfected with the same construct was included as a control (lane 7). Comparison of proliferation of 2F5-myc-ER cells

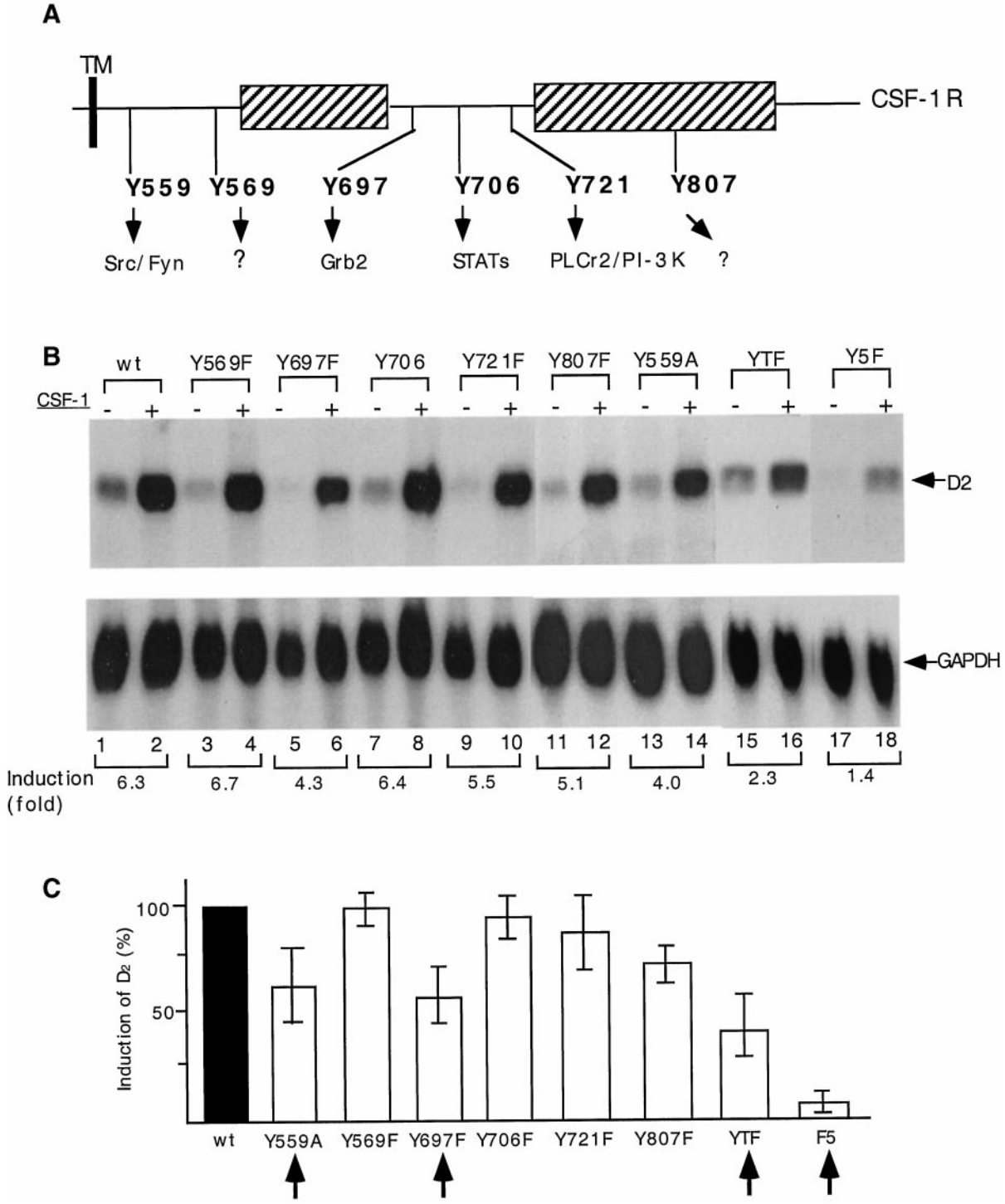

Figure 2. Effects of CSF-1R autophosphorylation mutations on CSF-1–induced cyclin D2 expression. FDC-P1 cells expressing the wild-type and the various mouse CSF-1R mutants (A) were starved in CSF-1–free medium with 10% fetal bovine serum for 16–18 h and untreated or treated with mouse CSF-1 (4.4 nM) for 5 h. Total RNA was isolated and analyzed for D2 (B, top) expression. GAPDH expression (B, bottom) was included as a control for RNA loading. Relative intensities of the bands were measured by scanning densitometry of each of the individual bands. Fold increases were given to each cell line after calibration with their corresponding GAPDH levels: band$^+$/band$^-$ X GAPDH$^-$/GAPDH$^+$ = Fold. "+" and "−" represent "with" and "without" CSF-1 stimulation, respectively. Percentages of induction of D2 by the various CSF-1R mutants in reference to the wild-type CSF-1R (100%) were based on results from three independent experiments (C), and data shown are mean ±SE of the scanning densitometry data of the three experiments.

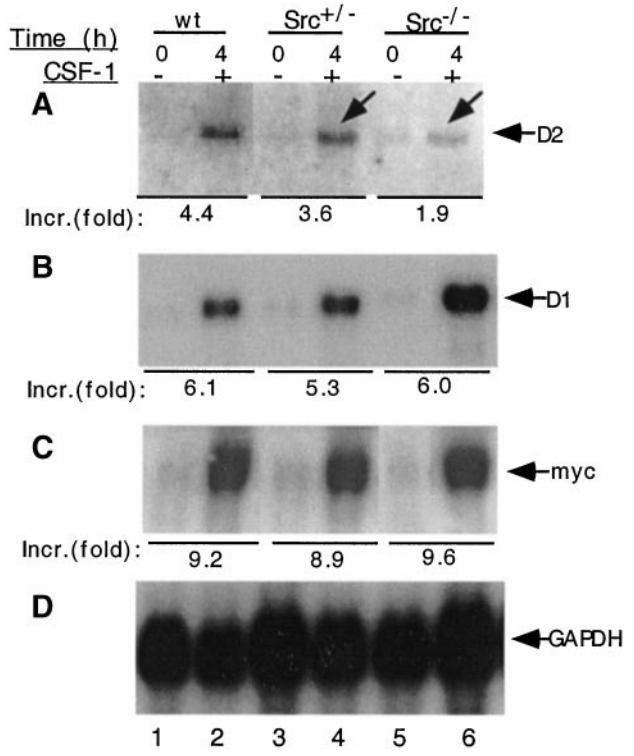

Figure 3. Src mediates CSF-1–induced cyclin D2, but not D1 or c-myc expression. BMMs were isolated from the wild-type, $Src^{+/-}$, and $Src^{-/-}$ mice. The 11th-d cultures were G1 arrested in CSF-1–free media and unstimulated or stimulated with CSF-1 (4.4 nM) for 4 h. Total RNA was isolated and CSF-1–stimulated D2 (A), D1 (B), c-myc (C), and GAPDH (D) gene expression was analyzed by Northern blot analysis (15 μg/lane). The relative fold increases (measured by scanning densitometry) were based on calibration with the corresponding GAPDH levels: $band^{+}/band^{-}$ X $GAPDH^{-}/GAPDH^{+}$ = Fold.

(clone 4) with parental BAC1.2F5 cells showed a similar growth rate in response to CSF-1 (Figure 4, C vs. A) and activation of the myc-ER by 4-HT or treatment of the cells with 4-HT together with CSF-1 neither enhanced nor decreased the rate of proliferation of 2F5-myc-ER cells (Figure 4C). These results are in disagreement with the previous report that overexpression of myc confers upon BAC1.2F5 cells with CSF-1–independent proliferation (Vairo *et al.*, 1995).

Clone 4 of 2F5-myc-ER cells lines was subjected to further studies. First, to confirm the myc-transactivating activity of myc-ER in these cells in response to 4-HT, induction of the well-known target gene for myc, ODC, was chosen as the positive control. It is shown in Figure 6C that expression of ODC was undetectable in parental BAC1.2F5 cells in the absence of CSF-1 (lane 4). CSF-1 stimulation caused a dramatic increase in ODC expression (lane 5). An increased basal level of ODC could already be detected in Bac1.2F5 cells even in the absence of CSF-1 or 4-HT (lane 1 vs. 4). This increased basal level was likely due to some constitutive activity ("leakage") of the overexpressed myc-ER. Nonetheless, as expected, both CSF-1 and 4-HT further enhanced ODC expression in these cells (lanes 2 and 3 vs. 1). These results establish that the ectopically expressed myc-ER could be transcriptionally activated by 4-HT. When the ODC probes on the same RNA blot were stripped off and reblotted sequentially with radioactively labeled D2 and D1 cDNA probes, the basal level of D2 mRNA was already elevated in the 2F5-myc-ER cells in the absence of 4-HT (Figure 6D, lane 1) in comparison to that of the parental BAC1.2F5 cells (lane 4). 4-HT treatment significantly increased D2 expression (lane 3). CSF-1 stimulation increased D2 gene expression in both 2F5-myc-ER cells (lane 2) and in the parental BAC1.2F5 (lane 5) cells. These data strongly suggest that myc acts upstream of D2, and this finding is consistent with two recent reports (Bouchard *et al.*, 1999; Perez-Roger *et al.*, 1999). In contrast, as shown in Figure 6E, no detectable increase of D1 expression was observed in either absence (lane 1) or presence (lane 3) of 4-HT. As expected, increased D1 expres-

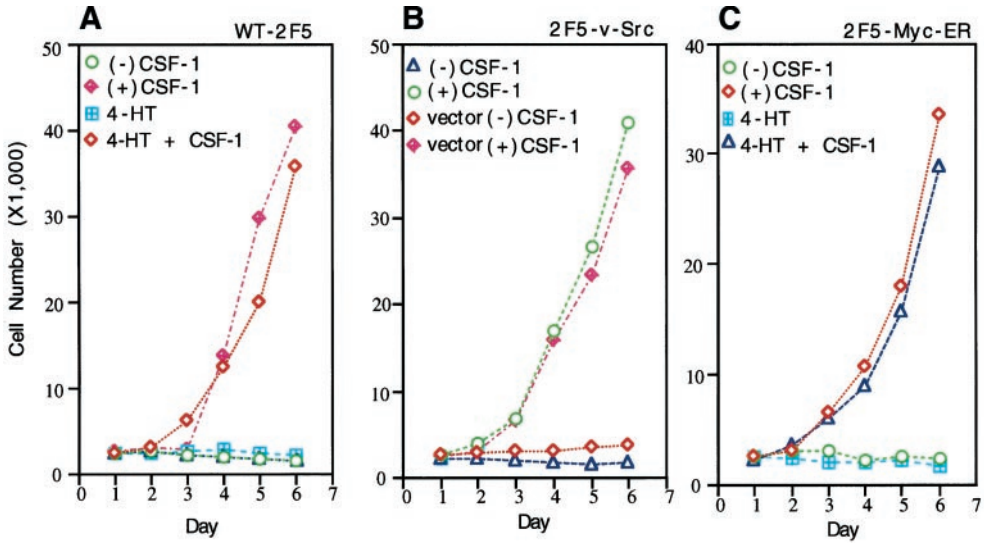

Figure 4. Establishment and growth curves of v-Src and myc-ER stable cell lines. BAC1.2F5 cells stably expressing v-Src or myc-ER were established by retrovival infection as described in MATERIALS AND METHODS. Equal numbers of the parental BAC1.2F5, 2F5-pMV7 (vector), 2F5-v-Src, and 2F5-myc-ER (clone 4) cells were seeded in duplicates, and proliferation under various conditions was compared for 6 d by counting the cells each day in triplicates and plotting the means (the SDs ranged from 4 to 8%). Results of one representative experiment are shown.

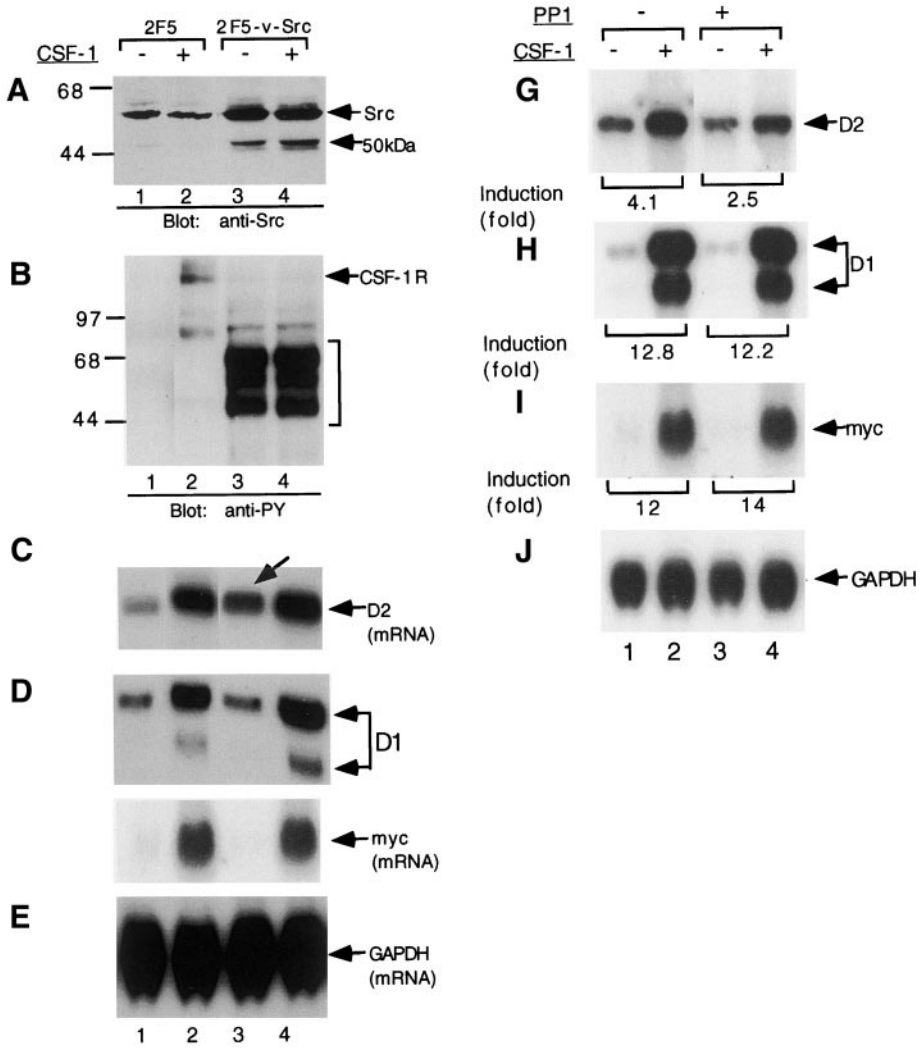

Figure 5. v-Src enhances D2 expression and PPI inhibits D2 expression. (A) Total lysates of 2F5-v-Src cells treated with or without CSF-1 (13.2 nM, 2 min for A and B) were analyzed for v-Src protein expression by anti-Src immunoblot. (B) The same lysates were tested for enhanced protein tyrosine phosphorylation by anti-PY immunoblot. (C–F) Total RNA was isolated from cells treated with or without CSF-1 (4.2 nM, 5 h) and analyzed for increased D2 (C), D1 (D), c-myc (E), and GAPDH (F) expression. (G–J) Cells were pretreated with PP1 (1 μM) for 4 h before addition of CSF-1 (4.4 nM, 5 h), and the total RNA was analyzed for D2 (G), D1 (H), c-myc (I), and GAPDH (J) expression. The relative fold increases (measured by scanning densitometry) were based on calibration with the corresponding GAPDH levels: band$^+$/band$^-$ $\times$ GAPDH$^-$/GAPDH$^+$ = Fold.

sion was detected in CSF-1–stimulated both the 2F5-myc-ER (lane 2 vs. 1) and the parental BAC1.2F5 (lane 5 vs. 4) cells. RNA sample loading was monitored by GAPDH expression (Figure 6F). It should be pointed out that because myc-ER is overexpressed in these cells, and it could cause significant increase in the amount of the myc-max complex leading to a higher transcription rate of target genes, the results from 2F5-myc-ER cells could not be used to quantitatively assess how much the myc pathway contributes to CSF-1–stimulated full induction of D2. Therefore, the observation that a similar or even higher induction of D2 expression by 4-HT (Figure 6D, lane 3) than that by CSF-1 (Figure 6D, lane 5) should not be interpreted as that myc alone can mediate the full induction of D2.

To further assess the role of c-myc pathway in D2 expression, we took advantage of our recent finding that blockade of PKCδ activation almost completely inhibits CSF-1–stimulated c-myc expression in BAC1.2F5 cells (Figure 6G, lane 3 vs. 2). Under these conditions, expression of D2 (Figure 6K), but not c-fos (Figure 6H) and D1 (Figure 6J), was partially but significantly blocked (lane 3 vs. 2). These results support the previous finding that c-myc acts upstream of D2 gene. Furthermore, the c-myc pathway is apparently independent of the Src pathway because induction of c-myc by CSF-1 was perfectly normal in Src$^{-/-}$ BMM cells in comparison with wild-type BMM cells (Figure 3) and inhibition of Src by PP1 had no effect on c-myc expression (Figure 5H), although we could

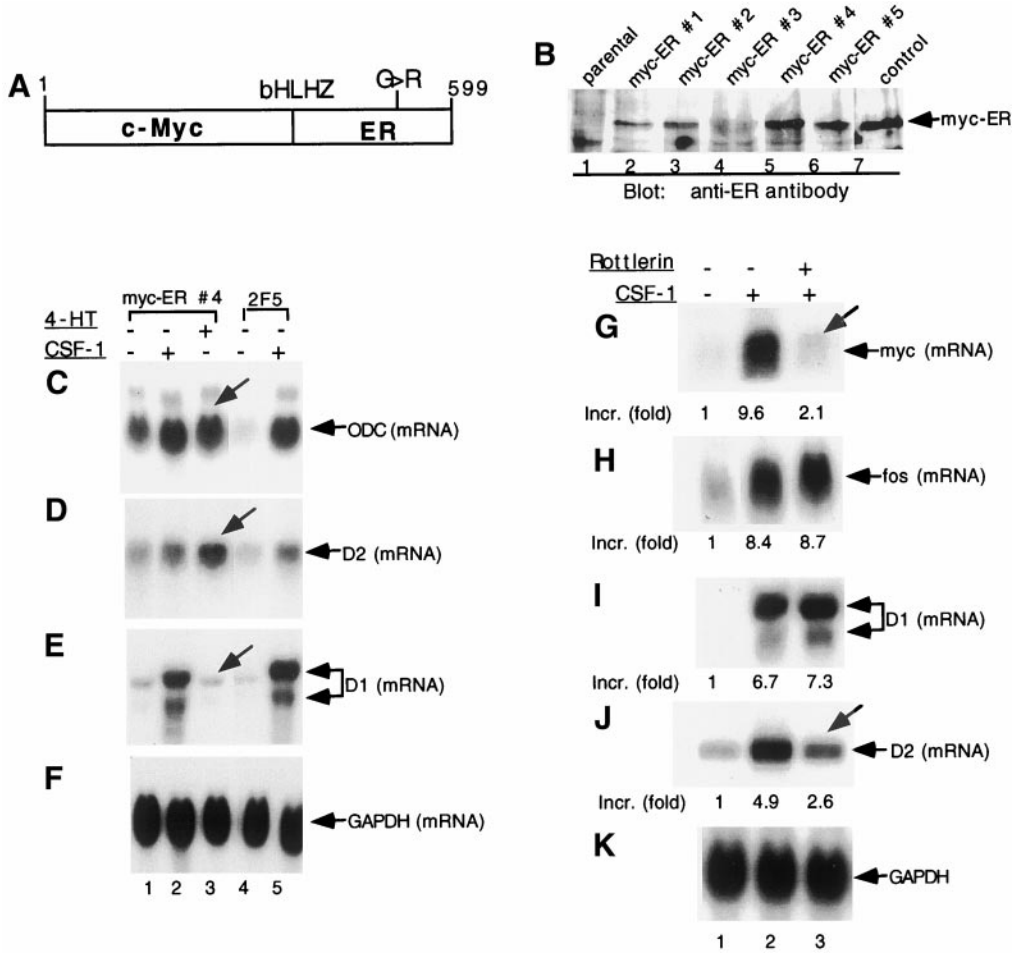

Figure 6. Activation of myc's transactivating activity induces ODC and cyclin D2, but not D1, and inhibition of c-myc expression correlates with inhibition of D2. BAC1.2F5 cell lines stably expressing myc-ER fusion proteins (as schematically shown in A) were established (see MATERIALS AND METHODS) and proliferation (clone 4) was studied (see Figure 4). Individual clones were isolated and tested for the myc-ER expression by anti-ER antibody immunoblot (B). Clone 4 was selected and either untreated or treated with CSF-1 (4.4 nM) or 4-HT (250 nM). Total RNA was isolated and subjected to Northern blot analyses (30 μg/lane). (C) ODC gene expression; (D) D2 gene expression; (E) D1 gene expression; and (F) GAPDH control. This is a representative experiment of three independent experiments by using the clone 4, 2, and 5. (G–K) BAC1.2F5 cells were treated with or without rottlerin (1 μM) in the presence or absence of CSF-1 (4.4 nM, 15 min for fos; 45 min for myc; and 5 h for D1 and D2), and total RNA was isolated and analyzed for expression of myc (G), D1 (H), fos (I), D2 (J), and GAPDH (K, GAPDH for D1 and D2). Similar levels of GAPDH control were obtained for fos and myc. The fold increases for c-fos and c-myc were calculated based on their own GAPDH levels.

not exclude the possibility that Src might regulate c-myc function by posttranscriptional mechanism.

Involvement of the MEK/ERK Pathway in Induction of D2 and D1 but Not c-myc

The reduced induction of D2 by the CSF-1R-Y697F mutant suggests that the Ras/Raf-1/MEK1/ERK pathway also contributes to the full induction of D2 in response to CSF-1. Because attempts to establish stable BAC1.2F5 cell lines expressing dominant-negative forms of Ras (N17-ras) or MEK1 (MEK1-2A) failed (due to the fact that products of these genes are essential for cell growth, and, furthermore, efficiency of transient transfection in BAC1.2F5 cells by all possible means so far available was <5% of the total cell population, we turned into a pharmacological approach). PD98059 is a kinase inhibitor that has been shown to have a specificity for MEK, the upstream activator of ERKs, but not for 18 other ser/thr protein kinases, PI3-K, and receptor tyrosine kinases (Alessi *et al.*, 1995; Dudley *et al.*, 1995), although no one can be absolutely sure it does not hit other untested cellular targets. We first examined what concentration of PD98059 completely blocks CSF-1–stimulated activation of ERK21 and ERK2 in BAC1.2F5 macrophages. As presented in Figure 7. CSF-1-stimulated ERK1 and ERK2 activation could be completely inhibited by 10 to 30 μM of PD98059 in intact cells (Figure 7A) (The in vitro IC50 is 2–7 μM; Alessi *et al.*, 1995). Under these conditions, 30–50% of

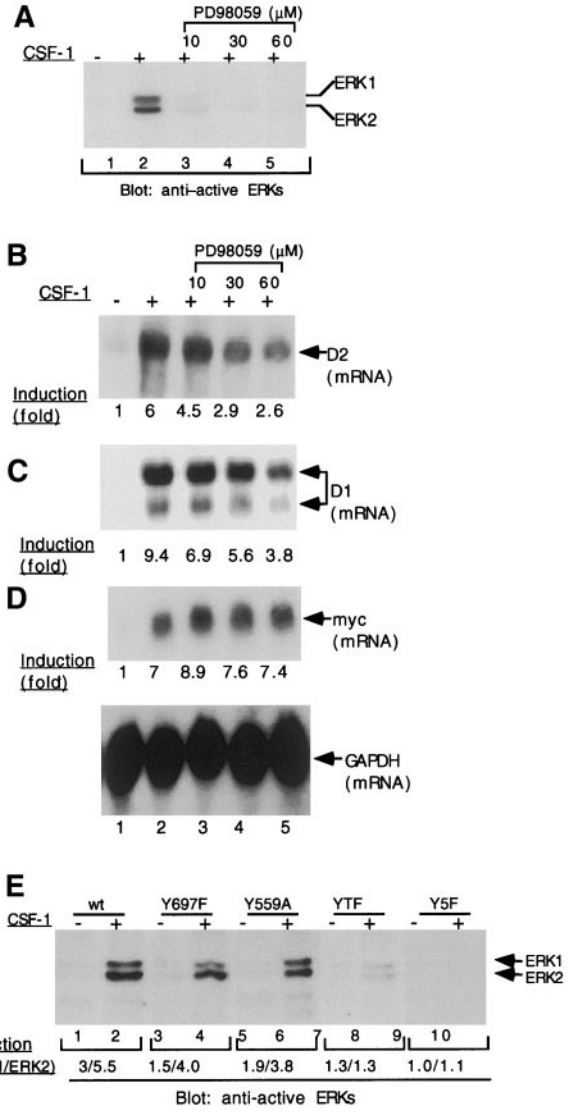

Figure 7. Roles for the MEK/ERK pathway in CSF-1–induced cyclin D2 gene expression. BAC1.2F5 cells were pretreated with or without indicated concentrations of MEK1 inhibitor PD98059 (15 min) before CSF-1 stimulation (13.2 nM, 5 min for A and F, and 5 h for B to E), and were subjected to either Western blot with antiactive ERK antibodies (A and F) or Northern blot analysis (B–E). (A) Effect of PD98059 on CSF-1–stimulated ERK1 and ERK2 activation. (B) Effect of PD98059 on D2 expression. (C) Effect of PD98059 on D1 expression. (D) Effect of PD98059 on c-myc expression. (E) GAPDH control. (F) CSF-1–stimulated activation ERK1 and ERK2 in cells expressing the four D2 expression-affected CSF-1R mutants. Induction was measured by scanning densitometry and was given in fold increase after calibration with corresponding GAPDH levels of each cell line: band$^+$/band$^-$ × GAPDH$^-$/GAPDH$^+$ = Fold. "+" and "−" represent "with" and "without" CSF-1 stimulation.

CSF-1–induced D2 (Figure 7B, lanes 3 and 4) and D1 (Figure 7C, lanes 3 and 4) expression was inhibited, whereas the induction of myc was unaffected, even at 60 μM (Figure 7D, lanes 3 to 5). The latter observation also suggests that the MEK/ERK pathway and the myc pathway are independent.

We then analyzed whether any of the four CSF-1R mutants, Y559A, Y697F, YTF, and Y5F, which showed either reduced or abolished induction of D2, affects CSF-1–stimulated activation of ERK1 and ERK2. It is shown in Figure 7F that CSF-1–stimulated activation of ERKs, especially the ERK1, was decreased by Y697F mutation, and activation of both ERK1 and ERK2 were dramatically blocked by the YTF triple mutation and completely abolished by the Y5F mutation.

Simultaneous Inhibition of Src, MEK/ERK, and c-myc Pathways Additively Inhibits CSF-1–stimulated D2 Expression

The above-mentioned studies indicate that CSF-1R uses at least three distinct pathways to induce D2. An important question then is, Do the three pathways additively or collaboratively contribute to induction of D2? We therefore tested whether simultaneous inhibition of the three pathways would have the strongest inhibition of CSF-1–stimulated D2 expression. We used two approaches to seek the answer of this question. First, cells were treated either individually or simultaneously with PP1, PD98059, and rottlerin. Total RNA was isolated and analyzed for CSF-1–induced expression of D2. It is shown in Figure 8A that inhibition of any one of the three individual pathways only partially blocked the CSF-1–stimulated D2 expression (lanes 4, 6, and 8 vs. 2). However, simultaneous inhibition of the three pathways inhibited >90% of CSF-1–induced D2 expression (lane 10 vs. 2). To ensure that it was due to direct effects of these inhibitors, we examined cell toxicity under these conditions (100 nM of PP1, 1 μM of rottlerin, and 20 μM of PD98059) for the entire period of the experiment (6 h). Both trypan blue staining and continued cell proliferation (after drug withdraw) assays indicated that only <8% of the triple drug-treated cells detached, and the remaining attached cells were able to continue proliferation in response to CSF-1 (our unpublished results). Second, we wanted to confirm the above-mentioned results by transfecting cells with the dominant-negative forms of Src (SrcK$^-$), PKCδ (PKCδ-K376R), and MEK (MEK-2A). As previously mentioned that due to the fact no one has been able to achieve >5% transfection efficiency in BAC1.2F5 cells, we compromised by using the NIH3T3-c-fms cells that stably express the human CSF-1R (Roussel *et al.*, 1990), in which we constantly achieved a transfection efficiency of 35–45% of the total cell population. Results of four independent transfection experiments were averaged as percentages of the D2 expression in vector alone transfected cells in response to CSF-1. It is shown in Figure 8B that expression of MEK-2A, PKCδ-K376R, and SrcK$^-$ alone (total DNA, 27 μg/100-mm dish) inhibited CSF-1–induced D2 by 18–28%, respectively. However, triple transfection of all the three constructs (total DNA, 27 μg/100-mm dish) inhibited >50% of the CSF-induced D2 expression. The relatively lower inhibitory effects by transfection than those by those chemical inhibitors may reflect the fact that less than a half of the cells were transfected.

DISCUSSION

It is becoming an emerging theme that cell surface receptors use multiple pathways to induce gene expression (Fam-

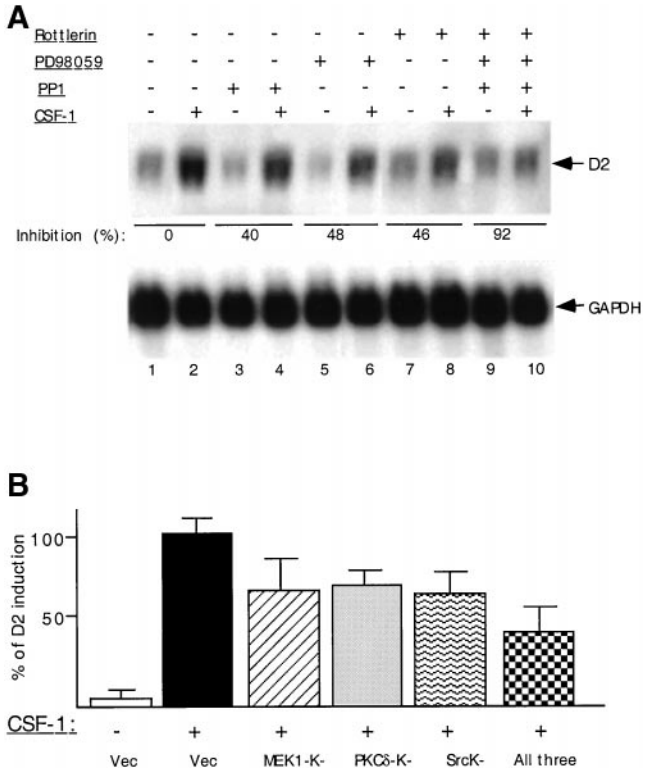

Figure 8. Simultaneous inhibition of Src, MEK1, and myc pathways additively blocks CSF-1–stimulated induction of D2. (A) Total RNA, isolated from BAC1.2F5 cells treated without or with 10 μM of PP1 (inhibitor of Src kinases, 2-h pretreatment) (lanes 3 and 4), 15 μM of PD98059 (inhibitor of MEK, 20 min pretreatment) (lanes 5 and 6), or 1 μM of rottlerin (inhibitors of c-myc expression, 30 min pretreatments) (lanes 7 and 8), or all three inhibitors (lanes 9 and 10) before CSF-1 stimulation (4.4 nM, 5 h), was subjected to Northern blot analysis for induction of D2. Percentages of inhibition by each of the single or combined inhibitors (measured by scanning densitometry of the bands) were calculated based on calibration with corresponding GAPDH levels of each of treatments: fold$^+$/fold$^-$ $\times$ 100% = %, in which "+" refers to with inhibitor(s) and "−" refers to without inhibitor(s). Approximately 8% of the cells died, as assayed by trypan blue staining, after the combined triple drug treatment. (B) NIH3T3-c-fms cells were transfected with either vector alone or the dominant-negative forms of MEK1 (MEK1K$^-$, 9 μg plus 18 μg of vector), PKCδ (PKCδK$^-$, 9 μg plus 18 μg of vector), or Src (SrcK$^-$, 9 μg plus 18 μg of vector), or all three genes (9 μg each, total 27 μg) by SuperFect. Cells were starved in 0.2% serum for 18 h and treated with or without CSF-1 for 6 h. Total RNA was isolated and subjected to Northern blot analysis. The percentages of the D2 induction were calculated against the vector alone (100%), and data shown are mean ± SE of the scanning densitometry data of three independent experiments.

brough *et al.*, 1999; Iyer *et al.*, 1999). Fambrough *et al.* (1999) showed that none of the phosphotyrosines alone in the platelet-derived growth factor (PDGF) receptor is responsible for full expression of any genes in response to PDGF, and even five combined mutations (Y5F) only quantitatively but not qualitatively affect the PDGF-induced gene expression profile (Fambrough *et al.*, 1999). However, it has not been demonstrated whether multiple pathways are indeed involved in full expression of a single gene. In this study, we have analyzed the signaling pathways that mediate CSF-1–stimulated induction of D2 gene. From analyses of the mouse CSF-1R autophosphotyrosine mutants, we found that only combined multiple mutations cause significant blockade of D2 induction, suggesting that CSF-1R uses multiple parallel signaling pathways to achieve the full induction of D2 (Figure 2). By screening inhibitors of various signaling pathways, three apparently independent pathways, the Src, the MEK1, and the myc pathways, were implicated. To further confirm these findings, we show that 50% of CSF-1–stimulated D2 gene induction was blocked in BMM cells isolated from Src-knockout mice (Src$^{-/-}$), whereas Src played no role in induction of D1 or myc. This finding is in conflict with previous reports that Src acts upstream of c-myc by either overexpression or antibody microinjection approaches in fibroblasts (Barone and Courtneidge, 1995; Roche *et al.*, 1995). The partial effect of Src is apparently not due to a compensating effect of other Src family kinases because treatment of the cells with PP1 at concentrations that would inhibit most other Src family kinases as well also partially (~ 40%) blocks CSF-1–stimulated induction of D2. Consistently, expression of v-Src caused increased D2 level even in the absence of CSF-1. To confirm that myc acts as an upstream of D2, we stably introduced myc-ER into BAC1.2F5 macrophages. We found that activation of the transactivating activity of myc-ER leads to an increased expression of ODC and D2 in the absence of CSF-1, whereas under the same conditions expression of D1 is not induced. This finding is consistent with recent reports from two independent groups that myc acts upstream of D2 gene (Bouchard *et al.*, 1999; Perez-Roger *et al.*, 1999). These investigators showed that the myc-induced D2 form complex with Cdk4, which in turn mediates myc-induced proliferation. The third pathway, MEK1/ERK pathway, also partially contributes to CSF-1–stimulated induction of D2. Inhibition of MEK1/ERK partially blocks induction of both D1 and D2, but not c-myc. Simultaneous inhibition of all the three pathways blocks 90% of D2 induction in response to CSF-1. Finally, coexpression of three dominant-negative forms of Src, PKCδ, and MEK additively inhibit CSF-1–induced D2 expression. A schematic representation of the signaling networks by which CSF-1 induces the full expression of D2 is shown in Figure 9. It should be pointed out that additional signaling pathway(s) may also be involved in induction of D2. Elucidation of exact percentage of contribution from each of the three pathways to induction of D2 and its biological significance will require detailed studies of the D2 gene promoter in the future.

As mentioned previously, results of our experiments have also established that Src, MEK1/ERK, and myc represent three nonlinear and possibly independent signaling pathways. Inhibition of MEK1 has no effect on CSF-1–stimulated c-myc induction, indicating that the MEK1/ERK pathway and the pathway leading to myc induction act in parallel. We found that Src does not mediate CSF-1–stimulated myc and c-fos (Ras-dependent) induction in macrophage, indicating that Src is nonlinear to Ras or myc. Inhibitor-screening study suggests that protein kinase C-δ plays an important role in CSF-1–stimulated induction of c-myc. Overexpresion of dominant-negative PKCδ inhibits CSF-1–induced myc expression (our unpublished results; She, Dey,

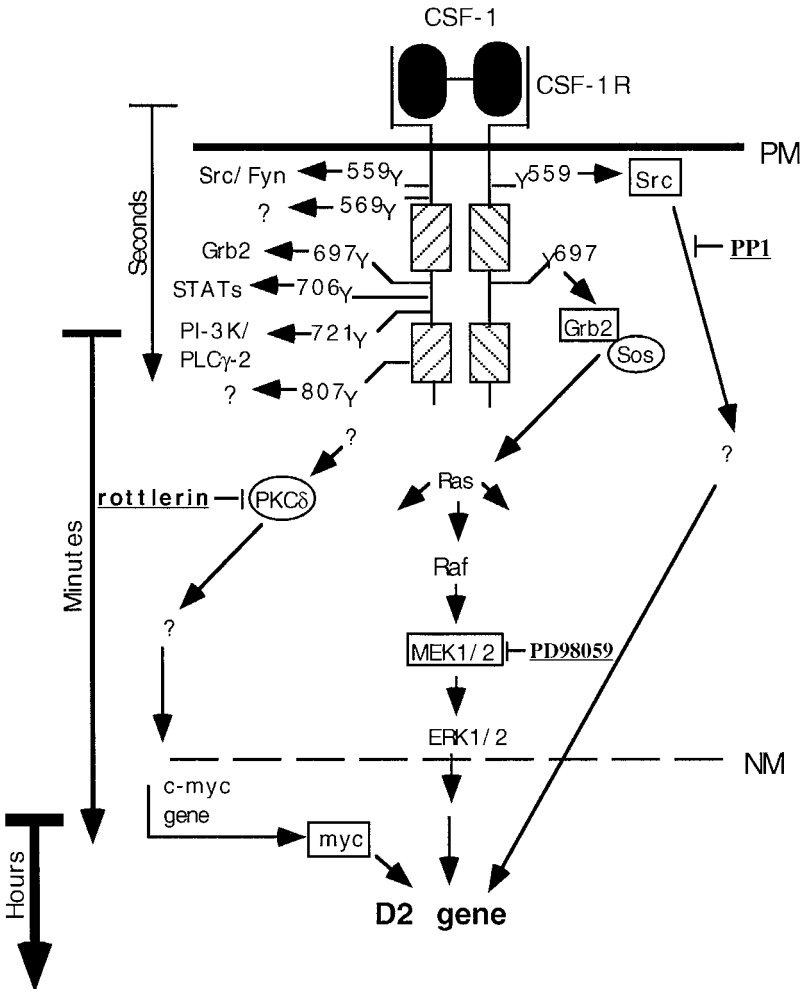

Figure 9. A schematic representation of CSF-1R signaling to cyclin D2 gene, in which at least three major pathways are involved. CSF-1 binding activates CSF-1R (shown as a dimer) and causes receptor autophosphorylation on tyrosines. The phosphotyrosines recruit cytoplasmic signaling molecules. Among them, CSF-1R engages three parallel pathways, Src (via Y559), Grb2/Sos/Ras/MEK/ERKs (via Y697), and c-myc (via an unknown site[s]), for full induction of D2. The mechanism by which CSF-1R activates PKCδ and the downstream effector for Src and PKCδ remain unknown. The approximate time zones show one-way travel once from the cell surface to the nucleus, and do not reflect any feedback signaling events. PM, plasma membrane; NM, nuclear membrane.

Lin, and Li, unpublished data). Finally, in support of the above-mentioned notion, simultaneous inhibition of all the three pathways shows an additive inhibitory effect on CSF-1–stimulated D2 induction.

It would be of great interest to compare the signaling mechanisms that mediate CSF-1–stimulated full induction of D2 with those for full induction of D1. Although inhibition of Ras could partially inhibit induction of D1, we found that neither Src nor myc plays any significant role in mediating CSF-1–induced D1 expression. Moreover, inhibition of the MEK1/ERK cascade has a moderate inhibitory effect on CSF-1–stimulated D1 induction. Treatment of the cells with wortmannin, a potent inhibitor for PI3-K, shows little inhibitory effect on either D1 or D2 induction (our unpublished results). These results suggest that the downstream signaling networks of PI3-K, such as AKT, Rho GTPases, and p38MAPK, are not required. Because Ras/Raf1/MEK/ERK pathway only plays a partial role in mediating CSF-1–stimulated induction of D1, other Ras-independent parallel pathway(s) must be involved. Our results so far suggest that CSF-1R uses both redundant and distinct signaling pathways to induce the two highly related cyclin genes. Because CSF-1 stimulates induction of D2 and D1 with different kinetics in cells, distinct signaling pathways would naturally be required to differentiate the induction of the two genes.

The above-mentioned finding also implicates that loss of any single one of the multiple signaling pathways for induction of an important growth control gene, such as D2, in cells may not face grave consequence. This may be the reason why the BMM cells isolated from $Src^{-/-}$ knockout mice, as well as fetal liver macrophages isolated from $Src^{-/-}$ and $Fyn^{-/-}$ double knockout mice, are still able to proliferate as the wild-type BMM cells in response to CSF-1 (our unpublished results). These observations indicate that Src is dispensable for CSF-1–stimulated macrophage proliferation. Hence, when it comes to such an important decision as cell proliferation, similar to having functionally redundant D1 and D2 gene, a cell acquires during evolution multiple parallel signaling pathways to secure induction of any one of these genes, even if engagement of some of the signaling pathways may fail.

This is the first study that has directly shown that multiple cytoplasmic signaling pathways are used by a cell surface RTK, CSF-1R, to achieve a full induction of a nuclear gene, D2. It provides an explanation for previous and repeated observations that none of the phosphotyrosine sites in RTKs

is essential for growth control and combined multiple mutations gradually diminish the function of the RTK. Recognition of this novel concept may help us to find better cure for human diseases and perhaps even cancer.

ACKNOWLEDGMENTS

We thank Dr. Larry Rohrschneider for FDC-P1 cell lines, Dr. Martine Roussel for cyclin D1 and D2 cDNAs, and Dr. E. Richard Stanley for BAC1.2F5 cells, mouse CSF-1, and helpful discussions. We thank Dr. Nissm Hay for c-myc and GAPDH cDNA. This study was supported in part by RO1 CA65567-05 (to W.L.).

REFERENCES

Alessi, D.R., Cuenda, A., Cohen, P., Dudley, D.T., and Saltiel, A.R. (1995). PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. J. Biol. Chem. *270*, 27489–27494.

Baldin, V., Lukas, J., Marcote, M.J., Pagano, M., and Draetta, G. (1993). Cyclin D1 is a nuclear protein required for cell cycle progression in G1. Genes Dev. *7*, 812–821.

Barone, M.V., and Courtneidge, S.A. (1995). Myc but not Fos rescue of PDGF signaling block caused by kinase-inactive Src. Nature *378*, 509–512.

Bouchard, C.K., Thieke, A., Maier, R., Saffrich, J., Hanley-Hyde, W., Ansorge, S., Reed, S., Sicinski, P., Bartek, J., and Eilers, M. (1999). Direct induction of cyclin D2 by Myc contributes to cell cycle progression and sequestration of p27. EMBO J. *18*, 5321–5333.

Bourette, R.P., Myles, G.M., Carlberg, K., Chen, A.R., and Rohrschneider, L.R. (1995). Uncoupling of the proliferation and differentiation signals mediated by the murine macrophage colony-stimulating factor receptor expressed in myeloid FDC-P1 cells. Cell. Growth Differ. *6*, 631–645.

Bourette, R.P., Myles, G.M., Choi, J.-L., and Rohrschneider, L.R. (1997). Sequential activation of phosphatidylinositol 3-kinase and phospholipase C-γ2 by the M-CSF receptor is necessary for differentiation signaling. EMBO J. *16*, 5880–5893.

Bourette, R.P., and Rohrschneider, L.R. (2000). Early events in M-CSF receptor signaling. Growth Factors *17*, 155–166.

Courtneidge, S.A., Dhand, R., Pilat, D., Twamley, G.M., Waterfield, M.D., and Roussel, M.F. (1993). Activation of Src family kinases by colny stimulating factor-1, and their association with its receptor. EMBO J. *12*, 943–950.

Dudley, D.T., Pang, L., Decker, S.J., Bridges, A.J., and Saltiel, A.R. (1995). A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc. Natl. Acad. Sci. *92*, 7686–7689.

Fambrough, D., McClure, K., Kazlauskas, A., and Lander, E.S. (1999). Diverse signaling pathways activated by growth factor receptors induce broadly overlapping, rather than independent, sets of genes. Cell *97*, 727–741.

Fantl, V., Stamp, G., Andrews, A., Rosewell, I., and Dickson, C. (1995). Mice lacking cyclin D1 are small and show defects in eye and mammary gland development. Genes Dev. *9*, 2364–2372.

Iyer, V.R., Eisen, M.B., Ross, D.T., Schuler, G., Moore, T., Lee, J.C.F., Trent, J.M., Staudt, L.M., Hudson, J.J., Boguski, M.S., Lashkari, D., Shalon, D., Botstein, D., and Brown, P.O. (1999). The transcriptional program in the response of human fibroblasts to serum. Science *283*, 83–87.

Kato, J.A., and Sherr, C.J. (1993). Inhibition of granulocyte differentiation by G_1 cyclins D2 and D3 but not D1. Proc. Natl. Acad. Sci. USA *90*, 11513–11517.

Lerner, E.C., Qian, Y., Blaskovich, M.A., Fossum, R.D., Vogt, A., Sun, J., Cox, A.D., Der, C.J., Hamilton, A.D., and Sebti, S.M. (1995). Ras CAAX peptidomimetic FTI-277 selectively blocks oncogenic Ras signaling by inducing cytoplasmic accumulation of inactive Ras-Raf complexes. J. Biol. Chem. *270*, 26802–26806.

Lerner, E.C., Zhang, T-T, Knowles, D.B., Qian, Y., Hamilton, A.D., and Sebti, S.M. (1997). Inhibition of the prenylation of K-Ras, but not H- or N-Ras, is highly resistant to CAAX peptidomimetics and requires both a farnesyltransferase and a geranylgeranyltransferase I inhibitor in human tumor cell lines. Oncogene *15*, 1283–1288.

Li, W., Yeung, Y.G., and Stanley, E.R. (1991). Tyrosine phosphorylation of a common 57-kDa protein in growth factor-stimulated and transformed cells. J. Biol. Chem. *26*, 6806–6814.

Lioubin, M.N., Algate, P.A., Schickwann, T., Carlberg, K., Aebersold, R., and Rohrschneider, L.R. (1996). $p150^{ship}$, a signal transduction molecule with inositol polyphosphate-5-phosphatase activity. Gene Dev *10*, 1084–1095.

Lioubin, M.N., Myles, G.M., Carlberg, K., Bowtell, D., and Rohrschneider, L.R. (1994). SHC, GRB2, SOS1 and a 150-kilodalton tyrosine-phosphorylated protein form complexes with Fms in hematopoietic cells. Mol. Cell. Biol. *14*, 5682–5691.

Littlewood, T.D., Hancock, D.C., Danielian, P.S., Parker, M.G., and Evan, G.I. (1995). A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. Nucleic Acids Res. *25*, 1686–1690.

Matsushime, H., Roussel, M.F., Ashmun, R.A., and Sherr, C.J. (1991). Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. Cell *65*, 701–713.

Morgan, C., Pollard, J.W., and Stanley, E.R. (1987). Isolation and characterization of a cloned growth factor dependent macrophage cell line, BAC1.2F5. J. Cell Physiol. *130*, 420–427.

Myles, G.M., Brandt, C.S., Carlberg, K., and Rohrschneider, L.R. (1994). Tyrosine 569 in the c-Fms juxtamembrane domain is essential for kinase activity and macrophage colony-stimulating factor-dependent internalization. Mol. Cell. Biol. *14*, 4843–4854.

Novak, U., Nice, E., Hamilton, J.A., and Paradiso, L. (1996). Requirement for Y706 of the murine (or Y708 of the human) CSF-1 receptor for STAT1 activation in response to CSF-1. Oncogene *13*, 2607–2613.

Orlofsky, A., and Stanley, E.R. (1987). CSF-1-induced gene expression in macrophages: dissociation from the mitogenic response. EMBO J. *6*, 2947–2952.

Pawson, T. (1995). Protein modules and signaling networks. Nature *373*, 573–579.

Pawson, T., and Schlessinger, J. (1993). SH2 and SH3 domains: protein modules that determine specificity of protein interaction in signal transduction. Curr. Biol. 3, 434–442.

Perez-Roger, I., Kim, S.H., Griffiths, B., Sewing, A., and Land, H. (1999). Cyclins D1 and D2 mediate myc-induced proliferation via sequestration of p27(Kip1) and p21(Cip1). EMBO J. *18*, 5310–5320.

Pines, J. (1995). Cyclins and cyclin-dependent kinases: a biochemical view. Biochem. J. *308*, 697–711.

Quelle, D.E., Ashmun, R.A., Shurtleff, S.A., Kato, J., Bar-Sagi, D., Roussel, M.F., and Sherr, C.J. (1993). Overexpression of mouse D-type cyclins accelerates G_1 phase in rodent fibroblasts. Genes Dev. *7*, 1559–15 71.

Reedijk, M., Liu, X-Q., and Pawson, T. (1990). Interaction of phosphatidylinositol kinase, GTPase-activating protein (GAP), and GAP-associated proteins with the colony-stimulating factor 1 receptor. Mol. Cell. Biol. *10*, 5601–5608.

Reedijk, M., Liu, X., van der Geer, P., Letwin, K., Waterfield, M.D., Hunter, T., and Pawson, T. (1992). Tyr721 regulates specific binding of the CSF-1 receptor kinase insert to P1 3′-kinase SH2 domains: a

model for SH2-mediated receptor-target interactions. EMBO J. *11*, 1365–1272.

Roche, S., Koegl, M., Barone, M.V., Roussel, M.F., and Courtneidge, S.A. (1995). DNA synthesis induced by some by not all growth factors requires Src family protein tyrosine kinases. Mol. Biol. Cell 15, 1102–1109.

Rohrschneider, L.R., Bourette, R.P., Lioubin, M.N., Algate, P.A., Myles, G.M., and Carlberg, K. (1997). Growth and differentiation signals regulated by the M-CSF receptor. Mol. Reprod. Dev. *46*, 96–103.

Rohrschneider, L.R., and Metcalf, D. (1989). Induction of macrophage colony-stimulating factor-dependent growth and differentiation after introduction of the murine c-fms gene into FDC-P1 cells. Mol. Cell. Biol. *9*, 5081–5092.

Roussel, M.F. (1997). Regulation of cell cycle entry and G_1 progression by CSF-1. Mol. Reprod. Dev. *46*, 11–18.

Roussel, M., Shurtleff, S.A., Dowing, J., and Sherr, C.J. (1990). A point mutation at tyrosine-809 in the human colony-stimulating factor 1 receptor impairs mitogenesis without abrogating tyrosine kinase activity, association with phosphatidylinositol 3-kinase, or induction of c-fos and junB genes. Proc. Natl. Acad. Sci. USA *87*, 6738–6742.

Roussel, M.F., Theodoras, A.M., Pagano, M., and Sherr, C.J. (1995). S rescue of defective mitogenic signaling by D-type cyclins. Proc. Natl. Acad. Sci. USA *9*, 6837–6841.

Sengupta, A., Liu, W.K., Yeung, Y.G., Yeung, D.C., Frackelton, A.R., Jr., and Stanley, E.R. (1988). Identification and subcellular localization of proteins that are rapidly phosphorylated in tyrosine in response to colony-stimulating factor-1. Proc. Natl. Acad. Sci. USA *85*, 8062–8066.

Sherr, C.J. (1993). Mammalian G1 cyclins. Cell *73*, 1059–1065.

Sherr, C.J. (2000). Cancer cell cycle revisited. Cancer Res. *60*, 3689–3695.

Sherr, C.J., and Stanley, E.R. (1990). Colony-stimulating factor 1 (macrophage colony-stimulating factor). In: Handbook of Experimental Pharmacology, Vol. 95/1, Peptide Growth Factors and Their Receptors I, Chpt. 15, ed. M.B. Sporn and A.B. Roberts, Heidelberg, Germany: Springer-Verlag, pp 667–698.

Shurtleff, S.A., Downing, J.R., Rock, C.O., Hawkins, S.A., Roussel, M.F., and Sherr, C.J. (1990). Structure features of the colony-stimulating factor 1 receptor that affect its association with phosphatidylinositol 3-kinase. EMBO J. *9*, 2415–2421.

Sicinski, P., Donaher, J.L., Gen, Y., Parker, S.B., Gardner, H., Park, M.Y., Robker, R.L., Richards, J.S., McGinnis, L.K., Biggers, J.D., Eppig, J.J., Bronson, R.T., Elledge, S.J., and Weinberg, R.A. (1996). Cyclin D2 is an FSH-responsive gene involved in gonadal cell proliferation and oncogenesis. Nature *384*, 470–474.

Sicinski, P., Donaher, J.L., Parker, S.B., Li, T., Fazeli, A., Gardner, H., Haslam, S.Z., Bronson, R.T., Elledge, S.J., and Weinberg, R.A. (1995). Cyclin D1 provides a link between development and oncogenesis in the retina and breast. Cell *8*, 621–630.

Soriano, P., Montgomery, C., Geske, R., and Bradley, A. (1991). Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. Cell *64*, 693–702.

Stanley, E.R. (1986). Action of the colony-stimulating factor-1, CSF-1. In: Biochemistry of Macrophages. Ciba Foundation Symposium 118, 29–41.

Tushinski, R.J., Oliver, I.T., Guilbert, L.J., Tynan, P.W., Warner, J.R., and Stanley, E.R. (1982). Survival of mononuclear phagocytes depends on a lineage-specific growth factor that the differentiated cells selectively destroy. Cell *28*, 71–81.

Vairo, G., Vadiveloo, P.K., Royston, A.K., Rockman, S.P., Rock, C.O., Jackowski, S., and Hamilton, J.A. (1995). Deregulated c-myc expression overrides IFN gamma-induced macrophage growth arrest. Oncogene *10*, 1969–1976.

van der Geer, P., and Hunter, T. (1991). Tyrosine 706 and 807 phosphorylation site mutants in the murine colony-stimulating factor-1 receptor are unaffected in their ability to bind or phosphorylate phosphatidylinositol-3 kinase but show differential defects in their ability to induce early response gene transcription. Mol. Cell. Biol. *11*, 4698–4709.

van der Geer, P., and Hunter, T. (1993). Mutation of Tyr697, a GRB2-binding site, and Tyr721, a PI 3′-kinase binding site, abrogates signal transduction by the murine CSF-1 receptor expressed in Rat-2 fibroblasts. EMBO J. *12*, 5161–5172.

Varticovski, L., Druker, B., Morrison, D., Cantley, L., and Rorberts, T. (1989). The colony-stimulating factor 1 receptor association with and activates phosphatidylinositol 3-kinase. Nature *342*, 699–702.

Wang, Y., Yeung, Y.G., Langdon, W.Y., and Stanley, E.R. (1996). c-Cbl is transiently tyrosine-phosphorylated, ubiquitinated, and membrane-targeted following CSF-1 stimulation of macrophages. J. Biol. Chem. *271*, 17–20.

Weinberg, R.A. (1995). The retinoblastoma protein and cell cycle control. Cell *81*, 323–330.