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CSF-1 activates MAPK-dependent and p53-independent pathways to induce growth arrest of hormone-dependent human breast cancer cells

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The CSF-1 receptor (CSF-1R) is expressed in >50% of human breast cancers. To investigate the consequence of CSF-1R expression, hormone-dependent human breast cancer cell lines, MCF-7 and T-47D, were transfected with CSF-1R. Unexpectedly, CSF-1 substantially inhibited estradiol (E_2) and insulin-dependent proliferation of MCF-7 transfectants (MCF-7*fms*) and prevented cyclin E/cdk2 and cyclin A/cdk2 activation, consistent with a G_1 arrest. In contrast, CSF-1 increased DNA synthesis in T-47D transfectants (T-47D*fms*) alone and with E_2 or insulin. In response to CSF-1, there was a marked and sustained upregulation of the cyclin-dependent kinase inhibitor, $p21^{Waf1/Cip1}$, in MCF-7*fms* but not T-47D*fms*. CSF-1 also markedly upregulated cyclin D1 in MCF-7*fms*. The coordinate increase in cyclin D1 and p21 had the effect of decreasing the specific but not absolute activity of cyclin D1/cdk4. p53 was not involved since CSF-1 induction of p21 was unaffected by dominant-negative p53 expression. ERK activation by CSF-1 was robust and sustained in MCF-7*fms* and to a much lesser extent in T-47D*fms*. Using pharmacological and transient transfection approaches, we showed that ERK activation was necessary and sufficient for p21 induction in MCF-7*fms*. Moreover, activated MEK inhibited E_2-stimulated cdk2 activity. Our findings indicate that the consequence of CSF-1R-mediated signals in human breast cancer cells is dependent on the genetic background of the particular tumor.

Keywords: colony-stimulating factor-1 receptor; p21Waf1/Cip1; cyclin D1; MAP kinase; breast cancer; estrogen

Introduction

Human breast cancer is characterized by genetic instabilities leading to loss of hormone dependence, activation of oncogenes and inactivation of tumor suppressor genes. These changes ultimately give rise to cellular lineages that are invasive, metastatic and lethal. Here we examine the consequences of activation of the colony stimulating factor-1 receptor (CSF-1R). This receptor tyrosine kinase (RTK) is not expressed in normal nonlactating mammary glands but recent reports by several laboratories indicate that 58–90% of invasive breast tumors may express CSF-1R (Kacinski *et al*., 1991; Tang *et al*., 1992; Scholl *et al*., 1994). An antibody that recognizes tyrosine phosphorylated CSF-1R stained 52% of the invasive tumors, indicating that CSF-1R is activated in breast cancer (Flick *et al*., 1997). Receptor activation is probably due to stimulation by its ligand, CSF-1, which is secreted by infiltrating monocytes at the tumor site (Scholl *et al*., 1994); in some tumors, CSF-1 is also co-expressed with its receptor (Tang *et al*., 1992). Given the ample data supporting a role for RTKs in cellular proliferation and transformation (Porter and Vaillancourt, 1998), it becomes important to establish the significance of CSF-1R expression in breast cancer and if it has prognostic value.

CSF-1R is the receptor for the macrophage growth factor, CSF-1 or M-CSF and the cellular homolog (c-*fms*) of the viral oncoprotein, v-*fms*, best characterized as a proliferative, survival and differentiation factor for cells of the monocyte/macrophage series (Roth and Stanley, 1992). In cells expressing CSF-1R, CSF-1 activates a multitude of signaling pathways, including those involving SHC/Ras/mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3-kinase), JAK/STAT and c-*cbl* (Lee, 1992; Roussel, 1994; Hamilton, 1997). In most instances, expression of the CSF-1R gene confers CSF-1-dependent mitogenesis (Rohrschneider and Metcalf, 1989; Kato *et al*., 1989) although there are exceptions (Ruden *et al*., 1991).

Most studies aimed at understanding the role of CSF-1R in breast cancer have utilized the estrogen receptor (ER)-negative human breast carcinoma cell line, BT20, which expresses low levels of endogenous CSF-1R that can be upregulated with glucocorticoid treatment. CSF-1 treatment was demonstrated to enhance cell invasiveness (Kacinski *et al*., 1991; Filderman *et al*., 1992; Sapi *et al*., 1998). Since breast cancers differ greatly in the genetic alterations they have sustained, we wished to investigate the consequence of CSF-1R expression in other breast cancer cells. In particular, those that are ER-positive are of interest since more than 60% of human breast cancers are ER-positive and about a third of metastatic cancers are hormonally-responsive (Dickson and Lippman, 1995). One study assessing CSF-1R expression in human breast cancers reported that 67% of the tumors they analysed were ER-positive while 58% were CSF-1R-positive (Scholl *et al*., 1994), implying that there are tumors positive for both ER and CSF-1R. The existing data suggest that cross-talk between growth factors and steroids can influence breast cell proliferation in both positive and negative ways. Progestin treatment of T-47D breast cancer cells upregulates various signal transduction pathways induced by the epidermal growth factor (EGF) and sensitizes cells to EGF's proliferative effects (Lange *et*

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al., 1998). On the other hand, fibroblast growth factor (FGF) inhibits the proliferation of E_2-dependent MCF-7 cells through a mechanism that involves induction of the cyclin kinase inhibitor, p21 (Wang *et al*., 1997; Johnson *et al*., 1998). However, the signaling pathways leading to growth inhibition are not well defined.

The present study was undertaken to investigate the effect of CSF-1R activation in ER-positive breast cancer cells, using as model systems the MCF-7 and T-47D cell lines into which the human CSF-1R gene (c-*fms*) was introduced. The data demonstrated that CSF-1 stimulation had opposing effects in the two cell lines: while CSF-1 was mitogenic for T-47D*fms*, it inhibited E_2 and insulin-stimulated growth of MCF-7*fms* cells, due to the sustained, MAPK-dependent and p53-independent induction of p21. These findings indicate that CSF-1R activation can have pleiotropic effects in human breast cancer cells.

Results

Expression of CSF-1R in MCF-7 human breast cancer cells inhibits estradiol and insulin-stimulated proliferation

We first examined the expression of CSF-1 and CSF-1R by Northern blotting in several well-studied human breast cancer cell lines (Figure 1a). Previous studies showed that CSF-1R transcripts in ER-negative BT20 and SKBr-3 were significantly upregulated after serum starvation and dexamethasone treatment (Kacinski *et al*., 1991). Total RNA was analysed from cells subjected to different conditions. Only BT20 had detectable CSF-1R transcript levels and then only after glucocorticoid treatment. MDA-MB-231, also ER-negative, was the only cell line that expressed CSF-1. We did not detect CSF-1R expression in SKBr-3 contrary to a previous report (Kacinski *et al*., 1991). To analyse the consequence of CSF-1R expression in a naive, ER-positive breast cancer cell line, we initially focused on MCF-7 which is well characterized as a model system for hormonally-responsive human breast cancer. Human CSF-1R was introduced into early passage MCF-7 cells and individual, stable clones selected. Two with the highest levels of CSF-1R expression were selected for further studies. By ^{125}I-CSF-1 binding, both expressed about 5×10^3 receptors/cell when compared to a myeloid cell line whose CSF-1R level was previously determined by Scatchard analysis (Lee and Nienhuis, 1990). Expression was also confirmed by Western blotting with an antibody directed against the kinase insert region of CSF-1R and by FACS analysis with an antibody that recognizes the CSF-1R ectodomain (Figure 1b).

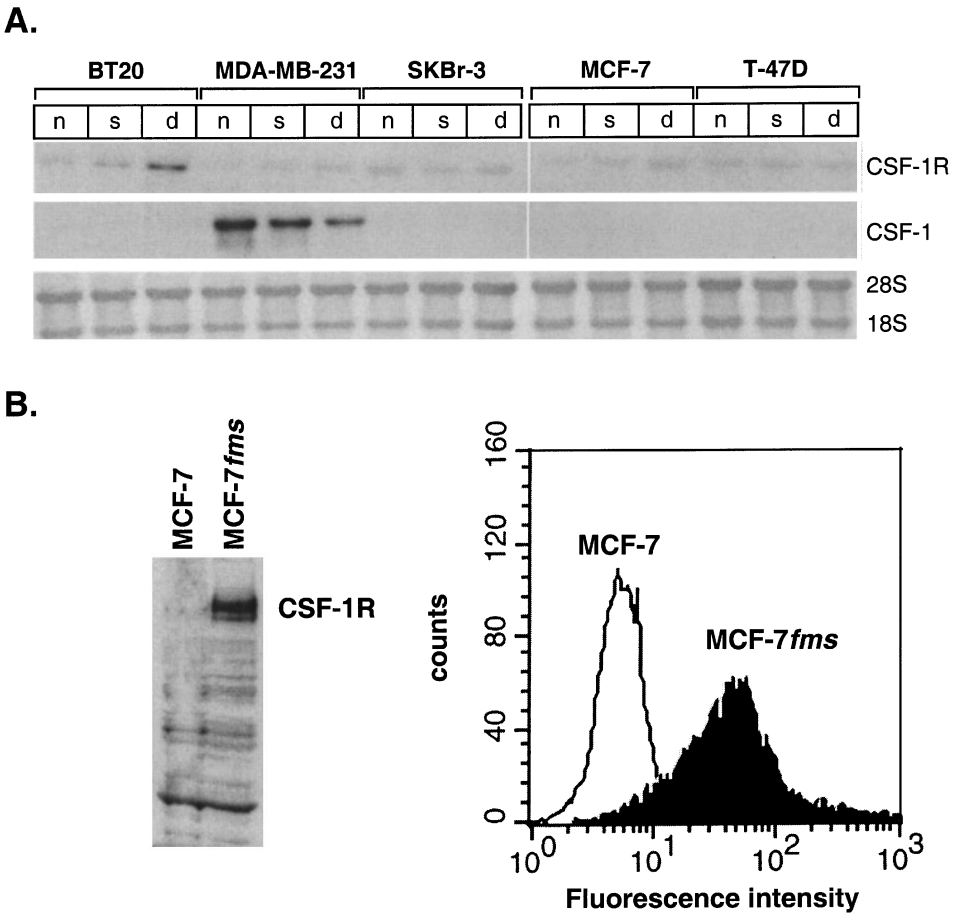

Figure 1 CSF-1 and CSF-1R expression in breast cancer cell lines. (**a**) Northern analysis of the indicated cell lines grown under different conditions: exponentially growing (n), starved for 24 h in serum-free medium (s) or starved and then treated with dexamethasone for another 24 h (d). Identical blots were probed for CSF-1R (top), CSF-1 (middle) or stained with methylene blue (bottom). (**b**) Western blot showing CSF-1R expression in parental MCF-7 and MCF-7*fms* cells (left). FACS analysis of surface CSF-1R expression in parental MCF-7 and MCF-7*fms* (right)

The effect of CSF-1 stimulation on cell number was next examined. A MCF-7 cell line transfected with the empty vector served as control. ER-positive cell lines, because of their exquisite dependence on E_2 for growth and the difficulty of complete removal of steroids from the medium, have generally not yielded large proliferative responses to E_2 or growth factors (Musgrove *et al*., 1993a; Prall *et al*., 1997). To enhance sensitivity, we utilized cells that were semi-synchronized by a 48 h incubation in serum/estrogen-free medium (SFM) or in phenol red-free medium containing CSS and 1 μM of the antiestrogen, tamoxifen. Staining with the DNA dye Hoechst 33258 showed no increased apoptosis associated with tamoxifen as evidenced by the absence of apoptotic bodies or cells with condensed nuclei. Representative growth curves are shown in Figure 2 for two methods of arrest-release (SFM and TAM-2). In both cases CSF-1 alone did not stimulate growth under conditions in which the potent mitogens, E_2 and insulin, supported a 3–5-fold increase in cell numbers over 5 days. Moreover, CSF-1 prevented this increase when given together with E_2 or insulin. Such an inhibition was not observed in the control cell line. Similar findings were obtained for the TAM-1 method of arrest-release and for a second clone of MCF-7*fms* (data not shown).

*CSF-1 inhibits E_2 and insulin-induced G_0-S transition and prevents Rb hyperphosphorylation in MCF-7*fms

We next determined if CSF-1's growth-inhibitory effect was due to a G_1 arrest. Cells were growth-arrested and released by the TAM-2 method. [^{3}H]thymidine uptake was determined and the results are shown (Figure 3a). Whereas CSF-1 had no effect on MCF-7 control cells, it inhibited DNA synthesis induced by E_2 or insulin in MCF-7*fms*. Comparable inhibition was observed for the TAM-1 method and for a second MCF-7*fms* clone (data not shown). To assess dose-response in [^{3}H]thymidine experiments, the concentration of CSF-1 was varied (0, 0.01, 0.1, 1, 10, 100 nM) and maximal inhibition was reached at 1 and 10 nM CSF-1 for insulin and E_2 respectively (data not shown). A dose of 10 nM CSF-1 was used in the experiments described. To monitor more closely the G_0/G_1-to-S transition, MCF-7*fms* cells were arrested by the TAM-2 method and pulse-labeled with ^{3}H-thymidine between 8 and 31 h after release. DNA synthesis peaked at 19 and 22 h for E_2 and insulin respectively (Figure 3b). CSF-1 alone did not stimulate S phase entry and when given with E_2 or insulin, primarily suppressed the number of cells incorporating [^{3}H]thymidine without affecting the time of entry. Thus, inhibition of DNA synthesis is not due to a delay in CSF-1-treated cells reaching S phase. To determine if CSF-1-induced growth arrest is associated with a failure to inactivate the retinoblastoma protein (pRb), total cell lysates were resolved by SDS–PAGE and Western blotted with an anti-Rb antibody (Figure 3c). Both E_2 and insulin induced pRb hyperphosphorylation. In the presence of CSF-1, there was a marked reduction of hyperphosphorylated pRb, consistent with the failure of CSF-1-treated cells to progress into S.

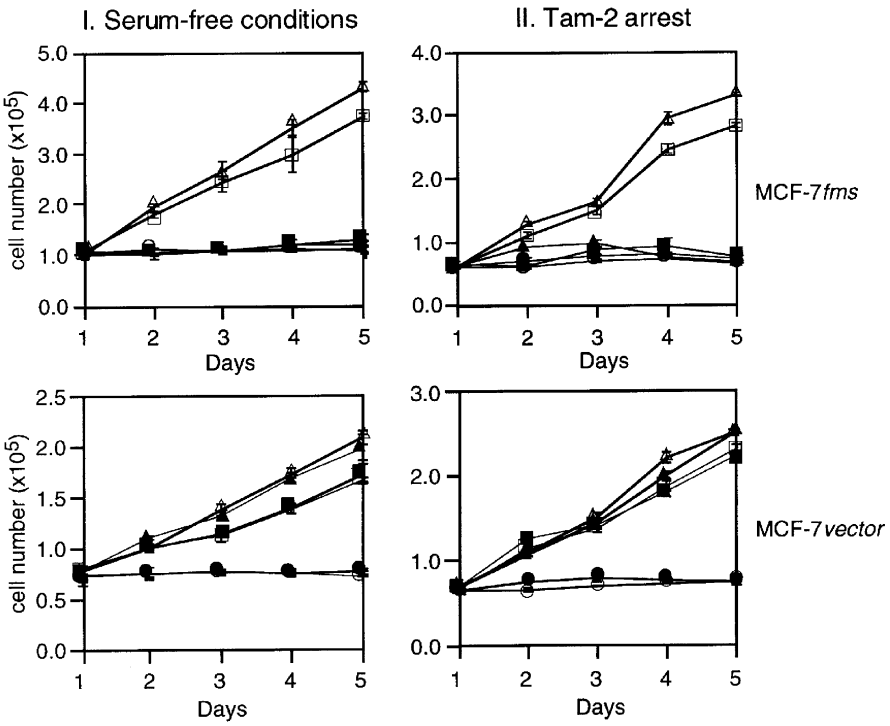

Figure 2 CSF-1 inhibits mitogen-stimulated proliferation in MCF-7*fms*. Representative growth curves are shown for MCF-7*fms* clone 1 (top row) and MCF-7 vector control (bottom row) grown in arrest medium only (circles) or supplemented with CSF-1 (filled circles), E_2 (triangles), E_2 + CSF-1 (filled triangles), insulin (squares) or insulin + CSF-1 (filled squares). Shown are the average total cell numbers and their standard deviations from duplicate wells. For details of the arrest and release conditions, see Materials and methods. For MCF-7 vector control, the curves representing growth in the presence or absence of CSF-1 essentially overlapped

*CSF-1 induces cyclin D1 and p21 expression in MCF-7*fms

The [^{3}H]thymidine uptake data indicate that a significant part of CSF-1's growth suppression is due to inhibition of the G_0/G_1-to-S transition. To investigate the nature of the G_1 arrest, lysates were prepared at different times after release from TAM-2 arrest and then immunoblotted for cell cycle proteins important during G_1 (Figure 4a). When given individually, insulin, E_2 and CSF-1 were all capable of inducing cyclin D1 expression, with CSF-1 inducing the most sustained levels. When CSF-1 was given in combination with E_2 or insulin, sustained levels were maintained throughout the 24 h period after release, well after cells had entered S phase. Cyclins D2 and D3 were not investigated since studies by others have shown these proteins to be undetectable or present at very low amounts in MCF-7 cells (Gorospe *et al*., 1996; Planas-Silva and Weinberg, 1997). Cyclin E expression appeared to be constant as was cdk2. Although not obvious in the blot shown in Figure 4a, all three agents stimulated a modest increase in cdk4 levels (see Figure 5). In the absence of notable suppression of G_1 cyclin and cdk levels, G_1 arrest could result from induction of cyclin-dependent kinase inhibitors (CKIs). p27 levels did not change with addition of any of the agents, however, CSF-1 induced a marked elevation in p21 expression, which was detected at the earliest time point in this series (6 h) and sustained for at least 24 h. The increase in p21 protein levels in CSF-1-treated cells over cells receiving ethanol control remained quite constant: 17-fold at 6 h and 21-fold at 24 h. Insulin also noticeably increased p21, but this was transient. The INK4 family of CKIs was not considered here since $p16^{INK4A}$ is homozygously deleted in MCF-7 and $p15^{INK4B}$ was reported to be present at very low levels and not affected by growth conditions (Musgrove *et al*., 1995; Prall *et al*., 1997).

To confirm that the induction of cyclin D1 and p21 was not specific to the arrest conditions, cells were also quiesced in SFM and monitored for expression of these two proteins in the presence or absence of CSF-1 or E_2 and combinations thereof. As shown in Figure 4b, CSF-1 supported sustained expression of cyclin D1 and p21 up to 48 h after release, irrespective of the presence of E_2. The mRNA levels of cyclin D1, p21 and p27 were next examined (Figure 4c). Cyclin D1 mRNA levels started to increase at 3 h after CSF-1 addition and attained a maximal fivefold increase at 12 h. Increased cyclin D1 levels were detected as early as 0.5 h after E_2 addition and reached maximum levels at

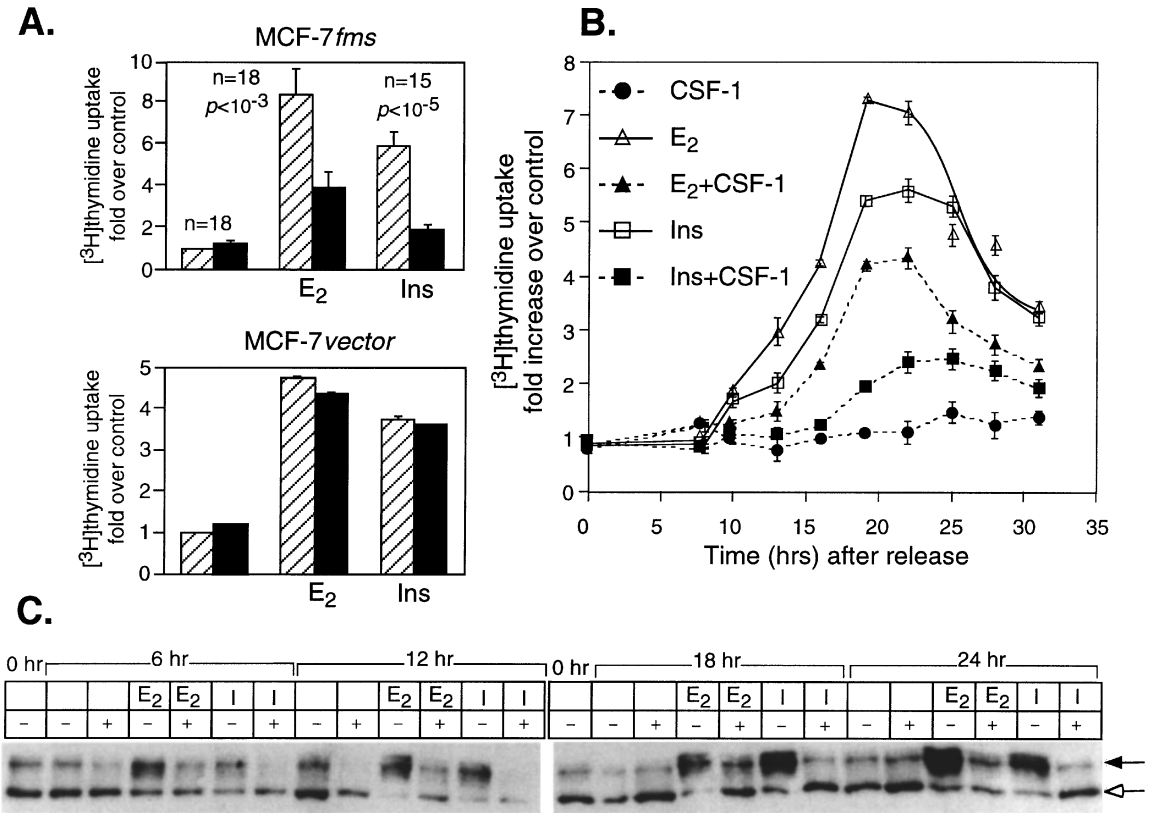

Figure 3 CSF-1 inhibits mitogen-stimulated DNA synthesis and pRb hyperphosphorylation. (**a**) DNA synthesis. MCF-7*fms* cells were arrested by the TAM-2 method and then stimulated for 20 h as indicated in the absence (cross-hatched bars) or presence (filled bars) of CSF-1. DNA synthesis was measured with a 4 h pulse of [^{3}H]thymidine. Shown are the averages and standard deviations based on *n* number of experiments and represented as fold increase over cells that received only ethanol solvent control. *P* values are calculated using the two-sided Student's *t*-test. The results of a representative experiment (out of two) are shown for the MCF-7 vector control where the averages and standard deviations are based on counts from triplicate wells. (**b**) G_0-S transition. MCF-7*fms* cells were arrested by the TAM-2 method and then released into the various agents as indicated. At the indicated times, cells were pulsed for 1 h with [^{3}H]thymidine. Results are the averages of counts from triplicate wells and represented as fold increase over cells receiving solvent control only. (**c**) pRb hyperphosphorylation. Cell lysates were prepared at the indicated times and analysed for pRb protein by Western blotting. '−' and '+' refer to the absence or presence of CSF-1; 'I' is insulin. Upper arrow points to the hyper- and lower arrow to the un-/hypo- phosphorylated pRb

3 h but these levels were lower than that induced by CSF-1 (3.5-fold). When both agents were present, the kinetics of cyclin D1 induction resembled that observed with E_2 alone but the maximum levels were comparable

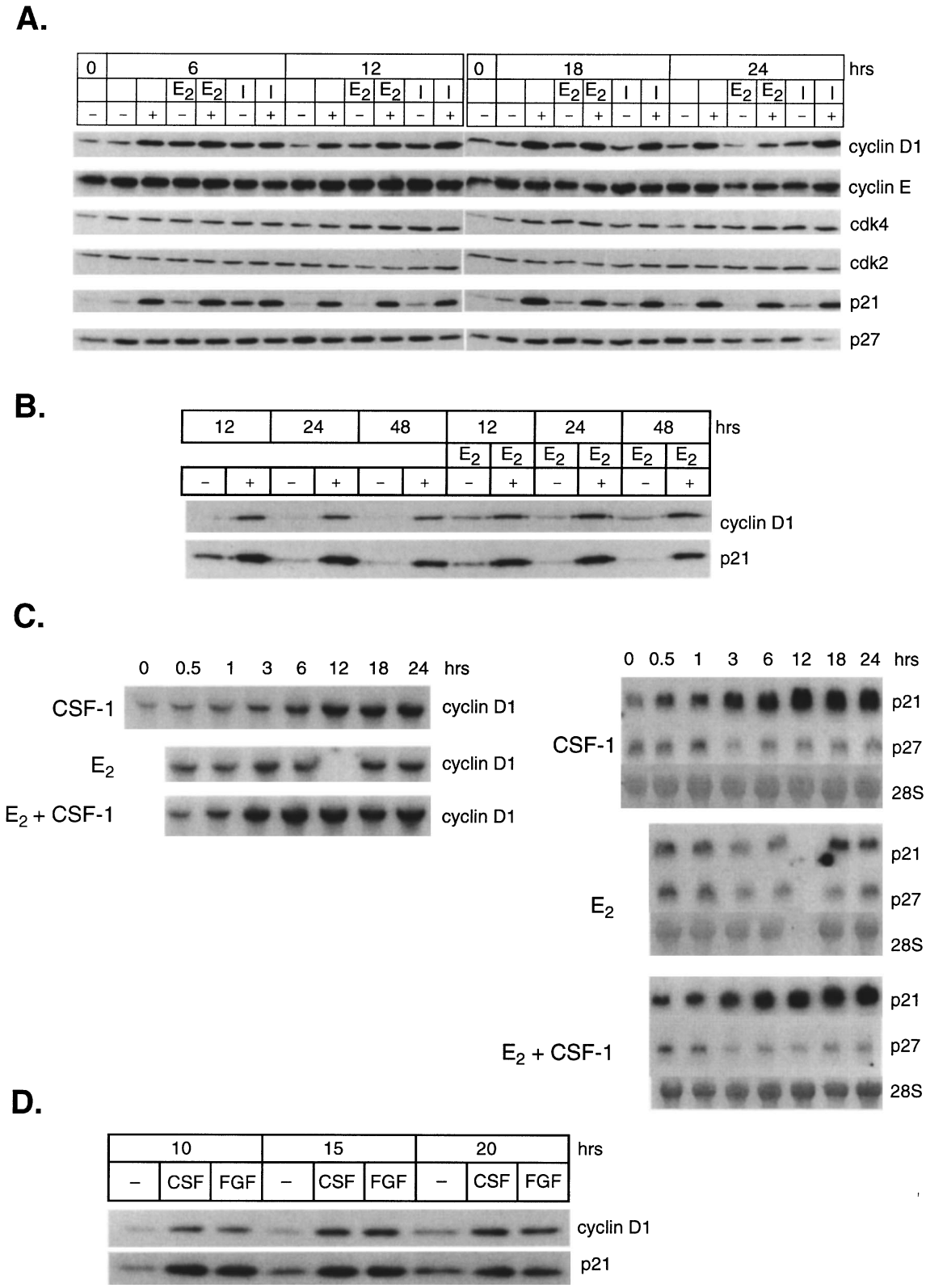

Figure 4 CSF-1 upregulates p21 and cyclin D1 RNA and protein levels. (**a**) G_1 and G_1/S cell cycle proteins. MCF-7*fms* cells were arrested by the TAM-2 method followed by stimulation (E_2 or insulin) or not in the absence ('−') or presence ('+') of CSF-1. Total cell lysates were prepared at the indicated times and Western blotted with specific antibodies. (**b**) MCF-7*fms* cells were arrested in SFM and treated or not with E_2 in the absence or presence of CSF-1. Lysates were Western blotted with antibodies to cyclin D1 and p21. (**c**) Northern blot analysis. MCF-7*fms* cells arrested in SFM were treated with CSF-1, E_2 or both and total cellular RNA harvested at the indicated times. Shown are Northern blots for cyclin D1, p21 and p27. (**d**) MCF-7*fms* cells arrested by the TAM-2 method were either left alone, or treated with CSF-1 or bFGF and harvested at the indicated times. Total cell lysates were blotted for cyclin D1 and p21

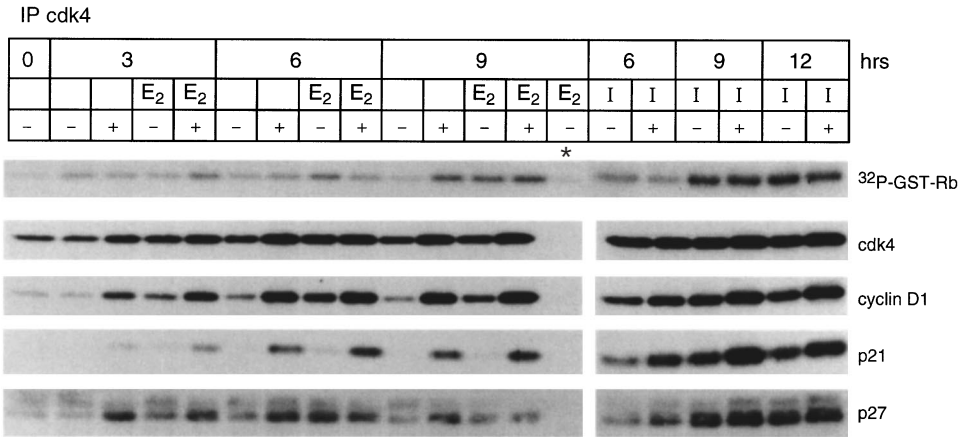

Figure 5 Cdk4 kinase activity and associated proteins. MCF-7*fms* cells arrested by the TAM-2 method were treated as indicated and lysed in Tween lysis buffer at the specified times. Lysates were immunoprecipitated with cdk4 antibodies. Specificity was confirmed by preincubating the cdk4 antibody with the antigenic peptide prior to immunoprecipitation (indicated by an asterisk). Kinase activity was measured using GST-Rb as a substrate. Cdk4 immunoprecipitates were Western blotted sequentially with antibodies against p21, cdk4, cyclin D1 and p27. '−' and '+' refer to the absence or presence of CSF-1 treatment

to that induced by CSF-1. Overall, the mRNA and protein levels appeared to track (compare Figure 4a,b with c). A detectable increase in p21 mRNA levels was observed 0.5 h after CSF-1 addition and reached a maximum of 4.5-fold at 12 h. In comparison, p21 protein levels increased as early as 1 h in some experiments (see Figure 8d), become maximal at 6 h and remained fairly constant thereafter, suggesting primarily a transcriptional or posttranscriptional mechanism. p21 was not affected by E_2 treatment and the time course of induction in the presence of E_2 and CSF-1 resembled that seen with CSF-1 alone. Similar to the protein expression levels, p27 mRNA levels also did not change in response to any of the agents tested.

It was recently found that basic FGF inhibited proliferation of MCF-7 cells and upregulated p21 levels (Wang *et al*., 1997; Johnston *et al*., 1998). To compare the effectiveness of CSF-1 and bFGF in their induction of cyclin D1 and p21, side-by-side experiments were carried out (Figure 4d). A concentration of 10 ng/ml of bFGF was used which others had shown to induce a maximal level of p21 (Wang *et al*., 1997). Very similar levels of cyclin D1 and p21 were found for both growth factors at all the time points examined. These results suggest that CSF-1 and bFGF may activate similar mechanisms to inhibit cell proliferation.

*CSF-1 increases cyclin D1/cdk4 complex formation and decreases the specific activity of cdk4 in MCF-7*fms

The next series of experiments examined the kinase activities and composition of cyclin/cdk complexes which mediate G_1 progression (cyclin D1/cdk4), the G_1-to-S transition (cyclin E/cdk2, cyclin A/cdk2) and S phase progression (cyclin A/cdk2) (reviewed in Sherr, 1996). Cells were arrested and released into different conditions and harvested at the times indicated (Figure 5). Lysates were immunoprecipitated with an antibody against cdk4 and assayed for activity against GST-Rb. The activity is specific since it was substantially reduced in the presence of the immunizing peptide. CSF-1 stimulated a slow, steady increase in cdk4 activity, reaching 3.3-fold at 9 h while E_2 stimulated a slightly more rapid increase but maximal activity was comparable. In insulin-treated cells, cdk4 activity reached a maximal activation of 4.9-fold at 12 h. No detectable change was observed in the cdk4 activity stimulated by E_2 or insulin when CSF-1 was given in combination. The amount of cdk4 in cdk4 immunoprecipitates was determined by Western blotting. Cdk4 increased modestly in response to all three agents compared to cells treated with ethanol control. Co-immunoprecipitating cyclin D1 was very low in control cells but increased significantly upon treatment with all three agents with the highest levels achieved for cells treated with CSF-1 and E_2 or insulin: at 9 h, there was $\approx$fivefold more cyclin D1/cdk4 in E_2 + CSF-1 than E_2-treated cells and at 12 h, there was twofold more cyclin D1/cdk4 in insulin + CSF-1 compared to insulin-treated cells. Since only cdk4 associated with cyclin D1 can be activated, and given that the Rb kinase activity was comparable between E_2 and E_2 + CSF-1-treated cells and between insulin and insulin + CSF-1 treated cells, we surmised that CSF-1 decreased the cdk4 specific kinase activity of E_2-treated cells by 4–5-fold and that of insulin-treated cells by $\approx$twofold.

As CSF-1 increased total p21 dramatically and p21 is a universal inhibitor of cdks, we next determined if p21 could contribute to the reduction in cdk4 specific activity. Cdk4 immunoprecipitates were Western blotted for the presence of p21. Whereas p21 was not detected in cdk4 immunoprecipitates from E_2-treated cells, p21 clearly bound to cdk4 when CSF-1 was given in combination with E_2. The situation with insulin is less obvious. Insulin alone induced a robust increase in cdk4-bound p21, consistent with a transient increase in total p21 (Figure 4a). Peak levels of p21 were present at a time when insulin-stimulated-cdk4 activity was highest (9 and 12 h). CSF-1 further increased the amount of p21 bound by 3–5-fold, but cyclin D1/cdk4 also increased by 40%–twofold, so it is unclear whether there are more p21 molecules bound to cyclin D1/cdk4 complexes in insulin + CSF-1 compared to insulin-treated cells. Since CSF-1 itself stimulated cdk4 activity in the face of increased p21 and total cdk4 kinase activity was not reduced when given in combination with E_2 or insulin, we conclude that cdk4 is unlikely to be the

primary target for p21's inhibitory action. Cdk4 immunoprecipitates were also blotted for p27. We were able to detect p27 only after a prolonged exposure (6 min compared to 30 s for p21), consistent with p27 being present at very low levels in MCF-7 cells (Parry *et al*., 1999). Essentially the amount of p27 in cdk4 immunoprecipitates correlated with the level of cyclin D/cdk4 complexes.

*CSF-1-induced p21 binds to cyclin E/cdk2 and inhibits E_2 and insulin-stimulated cyclin E-associated kinase activity in MCF-7*fms

A potential mechanism for CSF-1-dependent G_1 arrest could involve binding of p21 to cyclin E/cdk2 and inactivation. Cyclin E-associated kinase activity was measured in lysates of MCF-7*fms* cells harvested at different times after treatment (Figure 6a). In E_2-stimulated cells, cyclin E activity peaked at sixfold above control between 12–18 h. Insulin also stimulated a progressive increase in kinase activity reaching a maximum of 7.5-fold at 24 h, the last time point of this series. CSF-1 did not activate cyclin E/cdk2 and inhibited by 79% at 12 h and 54% at 18 h the activity induced by E_2 and by $>90\%$ that induced by insulin. The relative inhibition of E_2 and insulin-stimulated cyclin E/cdk2 kinase activity correlated with their respective levels of inhibition of [^{3}H]thymidine uptake by CSF-1. Cyclin E immunoprecipitates from lysates of the same experiment were probed for the presence of p21 (Figure 6b). In E_2-treated cells, p21 was found to be complexed to cyclin E at 6 h at levels comparable to control cells. However, p21 had disappeared by 12 h, coincidental with peak cyclin E kinase activity. In insulin-treated cells, a more persistent association of p21 with cyclin E was observed, but at 24 h when cyclin E kinase activity was maximal, p21 was absent from the complexes. The correlation of increased cyclin E kinase activity with decreased p21 bound to cyclin E/cdk2 provides an explanation for why insulin-stimulated cyclin E kinase activity lags behind that induced by E_2. In all cases, CSF-1 significantly increased the amount of p21 in cyclin E complexes, correlating with the much diminished kinase activity. Cyclin E-associated p27 was found to be barely detectable and cyclin-E-associated cdk2 level remained unchanged under all treatment (data not shown).

We next sought to demonstrate directly that CSF-1-treated lysates contained an activity that could inhibit active cyclin E/cdk2 complexes. Lysates from CSF-1-treated cells were either mock depleted with normal IgG or immunodepleted of p21 before mixing with lysates from E_2 or insulin-treated cells. Following incubation, cyclin E was isolated and its kinase activity determined. Mock-depleted CSF-1-treated lysates inhibited E_2 or insulin-stimulated cyclin E activity by $\approx 50\%$ and the inhibition was abolished when p21 was removed (Figure 6c). Given that p21 was markedly increased in CSF-1-treated cells, we were surprised to observe only 50% inhibition. Since the extent of inhibition depends on excess p21 not sequestered in complexes, our finding would suggest that not all of the p21 in CSF-1-treated cells was available to bind and inhibit active cyclin E/cdk2 during mixing.

Cdk2 activation requires phosphorylation of Thr 160 by CAK (cdk activating kinase) and dephosphorylation of Tyr 15 by cdc25A. In MCF10A human breast epithelial cells, transforming growth factor-β inhibited cell proliferation because of cdc25A downregulation (Iavarone and Massague, 1997). We determined if the observed reduction in cyclin E-associated kinase activity could be due to a CSF-1-mediated decrease in cdc25A phosphatase activity. Recombinant GST-cdc25A was used to reactivate cyclin E/cdk2 complexes isolated from cells treated with CSF-1, insulin or both agents. In some experiments, vanadate was used to inhibit cdc25A activity. Cdc25A increased the cyclin E-associated kinase activity of CSF-1 and insulin + CSF-1 treated cells in a dose-dependent manner and this increase was eliminated by vanadate (Figure 6d). However, incubation with cdc25A also resulted in a similar increase in cyclin E kinase activity from insulin-treated cells, indicating that deficient cdc25A activity was not the cause of the reduced cyclin E activity in the presence of CSF-1.

Cyclin A-associated activity is also required for S phase entry. Cyclin A/cdk2 kinase activity was determined in cyclin A immunoprecipitates from lysates prepared at different times. Activity increased steadily as E_2 or insulin-stimulated cells progressed towards S (Figure 6e). CSF-1 inhibited E_2-stimulated activity by 80% and completely abolished insulin-stimulated activity. In contrast to cyclin E, cyclin A protein levels increased significantly 24 h after E_2 or insulin addition. Cyclin A was much reduced in the presence of CSF-1 and was almost absent in cells stimulated with insulin + CSF-1 (Figure 6f), consistent with a G_1 arrest. There was a corresponding decrease in cdk2 associated with cyclin A. Thus the reduced cyclin A activity was a consequence of reduced cyclin A/cdk2 complexes.

*In CSF-1 treated MCF-7*fms*, most of cyclin D1 and cyclin E are bound to p21*

To determine what proportion of cyclin D1, cyclin E and cdk2 was bound to p21, immunodepletion was performed. Lysates from cells treated with CSF-1, E_2, or E_2 + CSF-1 for 12 h, or with CSF-1, insulin or insulin + CSF-1 for 24 h were subjected to two rounds of immunoprecipitation with antibodies to p21, p21 + p27 or with control IgG and the immunodepleted supernatants were Western blotted with specific antibodies. The time points corresponded to peak cyclin E-associated kinase activities for E_2 (12 h) and insulin (24 h). Figure 7a shows that p21 and p27 were completely removed after immunodepletion. In this set of lysates, both E_2 and insulin induced detectable amounts of p21 but CSF-1 further increased p21 by 7–8-fold. As shown earlier, there was no change in p27 levels with any of the treatments. In cells treated with E_2 or insulin, p21 partially depleted both the Thr-phosphorylated (faster-migrating) and inactive (slower-migrating) forms of cdk2. When cells were treated in combination with CSF-1, inactive cdk2 predominated and p21 depletion removed the majority of inactive cdk2 and all of the Thr-phosphorylated cdk2. This was reflected in the cdk2 that co-precipitated with p21 (Figure 7a, last row). Thus, in the presence of CSF-1, the majority of cdk2 is associated with p21 and is

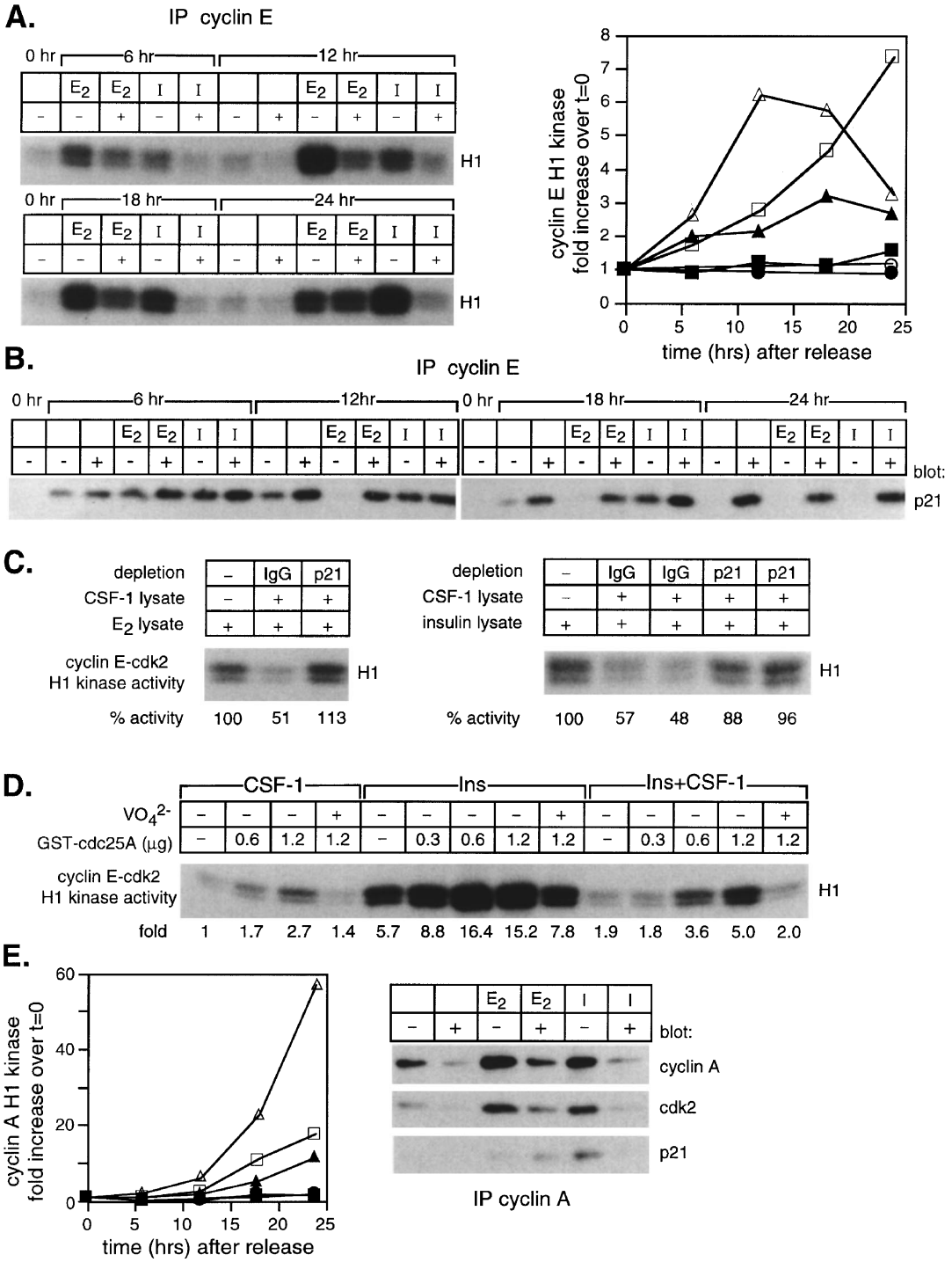

Figure 6 CSF-1 inhibits mitogen-stimulated cdk2 kinase activity through a mechanism involving p21 but not cdc25A. (**a**) Cyclin E associated kinase activity. Total cell lysates were immunoprecipitated with cyclin E antibodies and the immune complexes assayed for histone H1 kinase activity (left). The amount of ^{32}P incorporated into histone H1 was quantitated using a Molecular Dynamics Storm PhosphorImager and normalized to the activity present in control cells at time zero. '−' and '+' refer to the absence or presence of CSF-1 treatment. The meaning of the symbols is identical to that described in the legend to Figure 2. (**b**) Cyclin E-associated p21. Total cell lysates were immunoprecipitated with cyclin E antibodies and then Western blotted with αp21 antibodies. '−' and '+' have the same meaning as in (**a**). (**c**) Inhibitory activity in CSF-1-treated lysates. Lysates from cells treated with CSF-1 for 12 h were either mock-depleted or depleted of p21 prior to mixing with lysates from cells treated with E_2 for 12 h (left). Lysates from cells treated with CSF-1 for 24 h were similarly depleted before mixing with lysates from cells treated with insulin for 24 h (right). Following, the lysates were assayed for cyclin E-associated histone H1 kinase activity. Activity present in E_2- or insulin-stimulated cells was set to 100%. (**d**) cdc25A reactivation of cyclin E/cdk2. Cyclin E/cdk2 complexes were immunoprecipitated with cyclin E antibodies from cells treated with CSF-1, insulin or insulin+CSF-1 for 24 h. Immune complexes were subjected to treatment with various amounts of recombinant cdc25A in the absence or presence of Na_3VO_4. Complexes were then assayed for histone H1 kinase activity. Fold induction in kinase activity normalized to that observed in CSF-1-treated lysates is shown. (**e**) (Left) Histone H1 kinase activity was assayed in cyclin A immunoprecipitates as described in (**a**). (Right) Cyclin A was immunoprecipitated from cells treated with the indicated agents for 24 h and Western blotted for associated proteins. '−' and '+' have the same meaning as in (**a**)

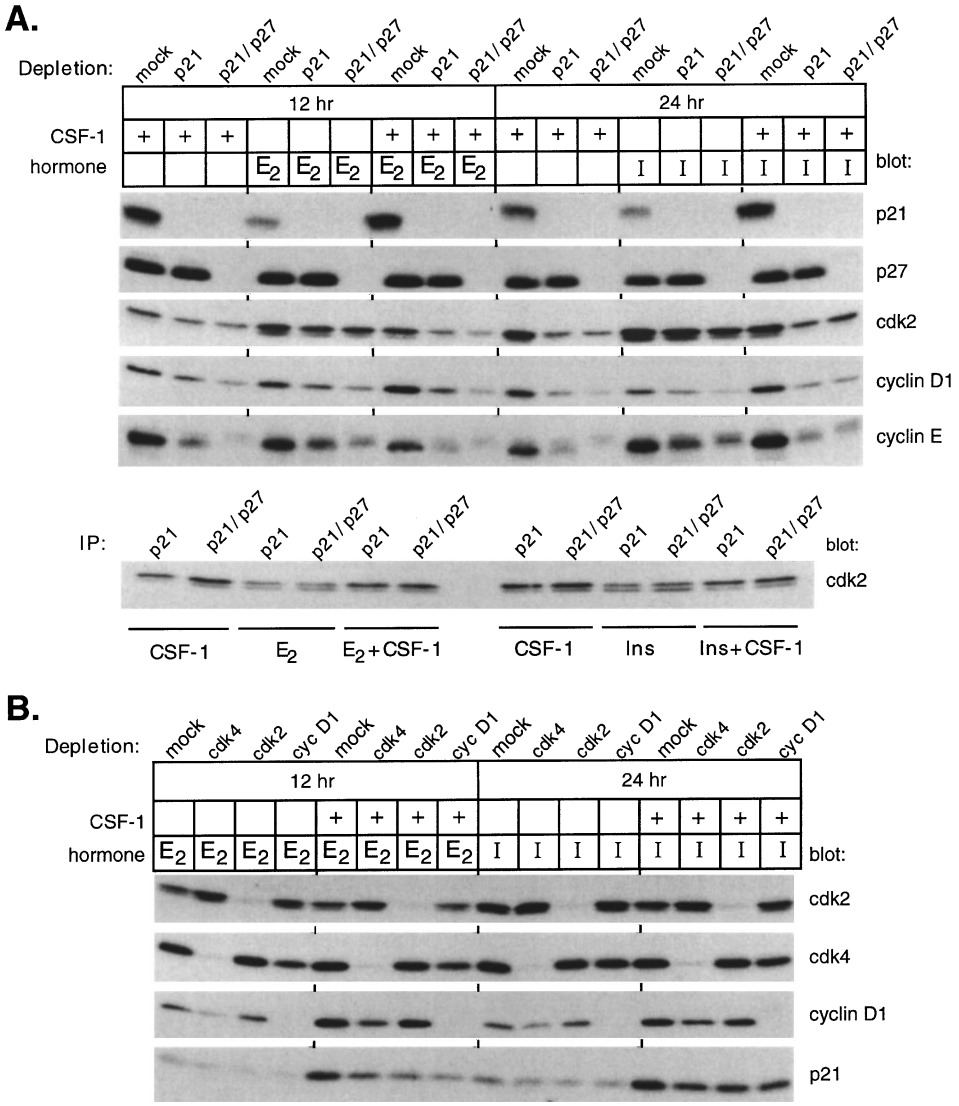

Figure 7 Distribution of p21. (**a**) Lysates from cells that have been treated with CSF-1, E_2 or E_2 + CSF-1 for 12 h and from cells treated with CSF-1, insulin (I) or insulin + CSF-1 for 24 h were subjected to immunodepletion with control IgG, p21 or p21 + p27 antibodies. The depleted lysates were then Western blotted with the indicated antibodies. Immunoprecipitates were also Western blotted with cdk2 antibodies to examine the phosphorylation state of cdk2 bound to p21 and p21 + p27 (bottom panel). (**b**) Lysates were similarly immunodepleted with control IgG or with antibodies to cdk2, cdk4 or cyclin D1 and then Western blotted

inactive, most likely because cdk2 phosphorylation is blocked by the p21 present in cyclin E/cdk2 complexes (Aprelikova *et al*., 1995). A minor population of cdk2 in CSF-1-treated cells is Thr 160 (CAK)-phosphorylated but exists only in complexes with p21 and its activation state is unclear. The inactive cdk2 not removed by p21 is probably free cdk2 usually in excess over cyclin E and A in cells and binds to p21 with much lower affinity (Harper *et al*., 1995).

In E_2 and insulin-treated cells, $\approx$50% of cellular cyclin D1 was immunodepleted by p21, in agreement with previous studies showing that p21 binds to cyclin D1 during E_2-stimulated G_1 progression (Planas-Silva and Weinberg, 1997; Prall *et al*., 1997). In the presence of CSF-1, 80–90% of cyclin D1 was depleted by p21. In all cases a minority of cyclin D1 was removed by p27 depletion. These results imply that almost all of the cyclin D1 in CSF-1-treated cells contained p21/p27. In E_2 and insulin-treated cells, $\approx$55% of cyclin E was removed by p21 depletion and another 25% by p27 depletion, leaving about 20% that was free of p21 and p27 (Figure 7a). We had earlier observed that p21 was essentially undetectable in cyclin E immunoprecipitates from E_2 and insulin-treated cells at these time points (Figure 6b). To reconcile this apparent discrepancy, we note that whereas it takes only one p21 molecule bound for the cyclin E/cdk2 complex to be depleted, it may take more p21 bound for p21 to be detected in the immunoprecipitates. In CSF-1-treated cells, 80–90% of cyclin E was removed by p21 depletion and a minority by p27 leaving 5–6% free of p21/p27. It was shown previously that the majority of cyclin E-associated kinase activity in E_2-stimulated MCF-7 cells is accounted for by the minor population of cyclin E/cdk2 that lacked p21/p27 (Prall *et al*., 1997; Planas-Silva and Weinberg, 1997). The observation that the p21/p27-free cyclin E population decreased from 20% to 5% when cells were treated with CSF-1 is consistent with the marked inhibition of cyclin E kinase activity in these cells.

We next determined the distribution of p21 between cdk4, cdk2 and cyclin D1 by immunodepletion and

Western blotting of depleted lysates (Figure 7b). In cells treated with CSF-1 and E_2 or insulin, cdk4 and cdk2 independently removed $\approx 50\%$ of p21. Since cdk4 and cdk2 do not co-exist in the same complex this implies that most of the p21 was bound, which explains why during *in vitro* mixing, CSF-1-treated lysates could only partially inhibit cyclin E/cdk2 isolated from mitogen-treated cells (Figure 6c). Cyclin D1 similarly depleted p21, as expected on the basis of its association with cdk4. Cdk4 depletion did not completely remove cyclin D1; possibly some of the cyclin D1 was dislodged during the prolonged incubation required for the immunodepletion experiments or that cyclin D1 is also complexed to cdk6 (Parry *et al*., 1999).

*CSF-1 induces low levels of p21 and functions as a mitogen for T-47D*fms

To investigate the generality of CSF-1's anti-proliferative effects in human breast cancer cells, human CSF-1R was similarly transfected into another well-characterized, ER-positive human breast cancer cell line, T-47D. Individual clones were isolated and two chosen for further analysis. Receptor expression was compared to MCF-7*fms* by binding of ^{125}I-CSF-1 to living cells and cold chase with unlabeled CSF-1 was used to demonstrate specificity. Expression levels were shown to be comparable (Figure 8a). [^{3}H]thymidine uptake was measured 20 h after addition of test reagents to T-47D*fms* cells that have been synchronized either by serum/estrogen starvation or by the TAM-2 method. We compared CSF-1 to E_2 and insulin, both mitogens for T-47D (Musgrove *et al*., 1993b). Results are shown for T-47D*fms* clone 1 (Figure 8b). In contrast to MCF-7*fms*, CSF-1 increased DNA synthesis 2–3-fold above cells receiving ethanol control, compared to 3–7- and 1.5–2-fold in response to E_2 and insulin respectively. Moreover, CSF-1 cooperated with insulin to enhance mitogenesis and to a lesser extent with E_2 as well. Similar results were obtained with T-47D*fms* clone 2 (data not shown). We correlated the [^{3}H]thymidine data with phosphorylation of pRb. Cell lysates harvested at different times were Western blotted for pRb (Figure 8c). CSF-1, E_2 and insulin all induced pRB hyperphosphorylation although with somewhat different kinetics. Since the induction of p21 by CSF-1 appeared to be the major determinant of growth arrest in MCF-7*fms* cells, a side-by-side experiment was carried out for T-47D*fms* and MCF-7*fms*. Whereas CSF-1 induced a robust increase in p21 levels as early as 1 h in MCF-7*fms* cells, only a slight increase was observed in T-47D*fms* and this occurred much later, at 12 h. These results support the possibility that the growth stimulatory effect of CSF-1 in T-47D*fms* cells is due to a lack of significant p21 induction.

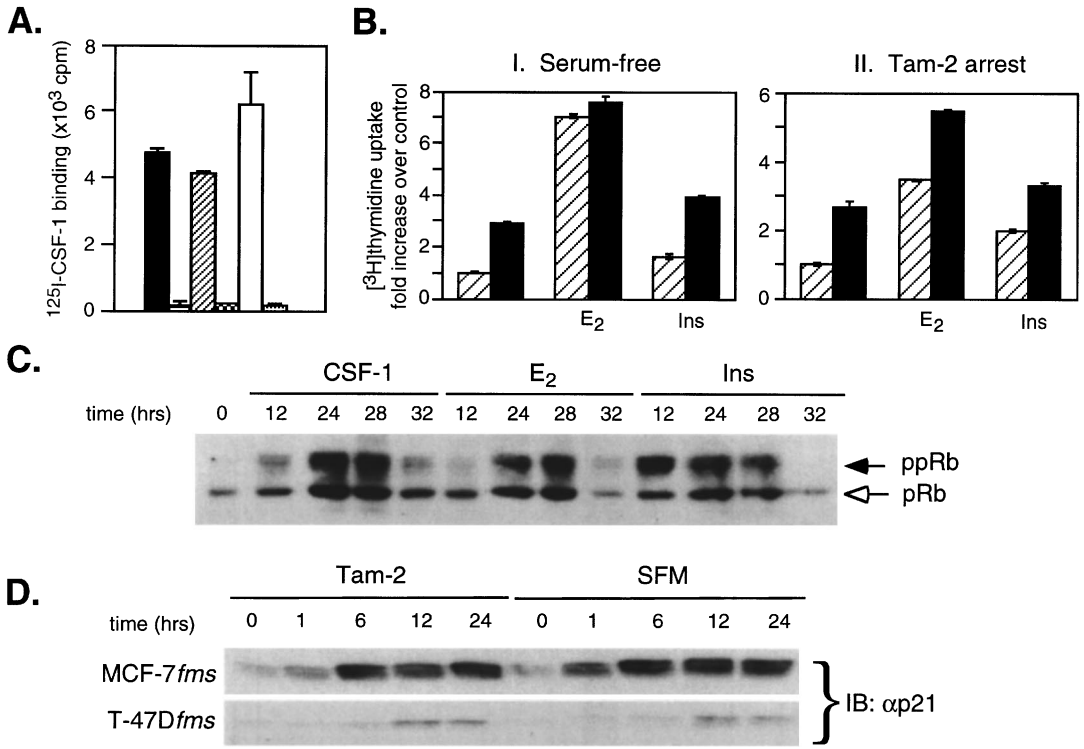

Figure 8 CSF-1 is mitogenic in T-47D*fms* and induces minimal levels of p21. (**a**) ^{125}I-CSF-1 binding. Cell surface receptor expression is assessed by binding of 2×10^5 living cells to ^{125}I-CSF-1 at 4°C (see Materials and methods). Shown are the averages and standard deviations from three independent determinations for MCF-7*fms* clone 1 (filled bar), T-47D*fms* clone 1 (cross-hatched bar) and T-47D*fms* clone 2 (open bar). The corresponding adjacent bar is the binding activity observed in the presence of excess unlabeled CSF-1. (**b**) DNA synthesis. [^{3}H]thymidine uptake was carried out as described for Figure 3. The corresponding filled bars denote the presence of CSF-1. Shown is a representative experiment for clone 1. The experiment has been repeated twice with similar results. (**c**) pRb hyperphosphorylation. T-47D*fms* cells were arrested by the TAM-2 method, released as indicated and total cell lysates Western blotted for pRb protein. (**d**) p21 induction. Side-by-side experiments were carried out for MCF-7*fms* and T-47D*fms*, arrested either by the SFM or TAM-2 method and then released into CSF-1. Lysates from different time points were Western blotted for p21

*CSF-1-mediated p21 induction in MCF-7*fms *is p53-independent*

We sought to understand the basis for the differential response of MCF-7*fms* and T-47D*fms* to CSF-1. One of the major differences between MCF-7 and T-47D is that the former contains wildtype p53 while the latter has only a single, mutated copy of p53 (Casey *et al*., 1991). Both p53-dependent and independent mechanisms have been described for the induction of p21 (el-Deiry *et al*., 1994; Jiang *et al*., 1994; Deng *et al*., 1995; Macleod *et al*., 1995; Bacus *et al*., 1996). To determine if p53 is required for CSF-1-mediated induction of p21, we made use of a p53 miniprotein (p53DD) which functions as a potent dominant-negative for wildtype p53 (Shaulian *et al*., 1992). MCF-7*fms* cells were transiently transfected with vector control, murine wildtype p53 or p53DD, arrested by the TAM-2 method and then treated with CSF-1, etoposide or solvent control. Etoposide is a DNA-damaging agent included as a control as it was previously shown to increase p53 and p21 and to transcriptionally activate p21 in a p53-dependent manner (Sheikh *et al*., 1994). Total cell lysates were Western blotted with an anti-p53 antibody that recognizes mouse p53 (Pab421), demonstrating marked overexpression of p53DD (Figure 9, top panel). The dominant-negative effect of p53DD results from oligomerization with endogenous p53 (Gottlieb *et al*., 1994). Using the DO-1 anti-p53 antibody which recognizes only human p53, endogenous p53 was found to be present at very high levels in cells transfected with p53DD (Figure 9, middle panel), indicating that the transfected p53DD was functional. In cells transfected with vector or wildtype p53, etoposide increased endogenous p53 levels as previously observed (Sheikh *et al*., 1994) but CSF-1 was without effect. p53DD expression reduced etoposide-induced but not CSF-1-induced p21 levels, demonstrating that p53 was involved in the former but not latter case.

*CSF-1 induction of p21 and cyclin D1 is MAPK-dependent in MCF-7*fms *and constitutively activated MEK induces p21, cyclin D1 and inhibits E_2-stimulated cdk2 activity*

Activation of the MAPKs, ERKs, has been most frequently associated with cell proliferation. Recently, sustained MAPK activity induced by differentiation-promoting factors such as nerve growth factor (NGF) (Pumiglia and Decker, 1997; Tombes *et al*., 1998) or by constitutively activated Raf (Lloyd *et al*., 1997; Sewing *et al*., 1997; Woods *et al*., 1997) or MEK (Lin *et al*., 1998), has been shown to produce growth arrest and senescence, as a consequence of p21 and/or $p16^{INK4A}$ induction. To address this possibility in MCF-7*fms*, we first compared activation of ERK/MAPK by CSF-1 and insulin. Our hypothesis was that if MAPK played a role, CSF-1 should induce sustained MAPK activity since it was growth-inhibitory and insulin should induce a transient activation since it was growth-promoting. Cells deprived of serum/estrogen were stimulated with CSF-1 or insulin and harvested at different times (Figure 10a). Lysates were immunoprecipitated with a polyclonal anti-ERK1/2 antibody and assayed for myelin basic protein (MBP) kinase activity. CSF-1 stimulated a robust increase in MAPK activity at 10 min followed by a temporary decline and reactivation at 3 h. Insulin also activated MAPK but only transiently. These results are thus consistent with the notion that sustained MAPK activity may play a role in mediating CSF-1's growth inhibitory action. In

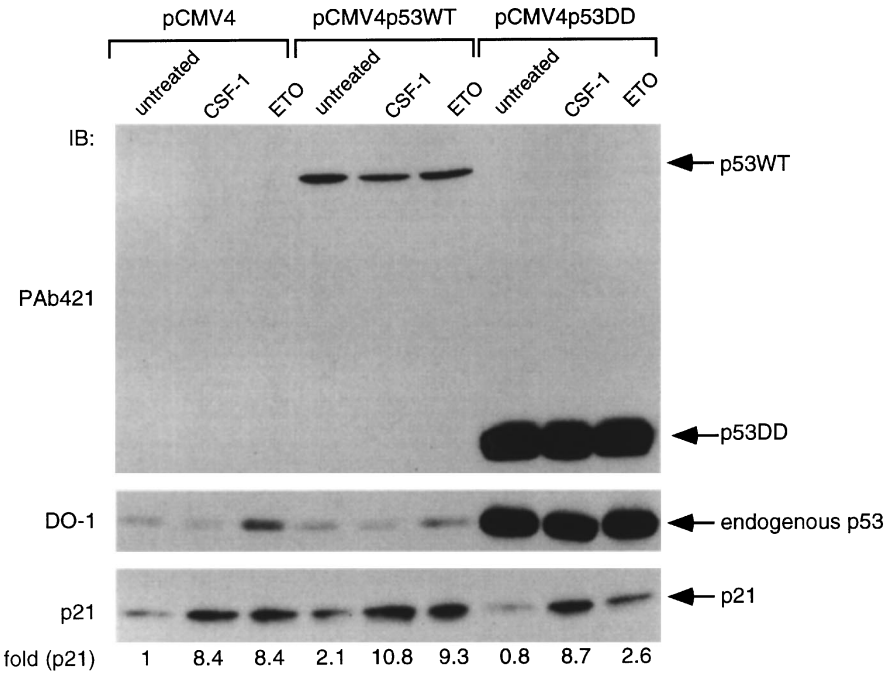

Figure 9 CSF-1 stimulation of p21 is not dependent on p53. MCF-7*fms* cells were transiently transfected with 10 μg of vector pCMV4, 10 μg of murine WTp53 or 10 μg of murine p53DD, arrested and treated with DMSO control (untreated), 10 nM CSF-1 or 10 μM of etoposide (ETO) for 6 h. Total cell lysates were Western blotted for murine p53 using Pab421, human p53 using DO-1 or for p21. Fold induction of p21 is normalized to p21 levels in untreated cells transfected with pCMV4

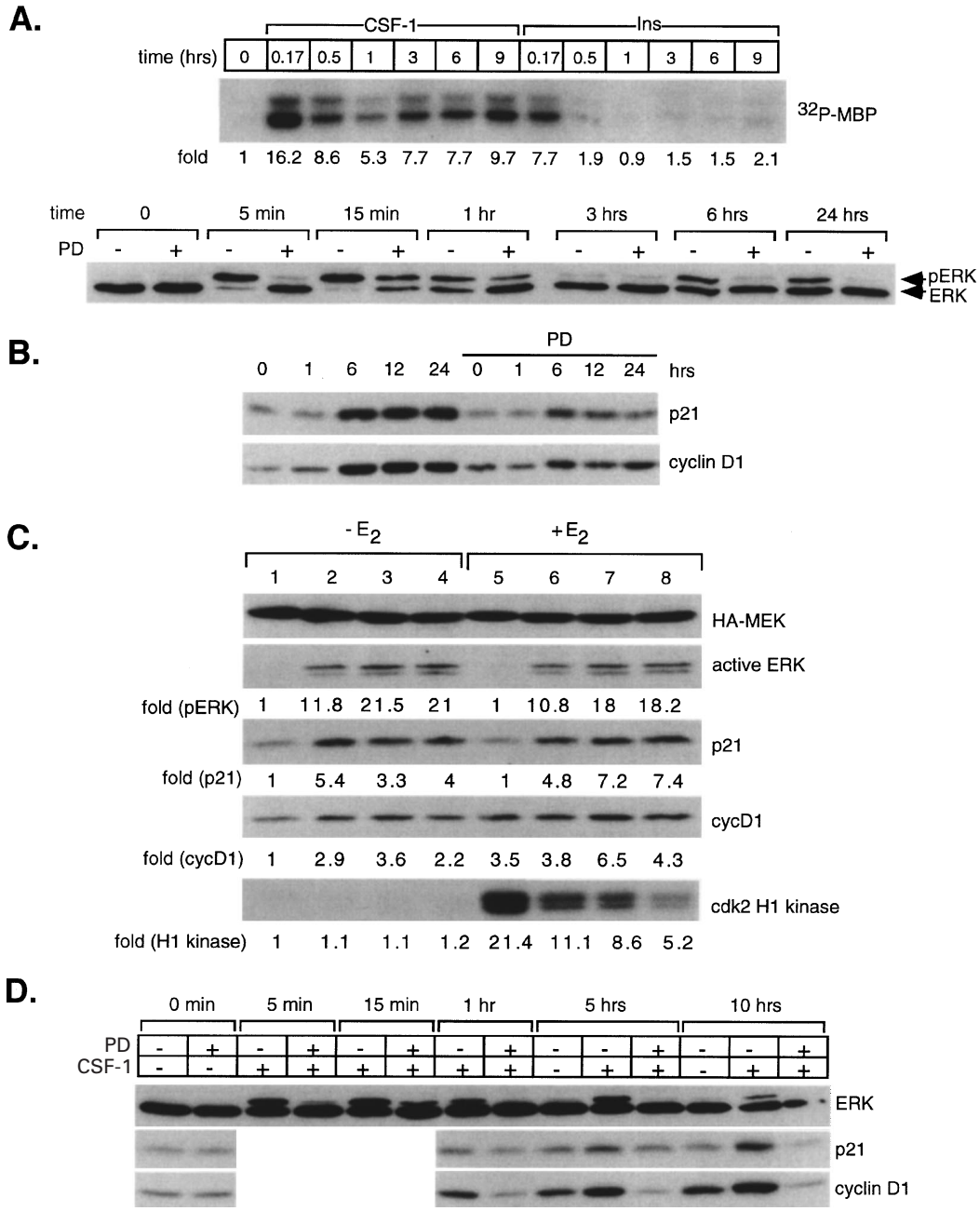

Figure 10 CSF-1 stimulates sustained MAPK activity in MCF-7*fms* which is required for p21 and cyclin D1 induction. (**a**) (Top) MCF-7*fms* cells arrested in SFM were stimulated with CSF-1 or insulin and lysates from different times were immunoprecipitated with ERK2 antibodies and assayed for myelin basic protein (MBP) kinase activity. (Bottom) MCF-7*fms* cells arrested by the TAM-2 method were stimulated with CSF-1 in the absence or presence of a 1 h-pretreatment with 50 μM PD98059 (PD). Total cell lysates were Western blotted with a monoclonal ERK1/2 antibody to demonstrate mobility shifts. (**b**) Cell lysates from cells pretreated or not with PD98059 followed by CSF-1 stimulation for the indicated times were Western blotted with specific antibodies. (**c**) MCF-7*fms* cells were transiently transfected with 5 μg each of WTMek1 and WTMek2 (lanes 1 and 5), 5 μg each of R4F and KW71 (lanes 2 and 6), 7.5 μg each of R4F and KW71 (lanes 3 and 7) or 10 μg each of R4F and KW71 (lanes 4 and 8) arrested and then treated either with ethanol control ($-E_2$) or with E_2 for 18 h before harvesting. Cell lysates were Western blotted with antibodies to HA (HA-MEK), dually-phosphorylated ERK (active ERK), p21 and cyclin D1. Cell lysates were also immunoprecipitated with cdk2 antibodies and assayed for histone H1 kinase activity. (**d**) T-47D*fms* were arrested by the TAM-2 method and then stimulated with CSF-1 with or without PD98059 pretreatment. Lysates were Western blotted for ERK (top panel) or for p21 (middle panel) and cyclin D1 (bottom panel)

contrast, CSF-1 induces only a transient activation in hematopoietic cells where it functions as a mitogen (Lee, 1999).

To more directly investigate the role of ERK/MAPK, we asked if MAPK activity was required for the induction of p21 by CSF-1. Cells arrested by the TAM-2 method were treated with the highly-specific MEK inhibitor, PD98059, or DMSO control prior to stimulation with CSF-1. We first determined ERK activation in the presence or absence of PD98059 by mobility shift on an ERK Western blot (Figure 10a). In the absence of PD98059, CSF-1 induced an upward shift in 95–98% (5 and 15 min), 53% (1 h), 36% (6 h) and 28% (24 h) of total ERK. When pretreated with PD98059, MAPK activity was inhibited by 80% (5 min), <50% (15 and 60 min) and by >50% (6

and 24 h). The extent of inhibition corresponded to the inhibition seen with MEK1 activation (data not shown). Similar results were obtained with different concentrations (40 or 60 μM) or source of PD98059 (New England BioLabs or Calbiochem) and independent of whether PD98059 was added 1 h, 30 min or 5 min before CSF-1 (not shown). The differential ability of PD98059 to inhibit MAPK activity was only evident when a detailed time course was performed which may explain why this phenomenon has not been widely reported in the literature, raising the question of precisely how PD98059 functions. We next determined the levels of p21 and cyclin D1 in total cell lysates. Results of a representative Western blot is shown (Figure 10b). Pretreatment with PD98059 resulted in a significant although not complete reduction in the levels of p21 and cyclin D1 induced by CSF-1. The average percent inhibition from two independent experiments indicates that inhibition of MAPK led to a 55–70 and 50–80% reduction in p21 and cyclin D1 respectively. The coordinate increase in p21 and cyclin D1 induced by MAPK activation is in agreement with reports using constitutively activated Raf and MEK.

The inhibition of p21 induction by PD98059 is indicative of an essential role for ERK/MAPK. We next determined if MAPK activity alone was sufficient for p21 induction. MCF-7*fms* cells were transiently transfected with a combination of wildtype MEK1 and MEK2 or with different amounts of constitutively activated MEK1 (R4W) and MEK2 (KW71). These plasmids have been described previously and the combination was shown to induce the highest level of endogenous ERK activity (Whalen *et al*., 1997). Cells were arrested by the TAM-2 method, stimulated or not with E_2 and harvested 18 h later. Lysates were first Western blotted with anti-HA antibody to confirm expression since all transfected proteins are hemaglutinin-tagged (Figure 10c). To determine if the active MEKs were able to activate endogenous ERK, lysates were blotted with an antibody that recognizes dually-phosphorylated ERK. Active MEKs increased phosphorylated ERK by 11–22-fold over wildtype MEKs which stimulated little activation. Activated MEKs also induced a 5–7-fold increase in p21 and 2–7-fold increase in cyclin D1, compared to a 3.5-fold induction of cyclin D1 by E_2. There was no effect on p27 (data not shown). Lysates were assayed for cdk2 kinase activity. Activated MEKs on their own were unable to activate cdk2. E_2 stimulation of cells transfected with wildtype MEKs showed a 21-fold increase in kinase activity whereas the E_2 stimulated activity was decreased by 51–79% in cells transfected with activated MEKs. The extent of inhibition increased with increasing amount of transfected MEK plasmids. Although we did not detect any significant increase in overall HA-MEK expression, the observed pattern could be accounted for by an increased number of transfected cells with increasing amounts of transfected MEK plasmids. These results demonstrate that in MCF-7 cells, expression of constitutively activated MEK leads to p21 and cyclin D1 induction and inhibition of E_2-induced cdk2 kinase activity.

We next analysed CSF-1-induced ERK/MAPK activation in T-47D*fms*. Cells were treated as described for MCF-7*fms*. To meaningfully compare the extent of MAPK activation between different cell types, we examined the percent of total ERK that was activated. CSF-1 stimulated MAPK in T-47D*fms* with kinetics similar to that observed for MCF-7*fms* (Figure 10d). Overall, the strength of activation was lower in T-47D*fms* compared to MCF-7*fms*: 33–35% of total ERK was activated at 5 and 15 min and $<10\%$ at 1 h. Interestingly, the pattern of inhibition by PD98059 was similar in the two cell types. Lysates were analysed for p21 and cyclin D1 induction. As shown earlier (Figure 8d), CSF-1 induced a small, delayed increase in p21 which was completely eliminated by PD98059, indicating that MAPK activation was the initiating event for p21 induction in both MCF-7*fms* and T-47D*fms*. These findings are consistent with the report that significant induction of p21 and inhibition of mitogenesis required high intensity activation of the Raf/MEK/MAPK pathway. Lower levels of MAPK activation induced cyclin D1 but p21 only minimally, resulting in a mitogenic outcome (Sewing *et al*., 1997; Woods *et al*., 1997).

Discussion

The growth factor receptor, CSF-1R, is expressed in a preponderance of malignant breast tumors yet its role is poorly defined. We have used as model systems, breast cancer cells which are estrogen-dependent and represent an early stage of malignant conversion (Dickson and Lipman, 1995). A transfection approach was undertaken so that comparison between naive versus CSF-1R-expressing breast cancer cells can be made on a common genetic background. Our data show that expression of a growth factor receptor does not uniformly lead to uncontrolled proliferation, even in a cancerous cell that has already acquired substantial genetic alterations. In MCF-7*fms* cells, CSF-1 treatment induced a G_1 arrest and substantially inhibited the proliferation in response to E_2 and insulin. The growth arrest induced by CSF-1 is a consequence of MAPK-dependent and p53-independent upregulation of the CDK inhibitor, $p21^{Waf1}$. The response of hormone-dependent breast cancer cells to CSF-1 is dependent on cellular context, since similar expression of CSF-1R in T-47D led to CSF-1-induced mitogenesis and a low level of p21 induction. Although our studies are confined to only two breast cancer cell lines, our data indicate that use of CSF-1R expression as a prognostic factor in human breast cancer is less clear than has been suggested previously (see Introduction) and should be considered in the context of other markers, such as MAPK activation, cyclin D1 and p21 levels.

Evidence in support of CSF-1 mediating a G_1 arrest in MCF-7*fms* cells treated with E_2 or insulin includes inhibition of DNA synthesis, pRb hyperphosphorylation and prevention of cyclin E/cdk2 and cyclin A/cdk2 kinase activation. Since E_2 and insulin act through vastly dissimilar receptors to stimulate divergent signaling pathways that ultimately converge on the cell cycle to induce mitogenesis, the most logical targets for CSF-1's inhibitory action would be one or more cell cycle proteins. In support of a predominant cell cycle effect, we had first determined that insulin-mediated early signaling events such as IRS-1 tyrosyl

phosphorylation and its association with Grb2 were not affected by CSF-1 and CSF-1 did not down-modulate estrogen receptor levels (data not shown). The major alterations in cell cycle proteins in response to CSF-1 treatment of MCF-7*fms* cells were increases in cyclin D1 and p21. p21 has been implicated in the G_1 arrest induced by a variety of agents, including DNA-damaging agents (el-Deiry *et al*., 1994; Poon *et al*., 1996), interferons (Mandal *et al*., 1998), heregulin (Bacus *et al*., 1996), NGF (Yan and Ziff, 1995; Pumiglia and Decker, 1997), FGF (Johnson *et al*., 1998) and high intensity activation of the Ras/Raf/MEK/MAPK pathway (Lloyd *et al*., 1997; Sewing *et al*., 1997; Woods *et al*., 1997; Lin *et al*., 1998). Also enforced expression of p21 under an inducible promoter in a variety of cell types has provided additional support for p21 playing a role in inhibiting the G_1-S transition (Niculescu *et al*., 1998; Sekiguchi and Hunter, 1998; Bates *et al*., 1998).

Several lines of evidence point to p21 being the causative agent in mediating CSF-1's inhibitory action in MCF-7*fms* cells: (1) p21 levels are markedly increased following CSF-1 addition, the induction occurs early on (1 h) and persists for >24 h; (2) the magnitude of the increase is comparable to that observed with FGF and etoposide, agents shown previously to induce p21 and implicated in mediating growth arrest of MCF-7 cells (Johnson *et al*., 1998; Sheikh *et al*., 1994); (3) CSF-1's growth inhibitory action in MCF-7*fms* is correlated with the induction of p21 and its mitogenic action in T-47D*fms* is correlated with the lack of significant p21 induction; (4) immunodepletion studies demonstrated that p21 was bound to almost all of the cyclin E in CSF-1 treated cells and only cyclin E immunoprecipitates from CSF-1-treated cells persistently contained p21; (5) mixing experiments demonstrated the presence of an activity in CSF-1-treated cells removable by p21 depletion that inactivated cyclin E/cdk2 immunopurified from E_2 or insulin-treated cells. On the basis of these observations, we conclude that p21 mediated CSF-1's growth inhibition. Our data point to cyclin E/cdk2 as being p21's major target since total cdk4 kinase activity was not reduced and inhibition of cyclin A/cdk2 activity was due to reduced cyclin A expression. This conclusion is consistent with the prevailing notion that *in vivo* inactivation of cyclin/cdk complexes by p21 can lead to G_1 arrest (Sherr and Roberts, 1995).

While the mitogen, insulin, also increases p21 in MCF-7*fms* cells, compared to CSF-1, insulin-mediated p21 induction is more transient and levels attained are lower. This implies that the G_1 arrest induced by CSF-1 depends on both the duration and magnitude of p21 upregulation. Similar transient increases in p21 have been described in other mitogenic responses, e.g. in quiescent cells released by serum addition (Li *et al*., 1994; Macleod *et al*., 1995). Our observations are in agreement with a previous report showing that p21 is modestly increased by the insulin-like growth factor I (IGF-I) in MCF-7 cells (Dufourny *et al*., 1997). These authors noted that the induction was not affected by inhibition of the ERK/MAPK pathway which is consistent with our findings that insulin activated MAPK only transiently while sustained MAPK activity is required for p21 induction. Thus our data and those of others suggest that insulin and IGF-I activate MAPK-independent pathways to induce p21 in MCF-7 cells.

Immunodepletion studies revealed that $\approx 50\%$ of p21 was bound to cyclin D1/cdk4. A reservoir function for cyclin D1 has been proposed to explain the mechanism of cyclin E activation induced by E_2. In this model, p21 is redistributed from cyclin E/cdk2 to cyclin D1/cdk4 when the latter increases in response to E_2 stimulation, leading to cyclin E/cdk2 activation (Prall *et al*., 1997; Planas-Silva and Weinberg, 1997). p21 has also been proposed to function as an assembly factor for cyclin D and cdk4 (LaBaer *et al*., 1997; Cheng *et al*., 1999). Below a certain threshold, increasing p21 expression increases cyclin D/cdk4 complex formation and kinase activity, much like what we observed with insulin treatment. Only after a certain point is the kinase activity inhibited, presumed to occur when more than one p21 molecule binds to cyclin D/cdk4 (Zhang *et al*., 1994; Harper *et al*., 1995). By analogy, we propose that in CSF-1-treated cells, the coordinate increase in cyclin D1 and p21 promoted cyclin D1/cdk4/p21 complex formation at the expense of a modest reduction in specific but not total kinase activity. Neither cyclin D1 nor cdk4 sequesters all of the p21. We cannot currently explain why the remaining p21 bound to cyclin E/cdk2 and not cyclin D/cdk4. A possibility might be that not all of the p21 is available to bind cyclin D1. However we have not been able to detect any gross difference in subcellular localization of the two proteins as indirect immunofluorescence of MCF-7*fms* cells treated with CSF-1 alone or in combination with E_2 or insulin showed predominant nuclear staining for both p21 and cyclin D1 (data not shown). Taken together, the induction of cyclin D1 by CSF-1 has the effect of mitigating the full inhibitory consequences of p21 upregulation by maintaining total cdk4 kinase activity and by sequestering a significant proportion of p21 away from cyclin E/cdk2. In this respect, cyclin D1 induced by CSF-1 fulfills the usual oncogenic role associated with its overexpression in breast cancers, a view derived from studies of human breast cancer specimens and transgenic mice (Wang *et al*., 1994; Hall and Peters, 1996). A prediction of our hypothesis is that the outcome of a given stimulus, be it mitogenesis or G_1 arrest, is dependent on the relative levels of cyclin D and p21/p27 induction.

In MCF-7*fms*, sustained MAPK activity was both necessary and sufficient for p21 induction. Also, FGF (Wang *et al*., 1997; Johnson *et al*., 1998), phorbol esters (Dufourny *et al*., 1997) and c-erbB2 overexpression (Giani *et al*., 1998) all stimulate sustained MAPK activity and inhibit the growth of MCF-7 cells. We suggest that any signaling system that stimulates sustained MAPK activity will induce growth arrest in MCF-7 cells. Our conclusion that MAPK is a major determinant of p21 induction stands in contrast to that reached by Johnson *et al*. (1998) in their analysis of FGF-mediated inhibition of MCF-7 cells. These authors observed that FGF activated MAPK in both MCF-7 and T-47D cells but FGF induced p21 in MCF-7 cells only. We also found that CSF-1 activated MAPK in T-47D*fms* with kinetics similar to that in MCF-7*fms* but that a significantly smaller proportion of total ERK was activated in T-47D*fms*. Using inducible Raf expression systems, Woods *et al*. (1997)

and Sewing *et al*. (1997) elegantly demonstrated that the mitogenic response as a function of intensity of Raf activation follows a bell-shaped curve with a sharp optimum. Raising MAPK activity by ≈twofold from the mitogenic optimum produced a precipitous drop in thymidine uptake as a consequence of increased p21 induction (Sewing *et al*., 1997). We propose that one possible explanation for why CSF-1 elicited a mitogenic response in one case (T-47D*fms*) and a growth-inhibitory response in another (MCF-7*fms*) is the difference in intensity of MAPK activation. It will be interesting to determine why T-47D*fms* did not support a similar level of MAPK activation, e.g. whether this could be due to higher levels of inactivating phosphatases in T-47D or whether CSF-1R activates the MAPK pathway in the two cell types by coupling to different signaling intermediates subject to different negative feedback mechanisms. In addition to differences in intensity of MAPK activation, MAPK may also stimulate overlapping but non-identical downstream pathways in MCF-7 and T-47D, only some of which can upregulate p21 levels. One or more of these pathways leading to p21 induction may be subverted in T-47D. Finally, even though high intensity MAPK activation may be a critical initiating event, it is important to emphasize that CSF-1 activates other signaling pathways as well and some of these will have an impact on the final outcome. For example, the magnitude of the subsequent p21 response could be modulated by activation of the JAK/STAT pathway which can also induce p21 (Chin *et al*., 1996), by activation of the small GTPase Rho which can block p21 induction (Olson *et al*., 1998) and by factors which influence transcript and protein stability, all of which are potentially cell type-dependent.

Our results also demonstrate that MAPK activity is necessary and sufficient for cyclin D1 induction. Increases in cyclin D1 protein levels occur as a consequence of increased transcription and/or enhanced protein synthesis or stabilization. CSF-1-mediates an increase in cyclin D1 mRNA which is consistent with reports demonstrating that the Ras/MEK/MAPK pathway promotes cyclin D1 transcription (Lavoie *et al*., 1996; Weber *et al*., 1997). Intriguingly, p21 and p27 also appear to enhance cylin D protein stability *in vivo* as was shown recently by Cheng *et al*. (1999). These authors hypothesized that CKI binding to cyclin D/cdk4 may protect cyclin D from degradation by masking a phosphorylation site on cyclin D (Thr 286 in cyclin D1) that targets it for degradation in the proteasomes. As it turns out, Thr 286 is also a target for regulation by the PI3-kinase/AKT pathway (Diehl *et al*., 1998). In our experiments, pretreatment with the MEK inhibitor did not completely abrogate the increase in cyclin D1 protein levels in response to CSF-1 raising the possibility that cyclin D1 induction in MCF-7*fms* cells is the result of the combined activities of the Ras/MAPK and the PI3-kinase/AKT pathways. However the relative contributions of the two pathways may be hard to assess since we were not able to eliminate ERK activation completely at all time points with the MEK inhibitor.

It is striking that expression of CSF-1R in MCF-7 and T-47D cells, both well-characterized *in vitro* models for ER-positive breast cancer, could have produced such contrasting behavior in response to CSF-1. CSF-1 has been proposed to play a role in the terminal differentiation of the mammary gland that occurs during the latter stages of pregnancy (Pollard and Hennighausen, 1994). It is tempting to speculate that what we observed with MCF-7*fms* more closely mimics the situation in normal breast during terminal stages of pregnancy since differentiation is frequently associated with growth arrest. In support of this possibility, we have observed that in MCF-7*fms* cells, CSF-1 induces extensive lipid accumulation, which is a marker of differentiation along the alveolar secretory pathway (AW Lee and JG Moffat, unpublished observations). On the other hand, the T-47D*fms* phenotype is more consistent with an oncogenic function for CSF-1R in invasive breast cancers. Additional comparative studies on MCF-7*fms* and T-47D*fms* and other breast cancer cells expressing CSF-1R will yield further insights into the differences in cellular context that ultimately dictate their response to CSF-1.

Materials and methods

Antibodies and reagents

Cell culture reagents and media were from GIBCO/BRL (Gaithersburg, MD, USA), PD98059 was from Calbiochem (La Jolla, CA, USA) or New England BioLabs (Beverly, MA, USA) and other reagents were from Sigma (St. Louis, MO, USA). Recombinant human CSF-1 was a gift of Genetics Institute (Cambridge, MA, USA), and basic FGF was from Collaborative Biomedical Products (Bedford, MA, USA).

Rabbit antiserum against the conserved kinase insert region of the CSF-1R was generated in our laboratory using a GST fusion protein containing residues 675–749 of the murine CSF-1R. Rabbit polyclonal antibodies against ERK2, ERα, cyclin D1, cyclin E, cyclin A, cdk2, cdk4, p21, p27 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and retinoblastoma protein (14001A) was from Pharmingen (San Diego, CA, USA). Monoclonal antibodies used were as follows: Ab-2 anti-CSF-1R directed against the extracellular domain (Oncogene Research Products, Cambridge, MA, USA), DO-1 anti-p53 (Santa Cruz, USA), Pab421 anti-p53 (Oncogene Research Products), anti-hemagglutinin (HA.11, BabCo, Richmond, CA, USA) and phospho-specific antibodies that recognize dually-phosphorylated ERK1/2 (New England BioLabs). Secondary antibodies were from Santa Cruz or GIBCO/BRL and protein A-horseradish peroxidase (HRP) was from Zymed (San Francisco, CA, USA).

Plasmids

Full length human CSF-1R cDNA was released from pSMc-fms (kindly provided by C Sherr, St. Jude Children's Hospital, Memphis, TN, USA) by *Bam*HI digestion and subcloned into pcDNAINeo (Invitrogen) to generate pcDNA*fms*. Constructs used as probes in Northern blots were human cyclin D1 cDNA (kindly provided by S Dowdie, Washington University, St. Louis, USA), p21, p27 (gifts of J Roberts, Fred Hutchinson Cancer Center, Seattle, USA), and human CSF-1 (gift of Genetics Institute). Constructs used to express murine wildtype p53 and dominant-negative p53 were generous gifts of M Oren (Weizmann Institute, Israel). Constructs to express hemagglutinin (HA)-tagged wildtype MEK1, MEK2 and constitutively activated MEK1 (R4F) and MEK2 (KW71) were kindly provided by N Ahn (U Colorado, Boulder). A construct to express GST-cdc25A in

bacteria was a gift of S Elledge (Baylor College of Medicine, Houston, TX, USA). pEGFP-N1 containing a green fluorescent protein in a mammalian expression vector was purchased from Clonetech (Palo Alto, CA, USA).

Cell culture and transfection

The early-passage, ER-positive MCF-7 human breast adenocarcinoma cell line, a gift from Edward Gelmann (Lombardi Cancer Research Center, Washington, DC, USA), was maintained in Dulbecco's modified Eagles medium (DMEM) with 5% fetal bovine serum (FBS). The ER-positive T-47D and ER-negative MDA-MB-231 human breast carcinoma cell lines were from the ATCC; T-47D was maintained in RPMI 1640 with 10% FBS and 7.4 μg/ml of bovine insulin and MDA-MB-231 in DMEM with 10% FBS. The ER-negative human breast carcinoma cell lines BT20 and SKBr-3 were kindly provided by M Flick and B Kacinski (Yale University, New Haven, CN, USA) and maintained in RPMI 1640 with 10% FBS and McCoy's 5A modified medium with 15% FBS respectively.

Stable MCF-7 clones expressing vector control or human CSF-1R were established by electroporating 10^7 cells with 10 μg of pcDNAINeo or pcDNA*fms* at 150 V and 960 μF using a Gene Pulser (Bio-Rad, Hercules, CA, USA). Stable T-47D clones were established by transfecting semi-confluent 6 cm dishes with 2 μg of pcDNAINeo or pcDNA*fms* and LipofectAMINE PLUS (GIBCO/BRL) according to the manufacturer's protocol. Clones were selected in G418, isolated by ring cloning, and screened for CSF-1R expression by binding ^{125}I-labeled human CSF-1 as described previously (Lee and Nienhuis, 1990). Transient transfections of MCF-7 cells were optimized with pEGFP-N1 and visualized daily for 4 days by fluorescence microscopy. Optimal transfection efficiency (50–90%) was obtained by electroporating 2×10^6 cells with 10 μg of plasmid DNA at 200 V and 960 μF. For expression of wildtype and mutant p53, cells were transfected as described above and split evenly into three dishes. The following day, cells were changed into TAM-2 arrest medium and 48 h later, treated for 6 h with 10 μM etoposide in DMSO, 10 nM CSF-1 and equivalent amount of DMSO or DMSO alone. For MEK expression, cells were transfected as for p53 and incubated in TAM-2 medium for 30 h before addition of 100 nM E_2 or ethanol control and analysis 18 h later.

^{125}I-CSF-1 binding

Ten μg of recombinant human CSF-1 was iodinated with Na^{125}I following a modified chloramine-T protocol as described (Stanley and Guilbert, 1981). Duplicate samples containing 2×10^5 cells released by scraping were incubated with 1 nM (saturating) of ^{125}I-CSF-1 for 16 h at 4°C, washed and cell-associated radioactivity determined in a γ-counter. To demonstrate specific binding, a 50-fold excess of cold CSF-1 was added to the cells 1 h before addition of ^{125}I-CSF-1.

Proliferation assays

For cell number analysis, $4-5\times10^4$ cells were seeded per well in 24-well dishes. All conditions were performed in duplicate. After 48 h in complete medium, cells were washed twice with phenol red-free (PRF) Hank's Balanced Salt Solution and put into quiescence medium. Three quiescence and release conditions were utilized. In the serum-free medium (SFM) method, cells were placed in SFM (PRF-DMEM with 0.1% BSA, 20 mM HEPES, 20 μg/ml transferrin, 1 mM sodium pyruvate and 10 nM sodium selenite) for 48 h and then test reagents added directly to the culture medium (10 nM CSF-1, 1 nM 17β-estradiol, 1.7 μM insulin or combinations thereof). In the TAM-1 method, cells were placed in TAM arrest medium (PRF-DMEM with 5% charcoal-stripped serum (CSS) (HyClone, Logan, UT, USA) and 1 μM tamoxifen) for 48 h. The medium was removed and replaced with fresh PRF-DMEM and 5% CSS supplemented with test reagents (10 nM CSF-1, 1 nM E_2, 0.17 μM insulin and combinations thereof). In the TAM-2 method, cells were placed in TAM arrest medium for 48 h and test reagents directly added to the medium (10 nM CSF-1, 100 nM E_2 and 1.7 μM insulin and combinations thereof). In all cases, cells were refed with fresh media and reagents on the third day. At the desired times, cells were detached with trypsin and counted by hemacytometer with dead cells excluded by Trypan Blue.

To monitor DNA synthesis, cells were seeded at 10^4 cells/well in 96 well plates with all conditions performed in triplicate. Cells were quiesced and released from arrest as described above. 20 h later, they were labeled for 4 h with 0.5 μCi/well [*methyl*-^{3}H]-thymidine. After rinsing with phosphate-buffered saline (PBS), cells were fixed to the wells by incubation with methanol for 5 min. DNA was precipitated with 10% trichloroacetic acid on ice for 60 min and solubilized with 0.1% SDS, 0.2 N NaOH. [^{3}H]thymidine incorporation was determined by liquid scintillation counting. In experiments to monitor the G_o-to-S transition, cells were arrested by the TAM-2 method and time points taken every 3 h starting at 8 h after release. At each time point, cells were pulsed with [^{3}H]thymidine for 1 h and then processed as described above.

Northern analysis

Total RNA was extracted as described (Chomczynski and Sacchi, 1987). Ten μg of RNA/sample was loaded onto formaldehyde gels, electrophoresed and blotted onto Hybond-N (Amersham, Arlington Heights, IL, USA). Restriction fragments used as probes were from coding regions and produced as follows: 1.2 kb *Hind*III fragment of cyclin D1, 2.1 kb *Xho*I fragment of p21; 2.1 kb *Eco*RI fragment of p27, 0.425 kb *Ava*I fragment of human CSF-1 and 0.408 kb *Pvu*II-*Xho*I fragment of human CSF-1R. cDNAs were labeled with α^{32}P-dATP using either Rediprime (Amersham) or the random-prime kit from GIBCO/BRL. Ribosomal RNA bands immobilized on membranes were stained with methylene blue to ensure equivalent loading and transfer.

Western blotting

Cell monolayers were washed with ice-cold PBS and lysed in RafLB as described (Lee, 1999) except that it contained 0.25 M NaCl. Protein content was determined using the Bio-Rad protein assay. Membranes were blocked in 5% (w/v) nonfat dry milk. Primary antibodies and HRP-conjugated secondary antibodies were used at the manufacturer's recommended concentrations. Detection was by enhanced chemiluminescence (Amersham) and quantitation was carried out by scanning multiple ECL exposures and integration of signals using NIH Image 1.6 software as described (Lee, 1999).

Immunoprecipitation and immunodepletion

For immunoprecipitations, cells were seeded at 8×10^5 per 10 cm dish and arrested by one of the methods described. At the indicated times, cells were lysed in RafLB and 200 μg of lysate was incubated with 1–2 μg of primary antibody for 2 h followed by a further 2 h incubation with 20 μl of protein A sepharose. Immunoprecipitates were washed four times with lysis buffer and once with HEPES-buffered saline before boiling in Laemmli sample buffer. To avoid overlapping signals between cyclin E or cyclin A and the IgG heavy chain on Western blots, these immunoprecipitates were not boiled but incubated at 37°C for 15 min and cyclin E and A

detected on Western blots with protein A-HRP. In immunodepletion experiments, 200 μg of lysate was subjected to two sequential rounds of immunoprecipitation with 1 μg of primary antibody or purified rabbit IgG as mock control. The protein content of the depleted lysate was determined again and 10–20 μg of depleted lysate was analysed by Western blotting.

Immune complex kinase assays

Cyclin and cdk For cyclin E, cyclin A and cdk2 kinase assays, lysates were prepared in RafLB at the indicated time points and immunoprecipitated as described above. Immune complexes were washed four times with RafLB, once with Tris-buffered saline, once with kinase buffer (25 mM HEPES, pH 7.4, 25 mM β-glycerophosphate, 1 mM dithiothreitol (DTT), 10 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM EGTA, 0.2 mM Na_3VO_4 and 0.03% Brij35) and resuspended in 25 μl of kinase buffer containing 2 μg histone H1 (Boehringer Mannheim, Indianapolis, IL, USA), 20 μM ATP and 10 μCi [γ^{32}P]ATP. The reaction was allowed to proceed for 15 min at 30°C and terminated by heating for 5 min at 90°C in Laemmli buffer. After separation by SDS–PAGE, gels were Coomassie-Blue-stained to locate substrate and IgG bands (to ensure equal loading), dried and incorporation into histone H1 quantitated by PhosphorImager.

Cdk4 assays were carried out essentially as described (Planas-Silva and Weinberg, 1997; Prall *et al*., 1997). At the indicated time points, cells were washed in PBS, detached by scraping and the drained pellets flash-frozen in liquid nitrogen and stored at −80°C until needed. At assay time, pellets were lysed by vortexing in Tween lysis buffer (Prall *et al*., 1997) with protease inhibitors and Na_3VO_4. Four hundred μg of lysate (equivalent to one 10 cm dish) was used per assay. After preclearing with rabbit IgG and protein A sepharose, cdk4 was immunoprecipitated and complexes washed thrice with Tween lysis buffer and thrice with kinase buffer (50 mM HEPES, pH 7.4, 2.5 mM EGTA, 10 M β-glycerophosphate, 10 mM $MgCl_2$, 1 mM NaF, 1 mM DTT, 0.2 mM Na_3VO_4). To control for nonspecific binding, some samples were incubated with anti-cdk4 previously bound to its immunizing peptide (Santa Cruz). Kinase reaction was initiated by the addition of 30 μl of kinase buffer containing 25 μM ATP, 15 μCi [γ^{32}P]ATP and 1 μg of GST-Rb(769-921) (Santa Cruz) as substrate and proceeded for 30 min at 30°C. The products were separated by 11% SDS–PAGE and the gel was cut between the 46 and 30 kDa molecular weight markers such that the top part was processed for GST-Rb phosphorylation and the bottom part for Western blotting with cdk4, p21 and p27 antibodies.

Mixing assay for inhibitory activity in cellular lysates Inhibitory RafLB lysates were prepared from cells treated with CSF-1 while active lysates were from cells treated with E_2 or insulin. 200 μg of inhibitory lysate was mixed with 100 μg of active lysate for 40 min at room temperature followed by cyclin E immunoprecipitation and histone H1 kinase assay. In some experiments, inhibitory lysates were immunodepleted of p21 before being used in the mixing assay.

MAPK (ERK) and MEK assays were carried out exactly as described previously (Lee, 1999).

cdc25A assay

GST-cdc25A was purified from bacteria using glutathione affinity chromatography following standard protocols. Cyclin E/cdk2 was immunoprecipitated with cyclin E antibody from 100 μg of lysate. The immune complexes were washed twice in 50 mM Tris pH 8, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, and 1 mM EGTA, twice in cdc25A buffer (50 mM Tris pH 8, 50 mM NaCl, 5 mM $MgCl_2$, 2 mM DTT) and then incubated in 30 μl of cdc25A buffer containing varying amounts of GST-cdc25A. Dephosphorylation proceeded for 30 min at 30°C, the immunoprecipitates were then washed twice with RafLB containing Na_3VO_4, once with Tris-NaCl and once with cdk kinase buffer followed by histone H1 kinase assay.

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