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Rescue of defective mitogenic signaling by D-type cyclins

(cyclin D/cyclin E/colony-stimulating factor 1 receptor/c-*myc*/mitogenesis/G_1 phase progression/cell cycle)

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ABSTRACT **Three gene products, including Myc and the D- and E-type G_1 cyclins, are rate limiting for G_1 progression in mammalian fibroblasts. Quiescent mouse NIH 3T3 fibroblasts engineered to express a mutant colony-stimulating factor (CSF-1) receptor (CSF-1R 809F) fail to synthesize c-*myc* and cyclin D1 mRNAs upon CSF-1 stimulation and remain arrested in early G_1 phase. Ectopic expression of c-*myc* or either of three D-type cyclin genes, but not cyclin E, resensitized these cells to the mitogenic effects of CSF-1, enabling them to proliferate continuously in liquid culture and to form colonies in agar in response to the growth factor. Rescue by cyclin D1 was enhanced by c-*myc* but not by cyclin E and was reversed by infecting cyclin D1-reconstituted cells with a retroviral vector encoding catalytically inactive cyclin-dependent kinase 4. Induction of cyclin D1 mRNA by CSF-1 was restored in cells forced to express c-*myc*, and vice versa, suggesting that expression of the two genes is interdependent. Cells reconstituted with c-*myc* were prevented from entering S phase when microinjected with a monoclonal antibody to cyclin D1, and conversely, those rescued by cyclin D1 were inhibited from forming CSF-1-dependent colonies when challenged with a dominant-negative c-*myc* mutant. Cyclin D mutants defective in binding to the retinoblastoma protein were impaired in rescuing mitogenic signaling. Therefore, Myc and D-type cyclins collaborate during the mitogenic response to CSF-1, whereas cyclin E functions in a separate pathway.**

Mammalian D- and E-type cyclins assemble with cyclin-dependent kinases (cdks) to form holoenzymes, whose activities are rate limiting for progression through the G_1 phase of the cell cycle (1–3). Cyclin E expression is periodic and maximal at the G_1 to S phase transition, where it regulates the activity of cdk2 (4, 5), a catalytic subunit whose functions are necessary for the onset of DNA replication (6–8). As cells enter the cycle following mitogenic stimulation, D-type cyclins (D1, D2, and D3) are expressed earlier than cyclin E in a lineage-specific manner (9), assemble into complexes with cdk4 and cdk6 (10–14), and execute their functions prior to the G_1/S transition (2, 9, 15). A critical substrate of the cyclin D-dependent kinases is likely to be the retinoblastoma protein (pRb) (10, 12, 13, 16), whose phosphorylation late in G_1 reverses its growth-suppressive function (17–20). Cyclin D1 appears not to be required in pRb-negative cells (21–23).

In macrophages, colony-stimulating factor 1 (CSF-1) initiates a mitogenic response by binding to the CSF-1 receptor (CSF-1R), thereby activating the receptor protein tyrosine kinase and signaling via multiple effector-mediated pathways. CSF-1 is required throughout G_1 to ensure entry of macrophages into S phase, and persistent CSF-1R kinase activity is necessary for the expression of both immediate early—e.g., c-*fos*, c-*jun*, and c-*myc*—and delayed early—e.g., D-type cyclins—response genes. Ligand-activated human CSF-1R can induce continuous proliferation when ectopically expressed in mouse NIH 3T3 fibroblasts, replacing their requirement for platelet-derived growth factor and insulin-like growth factor 1 (24). In contrast, a receptor mutant containing a phenylalanine for tyrosine substitution at codon 809 (here designated CSF-1R 809F) is mitogenically inactive (25), and ligand-stimulated cells remain arrested in early G_1 phase (26). Although cells expressing CSF-1R 809F exhibit receptor kinase activity and unimpaired *fos* and *jun* expression (25), c-*myc* is poorly induced, and expression of its mRNA is unsustained. Enforced expression of c-*myc* in these cells overcomes G_1 arrest and restores the proliferative response, demonstrating that c-*myc* is required for CSF-1 induced mitogenesis (27). Because expression of c-*myc*, like that of D- and E-type cyclins, is rate limiting for G_1 progression (28, 29), these genes might collaborate during the mitogenic response. To that end, we assayed the ability of G_1 cyclins to rescue CSF-1 responsiveness in cells bearing the CSF-1R 809F mutant.

MATERIALS AND METHODS

Expression Plasmids. Cyclin D expression plasmids were constructed as described (2). Cyclin D1 and D2 mutants disrupted in the LXCXE motif necessary for pRb binding but able to interact with cdk4 (16, 30–32) included pentapeptide deletions (ΔLXCXE) and mutation of glutamic acid-7 in cyclin D2 to lysine (D2-E7K) (32). A retrovirus vector encoding human cyclin E (1) was provided by James Roberts (Fred Hutchinson Cancer Center, Seattle) and a plasmid conferring resistance to puromycin (pJ6Ω-puro) was provided by Hartmut Land (Imperial Cancer Research Fund, London). A retrovirus encoding a *myc* dominant-negative mutant (In373) (33) that dimerizes with Max but is unable to bind to DNA (34) was provided by Charles Sawyers (University of California, Los Angeles). A cDNA encoding catalytically-inactive cdk4 (K35M) (32) was substituted for c-*myc* in the same vector.

Antibodies. Mouse monoclonal antibodies (mAbs) to human cyclin D1 (mAb DCS-6 cross-reactive to mouse cyclin D1) (35) and to mouse cyclin D1 (mAb 72-13G-11) (2, 36) but nonreactive toward cyclins D2 and D3 were used for microinjection, immunoprecipitation, and pRb kinase assays. Rat mAbs to mouse cyclins D2 (34B4-7) and D3 (18B6-10) (36) were used for immunoprecipitation. A mAb to human cyclin E was provided by Emma Lees (DNAX Corp., Palo Alto, CA). Sheep anti-BrdUrd polyclonal antibody was from Fitzgerald (Chelmsford, MA), and a fluorescein isothiocyanate (FITC)-conjugated mAb to BrdUrd was from Becton Dickinson. Biotinylated horse anti-mouse IgG, FITC-conjugated antibodies to mouse and sheep IgG, and Texas red-conjugated streptavidin were from Vector Laboratories.

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Abbreviations: cdk, cyclin-dependent kinase; pRb, retinoblastoma protein; mAb, monoclonal antibody; CSF-1, colony-stimulating factor 1; CSF-1R, CSF-1 receptor.

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Cells and Culture Conditions. Mouse NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum, 2 mM glutamine, and 100 units each of penicillin and streptomycin per ml. Derivatives expressing wild-type and mutant CSF-1R (27) are neomycin-resistant. Cells expressing CSF-1R 809F were cotransfected (37) with expression plasmids (15 μg) encoding cyclins D1, D2, D3, or their mutants, together with 5 μg of pJ6Ω-puro, and, after selection for 3 weeks with 7.5 μg of puromycin per ml, were maintained in culture with G418 (400 μg/ml) and puromycin (7.5 μg/ml). Replication-defective retroviral vectors encoding cyclin E, c-*myc* (In373), and cdk4 (K35M) were pseudotyped in 293T cells (a gift from David Baltimore, Massachusetts Institute of Technology) (34), and harvested virions were used to infect cells expressing CSF-1R or their derivatives. Infections were performed with unfrozen virus stocks at multiplicities >1 per cell, and cells were assayed for protein expression or CSF-1 responsiveness 72 h postinfection.

Polyclonal populations derived from virus-infected cells or from hundreds of puromycin- and G418-resistant colonies were seeded into semisolid medium (27, 38) in the absence or presence of 0.1 μg of purified human recombinant CSF-1 per ml (provided by Steve Clark, Genetics Institute, Cambridge, MA). Colonies derived from single cells were expanded and retested for CSF-1-dependent growth, reanalyzed for cyclin D expression, and assayed for their ability to proliferate in chemically defined medium containing human recombinant CSF-1 as the only added growth factor (24). Cells bearing wild-type CSF-1R and "rescued" clones expressing CSF-1R 809F and D-type cyclins proliferated at similar rates, with a doubling time of ≈22 h. For studies of synchronous S phase entry, cells grown to confluence for 2 days were starved for 18 h in medium containing 0.1% fetal calf serum, trypsinized, and replated at 60% confluence in either chemically defined medium containing CSF-1 or in DMEM plus 10% fetal calf serum. Entry into S phase was monitored by flow cytometric analysis of DNA content (9) and by analysis of BrdUrd incorporation into DNA (2).

Microinjection Studies. Quiescent cells on coverslips (60% confluence) were microinjected at pressures between 50 and 150 hPa (AIS Zeiss settings: angle "45," speed "10," and time "0.0" sec) (39) in 3.5-cm Petri dishes containing 3 ml of carbonate-free DMEM to avoid a decrease in pH. Immediately after injection, cells were stimulated with recombinant CSF-1 in serum-free medium containing BrdUrd (100 μM), and its incorporation into DNA was monitored 24 h later. Coverslips were rinsed in phosphate-buffered saline (PBS), fixed 10 min at −20°C in methanol:acetone (1:1), washed three times with PBS, and processed for immunofluorescence (15). Mouse mAbs to cyclin D1 were detected with biotinylated horse antibody to mouse IgG and Texas red-conjugated streptavidin, whereas BrdUrd was detected with a 1:50 dilution of sheep anti-BrdUrd followed by FITC-conjugated anti-sheep IgG or directly with undiluted FITC-mAb to BrdUrd. Counterstaining for DNA was performed by adding 1 μg of bisbenzimide (Hoechst 33258) per ml to the final PBS wash. The percent inhibition of BrdUrd incorporation was calculated as $N - I/N \times 100$, where N is the percent BrdUrd incorporation into 100 injected cells and I is the percent incorporation into 100 cells injected with mAb or control IgG. The numerical value is independent of experimental variations in the number of noninjected BrdUrd-positive cells.

Analytic RNA and Protein Analyses. Total cellular RNA was extracted (40), electrophoretically separated on agarose gels containing formaldehyde, transferred to nitrocellulose, and hybridized for 18 h with uniformly labeled c-*myc*, cyclin D1, or actin cDNA probes in buffer containing 0.6 M NaCl, 50% formamide, and 10% dextran sulfate (27). Filters were washed by step-wise increases in stringency, culminating in a final wash at 50°C in 0.02 M NaCl. All radiographic exposure times were 65 h. For metabolic labeling, 1×10^6 cells were starved in methionine-free medium for 30 min and cultured for 30 min with 200 μCi of Tran^{35}S-label per ml (1000 Ci/ml; 1 Ci = 37 GBq; ICN). Immunoprecipitated proteins from cell lysates were separated on denaturing polyacrylamide gels and visualized by autoradiography (41). Immunoblotting (42) was performed by using lysates (1×10^6 cells per sample) precipitated with rabbit antiserum to cyclin D1. Immune complexes were electrophoretically separated on denaturing gels, transferred to nitrocellulose, and blotted with mAb 72-13G-11 followed by ^{125}I-labeled protein A (Amersham). For visualization of cyclin E, lysates were separated directly on gels without prior immunoprecipitation.

RESULTS

D-Type Cyclins But Not Cyclin E Rescue Mitogenicity. NIH 3T3 fibroblasts expressing CSF-1R 809F did not form colonies in agar when stimulated by CSF-1 (Fig. 1 *A* and *B*), but those engineered to constitutively overexpress D-type cyclins grew in semisolid medium (Fig. 1 *C* and *D*) and proliferated continuously in serum-free liquid medium containing human recombinant CSF-1 (see *Materials and Methods*). Polyclonal populations formed colonies at frequencies approaching those obtained with *myc*-rescued cells (Table 1). Proliferating NIH 3T3 cells primarily express cyclin D1 (2), but the levels of exogenous cyclin D1 synthesis in transfected cells growing in serum-containing medium were 8- to 10-fold higher than those of the endogenous protein (Fig. 2*A*); equally high levels of ectopic expression of cyclins D2 and D3 were obtained (Fig. 2 *B* and *C*). Colonies picked at random, when grown up and reanalyzed, each expressed high levels of cyclin D (Fig. 2 *D* and *E*). All such subclones could be propagated in serum-free medium containing CSF-1 and formed colonies in agar at

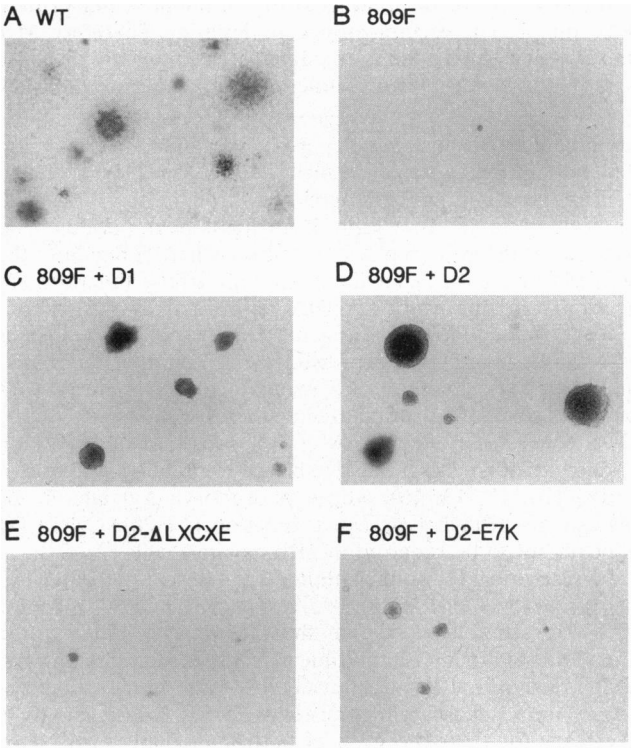

FIG. 1. Colony formation in semisolid medium. Cells expressing wild-type (WT) (*A*) or mutant (*B–F*) CSF-1R without (*B*) or with (*C–F*) the indicated cyclins were plated in agar containing CSF-1, and subsequent colonies were enumerated (Table 1) and photographed 3 weeks later. (*A–F*, ×40.)

Table 1. Colony formation in semi-solid medium

Cell line	Colony-forming efficiency, %
CSF-1R (wild-type)	15.0 ± 6.0
+ *myc*	21.5 ± 3.7
+ *myc* (In373)	5.9 ± 2.9
+ E	14.4 ± 4.8
+ D1	12.9 ± 3.4
+ cdk4	11.0 ± 3.5
+ cdk4 (K35M)	4.5 ± 0.9
CSF-1R (809F)	<0.1
+ *myc*	12.2 ± 2.7
+ D1	8.2 ± 3.4
+ D2	11.1 ± 4.5
+ D3	9.7 ± 3.7
+ D2 (E7K)	1.6 ± 1.5
+ D2 (ΔLXCXE)	0.9 ± 0.7
+ E	<0.1
+ D1 & *myc*	23.7 ± 3.2
+ D1 & *myc* (In373)	3.2 ± 1.4
+ D1 & E	9.2 ± 2.6
+ cdk4	<0.1
+ cdk4 (K35M)	<0.1
+ D1 & cdk4	10.9 ± 1.9
+ D1 & cdk4 (K35M)	3.7 ± 1.4

Cells expressing the indicated genes were seeded at 5000 cells per ml per plate into 35-mm diameter culture dishes in medium containing human recombinant CSF-1, and colonies were enumerated 3 weeks later. No colonies were observed in the absence of CSF-1. Those arising in cells transfected with cyclin D LXCXE mutants (Fig. 1) or in cells infected with either dominant-negative c-*myc* (In373) or cdk4 (K35M) mutants were characteristically much smaller than those obtained with cyclin D or c-*myc* alone. Representative colonies arising from cells expressing D-type cyclins expressed high levels of the proteins (Fig. 2) and formed CSF-1-dependent colonies in agar at ≈50% greater efficiencies when replated.

efficiencies about 50% greater than those of uncloned, parental transformants. Cyclin D synthesis was readily detected in growth factor-deprived transformants and at high levels throughout the cell cycle (Fig. 2*F*), and the cells expressed greatly elevated levels of cyclin D-dependent pRb kinase activity (Fig. 2*G*).

cdk4 interacts only with D-type cyclins (10, 16), and a catalytically inactive cdk4 mutant (K35M) was previously shown to reverse the phenotypic effects of cyclin D overexpression *in vivo*, where it presumably acts as a dominant-negative by sequestering cyclin D into inactive holoenzyme complexes (32). cdk4 (K35M) significantly inhibited colony formation in cells expressing wild-type CSF-1R and in CSF-1R 809F-bearing cells reconstituted by cyclin D1 (Table 1), verifying that CSF-1 responsiveness required the continuous presence of cyclin D.

Cells overexpressing cyclin D mutants disrupted in an N-terminal LXCXE pentapeptide necessary for pRb binding but not for cdk4 activation formed only few slowly growing colonies (Fig. 1 *E* and *F*; Table 1). Ectopic expression of the cyclin D1 ΔLXCXE mutant was equal to that of endogenous cyclin D1 (Fig. 2*A*), and the two biologically impaired mutant cyclin D2 proteins were produced in much greater amounts, approximating that of the ectopically expressed wild-type cyclin D2 gene product (Fig. 2*B*). Cells infected with a retrovirus encoding human cyclin E (1) also synthesized high levels of the corresponding protein (Fig. 2*H*, +E), as did representative single cell-derived subclones obtained by end point dilution (clones E1 and E2). During continuous passage in culture, the levels of ectopic cyclin E synthesis in the uncloned population increased gradually (Fig. 2*I*), implying

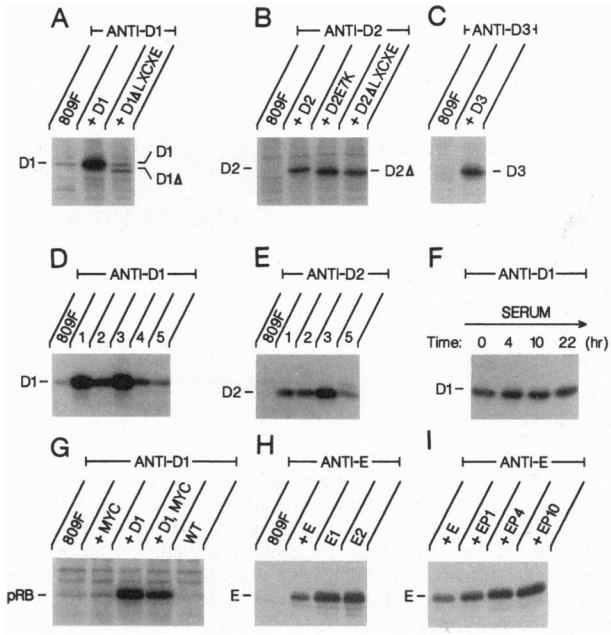

FIG. 2. Ectopic cyclin expression. Cells proliferating in DMEM and fetal calf serum and expressing CSF-1R 809F alone (809F) or together with the indicated cyclins were metabolically labeled and immunoprecipitated with mAbs directed against D-type cyclins (*A–F*). Results are shown with uncloned populations (*A–C*) and with numbered subclones derived from agar colonies (*D* and *E*). Cells transfected with the D1 ΔLXCXE mutant expressed a faster-migrating form of the protein (*A*), whereas the differences in mobility are less apparent for the more abundantly expressed D2 mutants (*B*). *F* demonstrates that cyclin D1 synthesis is constitutive throughout the cell cycle. Less than 5% of starved cells (time 0) were in S phase, and stimulated cells entered S phase synchronously with 24% in S phase by 10 h and >80% completing the cycle by 22 h. Cyclin D1 immune complexes prepared from untransformed cells (lanes 809F and WT) or from those expressing the indicated genes were tested for associated pRb kinase activity (*G*). At the exposure shown (30 min), little pRb kinase activity can be observed in cells expressing CSF-1R alone, but somewhat higher levels are observed in cells overexpressing c-*myc*. Human cyclin E overexpression (*H* and *I*) in a polyclonal population (+E) and in two subclones (E1 and E2) was documented by immunoblotting, but endogenous rodent cyclin E was not detected. Cells at different passage numbers (*I*) accumulated more cyclin E but failed to form colonies in agar.

positive selection for cells overproducing the protein. These cells also exhibited a robust cyclin E-associated histone H1 kinase activity that was >10-fold that detected in nontransfected controls (data not shown). Neither the passaged cyclin E-infected cells nor their subclones formed colonies in agar in response to CSF-1 (Table 1), and unlike cyclin D transformants, they did not proliferate in serum-free medium containing the recombinant growth factor.

Collaboration Between Cyclin D1 and c-*myc*. Because both cyclin D1 and c-*myc* rescued the CSF-1 response, we attempted to determine whether their activities were interdependent. Fibroblasts expressing wild-type CSF-1R or CSF-1R 809F were grown to confluence, starved in 0.1% serum, and then replated and stimulated either with fresh medium containing 10% serum or with serum-free medium containing human recombinant CSF-1. Both c-*myc* and cyclin D1 mRNA expression were induced by serum, but while cells expressing wild-type receptor responded to CSF-1 (Fig. 3*A*), those bearing CSF-1R 809F exhibited only weak transient induction of both mRNAs (Fig. 3*B*). More than 80% of cells expressing wild-type CSF-1R entered S phase in the first cycle after CSF-1 stimulation. In contrast, less than 9% of stimulated cells expressing CSF-1R 809F incorporated BrdUrd or expressed nuclear

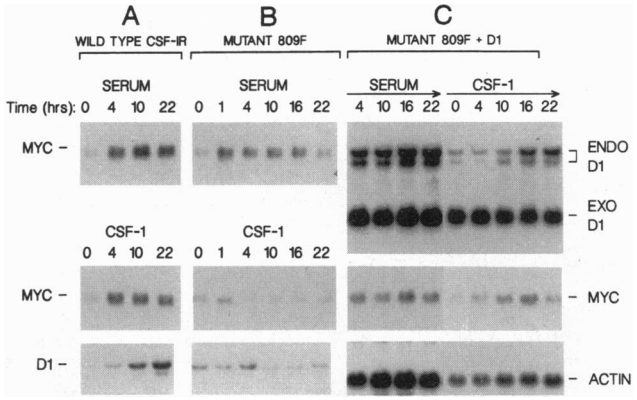

FIG. 3. Reconstitution of c-*myc* mRNA expression in cells overexpressing cyclin D1. Quiescent cells expressing wild-type (*A*) or mutant (*B*) receptors (time 0) were restimulated with serum (*Upper*) or CSF-1 (*Lower*) to reenter the cycle. RNA (10 μg) extracted at the indicated times was separated on gels, transferred to nitrocellulose, and hybridized sequentially with a c-*myc* and cyclin D1 probe. RNA (25 μg) from cyclin D1-rescued cells expressing CSF-1R 809F was subjected to a similar analysis (*C*), and an actin probe was used to control for RNA loading.

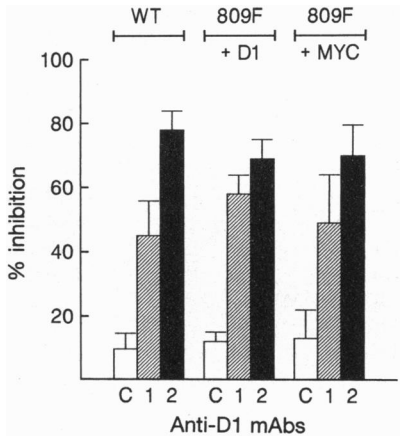

FIG. 4. Inhibition of S phase entry by microinjected antibodies to cyclin D1. Quiescent cells expressing wild-type (WT) or mutant receptors (809F) and reconstituted with either cyclin D1 (+D1) or c-*myc* (+MYC) were injected with control IgG (bars C) or with anti-D1 mAbs 13G-72-11 (bars 1) or DCS6 (bars 2). Injected cells were restimulated to enter the cell cycle with chemically defined medium containing recombinant CSF-1 and BrdUrd. BrdUrd incorporation into DNA was measured 24 h later. The values for percent inhibition represent averages from three independent experiments.

cyclin D1 (data not shown), with <0.1% of the latter yielding colonies in agar (see Table 1). Although c-*myc* and cyclin D1 are not appreciably expressed in quiescent cells (9, 27), cyclin D1 mRNA and protein synthesis were restored in CSF-1R 809F-bearing cells reconstituted with c-*myc* in a manner indistinguishable from that seen in cells expressing wild-type CSF-1R (see Fig. 3*A*; data not shown). Conversely, c-*myc* was induced by CSF-1 in cells forced to produce cyclin D1 (Fig. 3*C*). In four independent experiments, the maximal levels of c-*myc* mRNA induced in the first cycle in response to CSF-1 were 63% ± 17% lower than those obtained after serum treatment, when corrected for RNA loading by the use of a control actin probe. Nonetheless, both populations entered S phase at similar rates as judged by BrdUrd labeling (data not shown). Because the exogenous cyclin D1 mRNA species lacks most of its 3′ untranslated region, it was readily distinguished from endogenous cyclin D1 mRNAs (Fig. 3*C*). Constitutive expression of the exogenous cyclin D1 gene also restored growth factor-induced synthesis of endogenous cyclin D1 mRNA (Fig. 3*C*).

To determine if c-*myc*-rescued cells still depended upon cyclin D1 function for entry into S phase, quiescent cells were microinjected with antibodies to cyclin D1 and restimulated with recombinant CSF-1 in serum-free medium to reenter the cell cycle. BrdUrd was added with CSF-1, and its incorporation into DNA was determined 24 h later. Two monoclonal antibodies to cyclin D1 prevented S phase entry in cells bearing wild-type receptors, as well as in those rescued by either cyclin D1 or c-*myc*, whereas control IgG was without significant effect (Fig. 4).

It is unlikely that cyclin D1 simply acts downstream of Myc to bypass its function(s). Cotransfection of cyclin D1 and c-*myc* into cells expressing CSF-1R 809F reproducibly yielded a higher frequency of CSF-1-dependent colonies than either gene alone, indicating that their effects were additive (Table 1). Furthermore, introduction of a *myc* dominant-negative mutant into cyclin D1-rescued cells significantly inhibited colony formation (Table 1). Similar results were obtained when a vector encoding the Max dimerization partner Mad but not a Mad mutant incapable of associating with Max was introduced into these cells (M.F.R., D. E. Ayer, and R. N. Eisenman, unpublished data). Cyclin D1 and c-*myc* must either act in parallel cross-communicating pathways, or alternatively, Myc function may be required both upstream and downstream of cyclin D1 for cells to proliferate continuously.

DISCUSSION

D-type cyclins act as growth factor sensors to integrate extracellular signals with the cell cycle machinery (43). Synthesized as delayed early genes in response to treatment of quiescent cells with mitogens, their continued synthesis depends on growth-factor stimulation regardless of the cell's position in the cycle. Withdrawal of growth factors during G_1 phase leads to rapid cyclin D degradation correlating with the failure of cells to enter S phase, but its degradation after G_1/S has no effect on progression through the remainder of the cycle (9). Overexpression of D-type cyclins in fibroblasts shortens their G_1 interval, whereas microinjection of antibodies to cyclin D1 can prevent entry into S phase, as long as injections are performed prior to the G_1/S transition (2, 15). Therefore, cyclin D function is required during G_1 but is nonessential once cells commit to enter S phase. The requirement for cyclin D1 is dispensable in cells lacking pRb function (21–23), implying that pRb is the critical substrate of cyclin D-dependent kinases and that pRb phosphorylation facilitates G_1 exit.

The cyclin D1 gene responds to cooperating signals relayed via multiple CSF-1R-induced pathways. In NIH 3T3 cells expressing CSF-1R 809F, immediate early response genes, such as *fos* and *jun*, whose inductions are unimpaired, are insufficient for proper D1 expression. The c-*myc* response is attenuated and unsustained in these cells, but forced *myc* expression restores cyclin D1 synthesis and the cell's proliferative response to CSF-1. Yet, in c-*myc*-rescued cells, cyclin D1 induction remains CSF-1 dependent, so other CSF-1 responsive genes must also contribute. The c-*myc* and cyclin D1 genes are sequentially induced during the normal CSF-1 response, with cyclin D1 mRNA levels increasing in the face of maximal c-*myc* induction. Expression of inducible Myc proteins has been reported to positively (44) or negatively (45, 46) regulate the cyclin D1 promoter, or to have no effect on c-*myc* mRNA production while inducing cyclin D1 protein posttranscriptionally (47), so the manner by which ectopic c-*myc* expression contributes to D1 induction in cells expressing CSF-1R 809F remains unclear.

In turn, cyclin D1-mediated rescue of CSF-1-dependent mitogenicity restores c-*myc* expression, albeit with a lesser induction of c-*myc* mRNA in the first cycle after stimulation as compared with that realized after serum treatment. Induction of endogenous cyclin D1 mRNA is also restored, and the

rescued cells enter S phase with similar kinetics after serum or CSF-1 stimulation. Induction of c-*myc* by cyclin D1 might depend on an interaction of the cyclin D–cdk4 complex with pRb or other pRb-related proteins, thereby releasing pRb-tethered transcription factors, such as E2F, that are capable of activating c-*myc* transcription (48). Consistent with this hypothesis, cyclin D mutants disrupted in an LXCXE motif that facilitates pRb binding were significantly impaired in rescuing the CSF-1 proliferative response.

The activities of both cyclin D1 and Myc are required for reconstitution of the CSF-1 response in cells bearing CSF-1R 809F, although either alone is capable of rescue. Microinjection of antibodies to cyclin D1 inhibited CSF-1-dependent S phase entry in *myc*-reconstituted cells, whereas a dominant-negative *myc* gene interfered with D1-mediated rescue. Moreover, cotransfection of cells bearing CSF-1R 809F with both cyclin D1 and c-*myc* had an additive effect on their ability to form CSF-1 dependent colonies. In principle, the two genes could act in parallel pathways, with cross-talk contributing to the transcriptional regulation of one gene by the other. Cyclin D1 can collaborate with mutant *ras*, but not c-*myc*, in transforming fibroblasts, suggesting that D1 might replace certain c-*myc* functions (49, 50). However, cyclin D1 also synergizes with c-*myc in vivo* to accelerate lymphomagenesis in mice bearing both transgenes (51, 52). Although both cyclin D and Myc are rate-limiting governors of G_1 progression, Myc may also be required later in the cell cycle. In pre-B lymphocytes reconstituted with epidermal growth factor (EGF) receptors, EGF treatment enabled them to enter S phase but not to complete DNA synthesis; the latter deficiency was complemented by c-*myc* (53). These data argue against the idea that Myc solely functions upstream of cyclin D to drive cells through their division cycle.

Each of the D-type cyclins rescued CSF-1 induced mitogenicity in cells expressing the mutant receptor, even though cyclin D3 expression is not normally detected in these cells and the level of D2 synthesis is ordinarily much lower than that of D1. In marked contrast, high levels of ectopic cyclin E expression failed to reconstitute CSF-1 responsiveness. Overexpression of cyclin D1 or E in fibroblasts shortens their G_1 interval by about 30% (1–3), but the effects of their induced coexpression are additive, and only D1 induction leads to rapid pRb phosphorylation (54). Although microinjection of antibodies to cyclin D1 is without effect in pRb-negative cells (21–23), antibodies to cyclin E still prevent S phase entry in this setting (55). Evidently, the cyclin E-cdk2 complex must phosphorylate physiologic substrates other than pRb to regulate a distinct cell cycle transition.

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