Direct induction of cyclin D2 by Myc contributes to cell cycle progression and sequestration of p27

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Ectopic expression of Myc induces Cdk2 kinase activity in quiescent cells and antagonizes association of p27^{kip1} with Cdk2. The target gene(s) by which Myc mediates this effect is largely unknown. We now show that p27 is rapidly and transiently sequestered by cyclin D2–Cdk4 complexes upon activation of Myc and that cyclin D2 is a direct target gene of Myc. The cyclin D2 promoter is repressed by Mad–Max complexes and de-repressed by Myc via a single highly conserved E-box element. Addition of trichostatin A to quiescent cells mimics activation of Myc and induces cyclin D2 expression, suggesting that cyclin D2 is repressed in a histone deacetylase-dependent manner in quiescent cells. Inhibition of cyclin D2 function in established cell lines, either by ectopic expression of p16 or by antibody injection, inhibits Myc-dependent dissociation of p27 from Cdk2 and Myc-induced cell cycle entry. Primary mouse fibroblasts that are cyclin D2-deficient undergo accelerated senescence in culture and are not immortalized by Myc; induction of apoptosis by Myc is unimpaired in such cells. Our data identify a downstream effector pathway that links Myc directly to cell cycle progression.

Keywords: Cdk2/cyclin D2/Mad-1/Myc/p27

Introduction

The proto-oncogene *c-myc* encodes a transcription factor of the helix–loop–helix/leucine zipper family of proteins (for review, see Henriksson and Lüscher, 1996). The encoded protein, Myc, binds to specific sequences on DNA [so-called E-boxes with a central CAC(G/A)TG motif] as part of a heterodimeric complex with a partner protein, Max, and activates transcription. Max also forms heterodimers with a second group of related proteins, termed Mad-1, Mxi-1, Mad-3, Mad-4 and Mnt/Rox (Ayer *et al.*, 1993; Zervos *et al.*, 1993; Hurlin *et al.*, 1996, 1997; Meroni *et al.*, 1997). These complexes bind to identical

E-box elements, but repress transcription, at least in part, via recruitment of histone deacetylase activity (Alland *et al.*, 1997; Heinzel *et al.*, 1997; Laherty *et al.*, 1997; Sommer *et al.*, 1997). In mammalian cells, the relative levels of Myc and Mad proteins can determine the rate of cell proliferation and apoptosis (for review, see Bouchard *et al.*, 1998). For example, ectopic expression of Myc or activation of conditional alleles of Myc (MycER) (Eilers *et al.*, 1989; Littlewood *et al.*, 1995) can induce quiescent cells to enter the cell cycle and undergo apoptosis (Eilers *et al.*, 1991; Evan *et al.*, 1992). Conversely, ectopic expression of Mad-1 prevents growth factor-induced proliferation of fibroblasts (Roussel *et al.*, 1996). It is most likely, therefore, that this network of interacting proteins regulates a set of target genes important in controlling cell proliferation.

One downstream target of Myc activity is the cell cycle inhibitor p27^{kip1} and the related p21^{waf-1} protein. Several lines of evidence support this notion. First, activation of conditional alleles of Myc in quiescent fibroblasts rapidly induces cyclin E–Cdk2 kinase activity and loss of p27 from Cdk2 complexes (Steiner *et al.*, 1995; Müller *et al.*, 1997). Myc also up-regulates Cdk2 kinase activity at any point during the cell cycle of exponentially growing fibroblasts (Pusch *et al.*, 1997), demonstrating that the effects of Myc on Cdk2 are independent of its ability to stimulate quiescent cells to enter the cycle. Activation of Cdk2 is required for Myc to promote G₁ progression (Rudolph *et al.*, 1996). Secondly, expression of dominant-negative alleles of Myc arrests cell proliferation and suppresses cyclin E–Cdk2 activity (Berns *et al.*, 1997). Expression of cyclin E and Cdk2 is unimpaired in the arrested cells, suggesting that Myc function is required at a step after cyclin E synthesis. In fibroblasts carrying a homozygous null mutation of Myc, levels of p27 are elevated and cyclin E-dependent kinase activity is reduced (Mateyak *et al.*, 1999). Thirdly, ectopic expression of Myc partially antagonizes the growth suppressive activities of p27 (Vlach *et al.*, 1996); however, p27 is dominant when expressed at high concentration, demonstrating that Myc does not bypass p27 function (Rudolph *et al.*, 1996; Müller *et al.*, 1997). Ectopic expression of Myc also suppresses the p21-dependent growth arrest of temperature-sensitive alleles of p53 (Wagner *et al.*, 1994; Hermeking *et al.*, 1995). In both cases, it was suggested that Myc induces the ‘sequestration’ of the inhibitor into a heat-labile complex. The identity of this complex, however, remained unknown.

In order to identify Myc-induced p27-binding proteins, we began to purify p27-containing complexes from RAT1 fibroblasts that express a MycER protein. During the course of purification, we noticed that activation of Myc induces a rapid increase in the amount of cyclin D2–Cdk4–p27 complexes. We now provide evidence that

cyclin D2 is a direct target gene of Myc and that accumulation of cyclin D2 contributes to the sequestration of p27 that occurs in Myc-transformed cells.

Results

In order to purify Myc-induced p27-containing complexes, density-arrested RAT1-MycER cells were stimulated by the addition of 200 nM 4-hydroxy-tamoxifen (4-OHT) and extracts were prepared 8 h later. At this time point, p27 is largely dissociated from Cdk2 complexes but not yet degraded (Müller *et al.*, 1997). Upon gel filtration of either non-induced or induced extracts, p27 eluted as a single peak with a molecular weight of ~160 kDa, co-migrating with both cyclins E and D1 and with part of Cdk2 (shown for induced extracts in Figure 1A). In contrast, recombinant p27 eluted with a size of 60 kDa, suggesting that it forms a dimer. Also, a brief heat-treatment of cellular extracts caused p27 to migrate with the same molecular weight (data not shown). Thus, in RAT1 cells p27 is sequestered in heat-labile complexes before and after activation of Myc, suggesting that activation of Myc induces a redistribution of p27 among such complexes (Vlach *et al.*, 1996).

An immunodepletion procedure was used to remove known binding partners of p27. Extracts were depleted three times by a mixture of α -Cdk2, α -cyclin D1, α -cyclin D2 and α -cyclin D3 antibodies that had been covalently coupled to protein A-Sepharose. Controls showed that cyclin E, Cdk2, cyclins D1, D2 and D3 were each reduced by 90–95% in the depleted extracts relative to input (Figure 1B). Cyclin A was reduced by 50%, presumably because it can also form complexes with Cdc2 (Draetta *et al.*, 1989). As p27 interacts only weakly with Cdc2 complexes, we assume that this will not affect the results (Polyak *et al.*, 1994; Toyoshima and Hunter, 1994).

Depleted and non-depleted extracts were probed for the amount of p27 by Western blotting together with calibrated amounts of recombinant p27. The blots were developed by an enhanced chemiluminescence protocol and different exposures were scanned and quantitated. A representative Western blot is shown in Figure 1C, and the quantitation of three independent experiments is depicted in Figure 1D. Depletion of the known binding partners of p27 depleted >90% of cellular p27 both from induced and non-induced extracts. Fractionation revealed that the remaining p27 co-migrated with the bulk of p27 from non-depleted extracts (Figure 1A). Therefore, we did not obtain any evidence for an as yet unknown Myc-induced complex containing p27. While we cannot exclude the possibility that the remaining 10% of non-depleted p27 contains novel Myc-induced p27-binding proteins, the low levels of cyclin proteins remaining after depletion can also account for this amount of p27.

Since p27 dissociates from Cdk2 and we did not observe accumulation of cyclin D1- or cyclin D3-containing complexes (see Figure 2C) (Müller *et al.*, 1997), we repeated the depletion experiment omitting the α -cyclin D2 antibodies from the depletion mixture (Figure 1C and D). Under these conditions, 90% of p27 was depleted from the non-induced, but only 50% from the induced extracts, suggesting the presence of significant amounts of cyclin D2–p27 complexes in the induced extracts. We

concluded that activation of Myc promotes the formation of cyclin D2–p27 complexes.

Upon activation of Myc, p27 dissociates from Cdk2 within 4–6 h (Müller *et al.*, 1997). To assess whether the formation of cyclin D2–p27 complexes could account for this rapid dissociation, time course experiments were performed. Extracts were prepared from quiescent RAT1-MycER cells before, or at different time points after activation of Myc. At each time point, the amount of cyclin D2, cyclin A, Cdk2, p27, p27–Cdk2 and p27–cyclin D2 complexes was determined (Figure 2A).

Consistent with previous results, activation of Myc induced a rapid dissociation of p27 from Cdk2, which preceded overt degradation of p27 by several hours. In response to activation of Myc, levels of cyclin D2 and cyclin D2–p27 complexes strongly increased within 3 h and peaked between 6–9 h after addition of 4-OHT. Quantitation of the results showed that up to 20% of cellular p27 complexed with cyclin D2 after activation of Myc in this experiment and that the maximal amount of p27 bound to cyclin D2 exceeded the amount of p27 initially bound to Cdk2 (Figure 2B). We concluded that complex formation with cyclin D2 could account for the dissociation of p27 from Cdk2 after activation of Myc. Induction of cyclin D2 was transient and protein levels dropped in late G₁; a similar decrease has been observed previously in exponentially growing human cells (see also Figure 8) (Lukas *et al.*, 1995).

In several time course experiments, we noted a small but significant delay between the accumulation of the cyclin D2 protein and loss of p27 from Cdk2 (see time point at 3 h and also Figure 6). It is possible, therefore, that the apparent shift of p27 from Cdk2 to cyclin D2 results from binding of newly synthesized p27 to enhanced levels of cyclin D2 and loss of p27 from Cdk2 by turnover or degradation rather than from direct transfer of pre-existing p27 molecules from Cdk2 to cyclin D2. This is consistent with previous observations that mutants of p27 that cannot be phosphorylated by Cdk2, and therefore are not degraded, dissociate more slowly than wild-type p27 from Cdk2 after induction of Myc (Müller *et al.*, 1997).

In parallel experiments, we determined the change in each D-type cyclin–p27 and Cdk4–p27 complex (Figure 2C) and the amount of Cdk4 bound to different D-type cyclins before and after activation of Myc (data not shown). The amount of both cyclin D2 and Cdk4 bound to p27 increased in response to induction of Myc; in contrast, the amount of p27 bound to either cyclin D1 or D3 remained constant (Figure 2C; see also Müller *et al.*, 1997). Consistent with these findings, we observed a 3- to 4-fold increase in cyclin D2 bound to Cdk4 (data not shown); 30% of total Cdk4 was bound to cyclin D2 after induction of Myc. The remaining Cdk4 was bound to cyclin D1 (30% of total Cdk4) and to cyclin D3 (40%); the absolute amounts of these latter complexes remained constant during induction (data not shown). We conclude that the formation of ternary p27 cyclin D2–p27–Cdk4 complexes upon activation of Myc significantly contributes to sequestration of p27.

To assess whether induction of cyclin D2 by Myc occurs at the RNA level, total RNA was isolated from serum-starved murine fibroblasts that express a MycER protein and subjected to Northern blot analysis using a

Fig. 1. Analysis of Myc-induced p27 complexes. Extracts were prepared before and 8 h after addition of 200 nM 4-OHT to density-arrested RAT1-MycER cells. (A) Gel filtration analysis of cyclins E and D1, Cdk2 and p27 from induced RAT1-MycER cells. The position of marker proteins is indicated above the fraction number. The pool of small molecular weight Cdk2 indicates the presence of monomeric Cdk2 in these cells. His-tagged recombinant p27 was purified as described (Müller *et al.*, 1997). (B) Immunodepletion experiments. Western blots of extracts from induced and non-induced cells probed with the indicated antibodies before and after three rounds of depletion with a mixture of α -cdk2, α -cyclin D1, α -cyclin D2 and α -cyclin D3 antibodies. (C) α -p27 immunoblot of induced and non-induced extracts after depletion with a mixture of antibodies [as in (B)] either with or without α -cyclin D2 antibodies. As controls, aliquots of the non-depleted extracts ('input') and calibrated amounts of recombinant p27 were also blotted. (D) Quantitation of the amount of p27 in different extracts. The graph shows an average of three independent experiments (two experiments in the case of extracts depleted with a complete mixture of antibodies).

cloned murine cyclin D2 cDNA as probe. Expression of GAPDH was monitored as control (Figure 3A and C). Cyclin D2 was expressed at barely detectable levels in serum-starved cells and accumulated after stimulation with serum (data not shown) or addition of 4-OHT.

To determine whether cyclin D2 is directly induced by Myc, 4-OHT was added in the presence of 50 μ g/ml cycloheximide, which reduced protein synthesis by 97% (Figure 3B). Under these conditions, MycER proteins are degraded with a half life of ~30 min (M.Eilers, unpublished results). Consistent with a direct induction of cyclin D2 by Myc, addition of cycloheximide did not inhibit induction of cyclin D2 at early time points after induction (Figure 3C; the Northern blot is exposed longer to visualize the low

amounts of cyclin D2 mRNA present in resting cells). In contrast to cells induced in the absence of cycloheximide, no further induction of cyclin D2 mRNA was observed at time points later than 4 h after induction when MycER protein is no longer detectable. Addition of cycloheximide by itself did not affect cyclin D2 mRNA expression. A quantitation of an independent experiment in which parallel samples were induced either in the presence or absence of cycloheximide is shown in Figure 3D. Together, the data show that no intermediate proteins need to be synthesized that mediate activation of cyclin D2 expression by Myc, and that cyclin D2 is a direct target gene of Myc.

The human, rat and murine cyclin D2 promoters have been cloned; each promoter contains two high affinity

Fig. 2. Time course of cyclin D2-p27 complex formation. (A) Western blots of the proteins indicated and complexes after activation of MycER in RAT1-MycER cells. (B) Quantitation of cyclin D2-p27 and Cdk2-p27 complexes after activation of Myc. The results are shown relative to the amount of total p27 present at the start of the experiment. (C) Accumulation of cyclin D2-Cdk4 complexes. Western blots of the proteins indicated and complexes before and 8 h after activation of MycER (left and middle panels) and a quantitation of the results (right panel). Note the accumulation of both cyclin D2-p27 and Cdk4-p27 complexes.

Myc/Max-binding sites (Brooks *et al.*, 1996; Jun *et al.*, 1997; Yang *et al.*, 1997). One is conserved at -1200 base pairs (bp) relative to the start site of translation in all promoters and a second is located at -600 bp in the rodent promoters and -1000 bp in the human promoter (Figure 4A).

RAT1 cells are not easily amenable to transient transfections. Therefore, transient transfection experiments were performed in NIH 3T3 cells to assess whether Myc, Max or Mad proteins can regulate the murine cyclin D2 promoter (Figure 4B-F). Ectopic expression of neither Max, Myc (data not shown) nor Myc and Max together significantly affected promoter activity (Figure 4B). In contrast, ectopic expression of Mad-1 repressed the cyclin D2 promoter; repression by Mad-1 was further enhanced by co-expression of Max, suggesting that complexes of Mad-1 with Max can repress the cyclin D2 promoter. Consistent with this view, disruption of the leucine zipper of Mad-1, which is required for dimerization with Max, strongly diminished its ability to repress the cyclin D2 promoter (Figure 4C). Efficient repression by Mad-1 also required the integrity of an N-terminal domain which interacts with the Sin3 protein, suggesting that recruitment of Sin3 is required for repression (Ayer *et al.*, 1995; Schreiber-Agus *et al.*, 1995).

In order to determine whether repression by Mad-1 requires direct binding to the cyclin D2 promoter, reporter constructs were generated in which the E-boxes had been individually mutated to CGCGTG instead of CACGTG sequences (Figure 4A); *in vitro* bandshift assays showed

that the mutation severely impaired binding by Max proteins (data not shown). These experiments revealed that the E-boxes were not equivalent (Figure 4D). Disruption of the conserved E-box at -1200 (mut3) essentially abolished repression by Mad-1-Max complexes. In contrast, disruption of the more proximal E-box (mut4) did not significantly affect repression by Mad; we concluded that repression of the cyclin D2 promoter by Mad requires direct binding of a Mad-Max complex to the more distal E-box element.

While ectopic expression of Myc did not affect promoter activity by itself, Myc was able to partly revert repression by Mad-1-Max complexes; in contrast, ectopic expression of USF, another E-box binding transcription factor (Sawadogo *et al.*, 1988) did not relieve repression by Mad-1 (Figure 4E). De-repression by Myc did not depend on the integrity of the promoter-proximal E-box. Deletion of the leucine zipper or the helix-loop-helix domain of Myc abolished de-repression (Figure 4F). Furthermore, a mutant lacking 37 amino acids encompassing Myc-box II ($\Delta 106-143$) poorly de-repressed the cyclin D2 promoter; *in vivo*, ($\Delta 106-143$) MycER chimeras failed to activate the endogenous cyclin D2 gene (A.Kiermaier, unpublished results). Smaller mutants in the same region gave partial effects. In contrast, de-repression was normal in mutants lacking Myc-box I ($\Delta 45-63$). This behaviour closely parallels the ability of these mutants to activate cyclin E-Cdk2 kinase in quiescent cells when stably expressed as ER chimeras (Steiner *et al.*, 1995). The data also show

Fig. 3. Regulation of cyclin D2 mRNA expression by Myc. Northern blots documenting expression of cyclin D2 and of GAPDH in murine fibroblasts expressing a MycER protein. (A) Time course of cyclin D2 mRNA induction after addition of 200 nM 4-OHT to serum-starved cells. (B) Inhibition of protein synthesis by 50 μ g/ml cycloheximide as measured by incorporation of [³⁵S]methionine. (C) Induction of cyclin D2 mRNA by Myc is resistant to inhibition of protein synthesis. In all samples, cycloheximide was added to a final concentration of 50 μ g/ml a few minutes before addition of 4-OHT. One time point is shown for addition of cycloheximide alone (2 h); however, a complete time course was performed showing no induction of cyclin D2 mRNA. (D) Quantitation of cyclin D2 mRNA after addition of 4-OHT in the presence and absence of cycloheximide (CHX).

that de-repression is not simply due to competition between Myc and Mad-1 for access to Max.

The data suggest that activation of cyclin D2 expression in response to activation of MycER occurs by de-repression of a Mad–Max-mediated repression of the cyclin D2 promoter in quiescent cells. Repression of transcription by Mad-1 *in vivo* depends, at least in part, on the recruitment of histone deacetylase activity to target sites on DNA and can be inhibited by addition of the histone deacetylase inhibitor, trichostatin A (Laherty *et al.*, 1997; Sommer *et al.*, 1997).

We therefore tested whether addition of trichostatin A up-regulates expression of cyclin D2 in quiescent cells. Serum-starved mouse embryo fibroblasts were treated with trichostatin A and the expression of cyclin D2 was monitored. As controls, the extracts were probed for expression of Cdk4 and cyclins D3, D1 and A (data not shown). Addition of trichostatin A specifically up-regulated expression of cyclin D2, but not of other cyclins or Cdk4, in a dose- and time-dependent manner (Figure 5); similar results were obtained in RAT1 cells (data not shown). We concluded that cyclin D2 is repressed in a histone deacetylase-dependent and Myc-reversible manner in quiescent cells. Taken together, the data strongly suggest a role for Mad and Myc proteins upstream of cyclin D2 in quiescent cells.

Two sets of experiments were performed in order to obtain functional evidence that induction of cyclin D2 mediates sequestration of p27 in response to activation of Myc. First, RAT1-MycER cells were superinfected with

retroviruses encoding p16, as binding of p27 to cyclin D–Cdk4 complexes is blocked by p16 (Reynisdottir *et al.*, 1995). Individual MycER clones expressing p16 were isolated and propagated; such clones proliferated more slowly than wild-type cells both in the absence and presence of hormone, and showed delayed hyperphosphorylation of p130 in response to addition of serum (data not shown). Western blotting confirmed that the cells expressed moderate amounts of p16 (Figure 6, upper panel). Upon induction of Myc, cyclin D2 was induced with similar kinetics both in control and p16-expressing cells (Figure 6, lower left panel). In control cells, p27 was rapidly lost from Cdk2 complexes, as described before. In cells expressing p16, Cdk2–p27 complexes were significantly more stable after induction of Myc (Figure 6, lower middle panel). Kinase assays revealed that induction of Cdk2 kinase was delayed in cells expressing p16 upon activation of Myc (lower right panels). Similar results were obtained with a pool of MycER cells expressing p16, demonstrating that the failure to induce Cdk2 kinase activity was not due to clonal variation (data not shown). We concluded that ectopic expression of p16 inhibits Myc-induced dissociation of Cdk2–p27 complexes and induction of Cdk2 kinase activity.

Secondly, monoclonal antibodies known to ablate the function of cyclin D2, but not D1, were affinity-purified and microinjected into MycER cells (Lukas *et al.*, 1995); as control, equal amounts of affinity-purified, isotype-matched control antibodies were injected. For these experiments, we used a mouse 3T3 cell line stably expressing

a MycER chimera, as the available antibodies recognize mouse cyclin D2 more easily than rat cyclin D2 (Figure 7A). Similarly to RAT1 cells, all three D-type cyclins were induced upon addition of growth factors to serum-starved cells, but only cyclin D2 was induced upon activation of Myc. Cell cycle progression was monitored by staining with antibodies directed against cyclin A. Three independent experiments were performed (Figure 7B). In each experiment, we observed a significant inhibition of cyclin A induction by Myc when antibodies directed against cyclin D2, but not control antibodies, were injected. We concluded that cyclin D2 function is required for Myc to promote G₁ progression efficiently in established cell lines.

Previous work has shown that Myc is essential for the proliferation of primary mouse fibroblasts; in contrast, the phenotype of cyclin D2-deficient fibroblasts has not been characterized (Davis *et al.*, 1993; Sicinski *et al.*, 1996). Therefore, primary fibroblasts were isolated from cyclin D2-deficient mice and wild-type littermates; as controls, we used primary fibroblasts from cyclin D1^{-/-} mice. In culture, both cyclin D1^{-/-} and cyclin D2^{-/-} cells showed a reduced rate of proliferation relative to wild-type cells (Figure 8A); this phenotype was observed with fibroblasts from different embryos, demonstrating that it was not due to variation between individual litters (data not shown). In order to identify the reason for this defect more precisely, we measured the growth factor-dependence of DNA replication by determining the percentage of cells incorporating BrdU dependent on the concentration of fetal calf serum in the culture medium (Figure 8B). In both cyclin D^{-/-} and wild-type cells, the percentage of replicating cells was strictly dependent on the concentration of fetal calf serum; half-maximal incorporation was observed at ~2% fetal calf serum in all cell types. However, even at the highest concentration of serum, a significantly lower percentage of cyclin D2^{-/-} and cyclin D1^{-/-} cells incorporated BrdU relative to wild-type cells. The data suggest that the lower rate of proliferation of cyclin D^{-/-} cells is not due to a reduced sensitivity to serum growth factors.

Primary cells have a finite lifespan in culture and undergo senescence after a number of passages. In order to assess whether cyclin D2^{-/-} cells undergo premature senescence, we determined the percentage of senescent cells at each passage for wild-type and cyclin D^{-/-} cells by staining for acidic β -galactosidase, an established marker for senescent cells (Figure 8C) (Dimri *et al.*, 1995). Both cyclin D1^{-/-} and cyclin D2^{-/-} showed a markedly accelerated start of senescence, suggesting that accelerated senescence causes their lower rate of proliferation.

Ectopic expression of Myc prevents senescence and

immortalizes primary rodent fibroblasts. In order to test whether expression of Myc can bypass the accelerated senescence observed in cyclin D-deficient cells, we infected wild-type and cyclin D-deficient cells with either control viruses or retroviruses expressing human Myc. Controls using a GFP-expressing retrovirus showed that both cell types could be infected with comparable efficiency (data not shown). Resistant cells were selected and the plates inspected for several weeks. After 10–14 days, individual colonies emerged on wild-type cells infected with retroviruses expressing Myc, but few or no colonies were recovered in any of the other plates (Figure 9A, left panel; a quantitation of the results of three independent experiments is shown on the right). Similarly, plating of an equal number of resistant cells recovered after infection showed that both cyclin D2^{-/-} and cyclin D1^{-/-} cells were unable to proliferate after infection by Myc (data shown for cyclin D2^{-/-} cells in Figure 9B). We concluded that immortalization of primary cells by Myc requires the presence of both cyclins D1 and D2. All cell types showed signs of apoptosis when infected with retroviruses that express both human Myc and H2B-GFP (data not shown), suggesting that the ability of Myc to induce apoptosis does not require D-type cyclins.

We established cell lines from colonies emerging from wild-type cells infected with Myc; these lines were essentially immortal, as they could be cultured for many passages without signs of senescence (Figure 9C). Western blots using a human specific anti-Myc antibody (9E10; Evan *et al.*, 1985) documented that these cell lines expressed human Myc protein (Figure 9D). Cells immortalized by Myc, expressed elevated levels of cyclin D2 protein in a growth factor-independent manner, whereas the expression of cyclin D2 was lower and dependent on the presence of external growth factors in the primary cells before immortalization. The expression of cyclin D1 was identical in primary and Myc-immortalized cells. The data show that ectopic expression of Myc is sufficient to up-regulate cyclin D2 expression in primary mouse fibroblasts; induction of cyclin D2 is, therefore, not an artificial property of MycER chimeras. Similar observations have previously been published for established Balb/c-3T3 and RAT1 cells (Jansen-Dürr *et al.*, 1993; Vlach *et al.*, 1996).

Finally, we were curious whether up-regulation of cyclin D2 is sufficient to account for the mitogenic properties of Myc in established cell lines. To assess this, we used a retroviral infection protocol in which ectopic expression of Myc has been shown to confer enhanced resistance to the cell cycle inhibitors, p16 and p27 (Vlach *et al.*, 1996; Alevizopoulos *et al.*, 1997). Therefore, we asked whether cells ectopically expressing cyclin D2 show enhanced resistance to ectopic expression of p27 and p16.

Fig. 4. Regulation of the cyclin D2 promoter by Mad and Myc proteins. (A) The left drawing shows the murine, rat and human cyclin D2 promoters indicating the location and conservation of the two consensus E-box elements. The dashed E-box element located at -1000 in the rat and mouse promoter has the core sequence CACATG, which is a low-affinity Myc/Max binding site (Blackwell *et al.*, 1993). On the right, a scheme of the mouse promoter indicating which E-boxes are disrupted in the reporter constructs mut3 and mut4. (B) Co-expression of Mad and Max represses the murine cyclin D2 promoter. Results of transient transfection assays in NIH 3T3 with the indicated amounts (μ g) of CMV-driven expression plasmids and a murine cyclin D2 promoter reporter construct. (C) Repression by Mad-Max complexes requires the integrity of the Mad leucine zipper and N-terminal Sin3 interaction domain. Mad Δ LZ refers to a mutant lacking the leucine zipper, Mad Δ N to a mutant lacking the N-terminal Sin3-interaction domain (Sommer *et al.*, 1997). (D) Repression requires the integrity of E-box 3, but not E-box 4 in the murine cyclin D2 promoter. (E) Expression of Myc, but not USF, antagonizes repression of the cyclin D2 promoter by Mad. (F) Domains of Myc required for de-repression of the cyclin D2 promoter. The numbers indicate the amino acids deleted in each construct.

Fig. 5. Inhibition of histone deacetylases de-represses cyclin D2 expression in serum-starved cells. Western blots probed with the antibodies indicated of extracts from serum-starved mouse embryo fibroblasts after incubation for the indicated times with 50 ng/ml trichostatin A (TSA) or with the concentrations indicated of TSA for 24 h.

Fig. 6. Ectopic expression of p16 inhibits Myc-induced dissociation of p27 from Cdk2. The upper panels document expression of p16 and MycER protein in control RAT1-MycER cells and in a clone of RAT1-MycER cells infected with a retrovirus expressing p16. The lower panels document the amount of cyclin D2 protein (left), Cdk2-p27 complexes (middle) and Cdk2 kinase activity (right) in either control cells (upper row) or cells expressing p16 (lower row) at the times indicated after activation of MycER.

Two retroviruses were constructed: one expressing cyclin D2, the second expressing Cdk4 together with cyclin D2 using an internal ribosomal entry site (IRES) element. Western blots documented that cells infected with these retroviruses expressed the encoded proteins (Figure 10B). No cells were recovered when control cells were infected with a retrovirus encoding p16 (data not shown). In contrast, a few colonies were recovered in infections of cyclin D2 or cyclin D2-Cdk4-expressing cells. Cells expressing p16 proliferated very slowly relative to control cells, demonstrating that there is at best a marginal rescue of proliferation (Figure 10A). The rate of proliferation of cells expressing p27 either without or together with cyclin D2 or cyclin D2-Cdk4 was essentially identical (Figure 10A). We concluded that ectopic expression of cyclin D2 is not sufficient to overcome p16- and p27-induced arrests of proliferation.

Discussion

One hallmark of the *c-myc* proto-oncogene is its ability to induce cell cycle entry and progression of resting cells when expressed in a deregulated manner. Previous work has shown that activation of Myc rapidly up-regulates

Fig. 7. Cyclin D2 is required for Myc-induced G₁ progression. (A) Expression of cyclins D1, D2, D3 and Cdk4 after addition of either serum or 4-OHT to serum-starved mouse 3T3 fibroblasts expressing a MycER protein. Western blots of extracts prepared at the times indicated. (B) Antibodies directed against cyclin D2 inhibit Myc-induced G₁ progression. Mouse MycER cells were serum-starved for 24 h and injected with affinity-purified antibodies before addition of 4-OHT. Twenty-two hours later, cells were fixed and stained with anti-cyclin A antibodies and secondary anti-mouse antibody to visualize the injected cells. The percentage of cyclin A positive cells from three independent experiments is shown.

cyclin E-Cdk2 kinase and antagonizes association of p27 with Cdk2. We now extend these findings in three directions.

First, we have partially purified p27-containing Cdk complexes from RAT1-MycER cells at a time point briefly after activation of Myc when p27 is completely dissociated from Cdk2 but not yet degraded. At the level of resolution of these experiments (which we estimate to be ~5–10% of the total cellular pool of p27), cellular p27 is bound to

Fig. 8. Phenotypes of primary cyclin D1^{-/-} and D2^{-/-} mouse embryo fibroblasts and of cells from wild-type littermates. For each panel, cells were tested from at least two independent embryos (not shown). (A) Growth curve: 5×10^4 cells (passage 3) were seeded into 60-mm plates and the cell number of triplicate dishes was recorded each day. (B) Percentage of BrdU positive cells dependent on serum concentration at passage 3. (C) Percentage of cells staining positive for acidic β -galactosidase in dependence of passage number.

cyclin D–Cdk4 complexes under these conditions. No evidence was obtained for as yet unknown, novel p27-binding proteins that sequester p27 in response to activation of Myc. Moderate expression of p16 from a retrovirus led to a delayed dissociation of p27 from Cdk2 and a delayed activation of Cdk2 kinase activity in response to

activation of Myc, providing direct functional evidence that cyclin D–Cdk4 complexes contribute to sequestration of p27 in response to activation of Myc. We suggest that these observations explain the ability of p16 to inhibit cell cycle induction and transformation by Myc when expressed at sufficiently high levels (Serrano *et al.*, 1995; Rudolph *et al.*, 1996; Müller *et al.*, 1997). The findings are consistent with a model suggesting a general role for D-type cyclins as proteins inactivating p27 in response to mitogenic stimulation (Sherr and Roberts, 1995).

Secondly, the finding that cyclin D2 is directly regulated by Myc identifies one pathway that leads to the accumulation of cyclin D–Cdk4 complexes in response to activation of Myc. Several lines of evidence show that cyclin D2 is a direct target gene of Myc; for example, cyclin D2 mRNA accumulates rapidly and in a cycloheximide-resistant manner after induction of a MycER chimera. The promoter of the cyclin D2 gene is repressed by Mad–Max complexes via an E-box element that is conserved in human and rodent promoters; repression by Mad is specifically antagonized by Myc. Finally, cyclin D2 protein is expressed in a growth factor-independent manner in mouse fibroblasts expressing a constitutive allele of Myc. Clearly therefore, up-regulation of cyclin D2 is not a cellular response limited to the use of a conditional system to regulate Myc activity.

The mode of regulation of cyclin D2 by Myc differs from previously defined target genes of Myc, e.g. from prothymosin- α (Desbarats *et al.*, 1996). For example, we did not observe any activation of the cyclin D2 promoter by co-expression of Myc and Max under conditions where expression of a prothymosin- α reporter construct was activated 50-fold (data not shown). Conversely, we have been unable to repress the basal level of prothymosin- α expression by co-expression of Mad-1 (L.Desbarats and M.Eilers, unpublished results). Our data suggest therefore, that Myc induces cyclin D2 expression solely by de-repression rather than true activation. This is further supported by the observation that the effect of Myc can be mimicked by the addition of trichostatin A to resting cells. Of the Mad protein family members, Mxi, Mnt-1/Rox and Mad-4 are expressed in murine fibroblasts and we suggest a model in which these proteins contribute to repression of cyclin D2 in resting cells (Hurlin *et al.*, 1997; Meroni *et al.*, 1997; Schreiber-Agus *et al.*, 1998; B.Lüscher, personal communication). We are currently testing this model using Mxi-deficient mice. The findings further suggest that the mode of gene induction by Myc *in vivo* varies from de-repression to true activation gene depending on the exact target gene analysed, and that the set of genes regulated by either Myc or Mad proteins is not identical.

De-repression of cyclin D2 depends on the presence of a conserved domain termed Myc-box II, but not strictly on the presence of further N-terminal domains of Myc. The analysis suggests that de-repression requires the TRRAP protein that interacts with Myc-box II and is required for transformation by Myc (McMahon *et al.*, 1998). In several rapidly growing cells and tumours, high levels of a stable fragment of Myc (termed s-Myc or c-mycS) accumulate, which initiate at a downstream ATG codon. These forms lack a transactivation domain, but retain Myc-box II, and have originally been suggested to

Fig. 9. Cyclin D1^{-/-} and D2^{-/-} fibroblasts are resistant to immortalization by Myc. **(A)** A photograph of Giemsa-stained plates of wild-type, cyclin D2^{-/-} and cyclin D1^{-/-} cells infected with control ('Hygro') or Myc-expressing virus ('Hygro-Myc') after selection and incubation for 2 weeks. The right panel shows a quantitation of three independent experiments. **(B)** Growth curve of wild-type and cyclin D2^{-/-} cells recovered after infection with the viruses selection indicated; 5×10^4 drug-resistant cells were plated at the start of the experiment. **(C)** Percentage of senescent cells in a stably growing clone obtained from Myc-infected wild-type cells (passage 9 after selection) and in control non-infected cells at passage 5 after initial plating. **(D)** Expression of human Myc and cyclins D1 and D2 in either wild-type or cyclin D2^{-/-} MEFs and in a clone recovered after infection of wild-type cells with Hygro-Myc. 'exp' indicates exponentially growing cells, 'sta', serum-starved cells and 'resti', restimulated cells.

Fig. 10. Ectopic expression of cyclin D2 is not sufficient to account for the mitogenic properties of Myc in established cells. **(A)** Growth curves of RAT1 cells sequentially infected with either retroviruses encoding cyclin D2, cyclin D2 together with Cdk4 or resistance marker alone as indicated. After brief selection, resistant cells were pooled and infected with retroviruses encoding p16 or p27. Panels show growth curves of double-resistant cells after plating at low density in medium containing 10% FCS. Control cells super-infected with a virus encoding p16 were not recovered. **(B)** Western blots documenting expression of cyclin D2, Cdk4, p27 and p16 in the infected cells.

represent a dominant-negative form of the protein (Spotts *et al.*, 1997). More recently, s-Myc has been shown to retain transforming ability suggesting that it still retains

critical gene regulatory functions (Xiao *et al.*, 1998); one possibility based on our findings is that s-Myc may still de-repress target genes such as cyclin D2 and thereby

promote proliferation even in the absence of a transactivating domain.

Thirdly, our results indicate that cyclin D2 function is required for two gain-of-function phenotypes of Myc. Microinjection of monoclonal antibodies strongly indicates a role for cyclin D2 in Myc-induced cell cycle entry in an established murine fibroblast cell line. Similarly, ectopic expression of p16 blocked Myc-induced sequestration of p27 in RAT1 fibroblasts suggesting a role for cyclin D2 in cell cycle entry in response to activation of Myc in this cell line too.

Using fibroblasts from cyclin D2^{-/-} animals, we found that cyclin D2 is required for Myc to immortalize primary mouse embryo fibroblasts. Cyclin D2^{-/-} cells could easily be infected with retroviruses expressing either H2B-GFP or human Myc together with H2B-GFP; however, cyclin D2^{-/-} failed to proliferate after expression of Myc, and stable cell lines could only be established from wild-type, not from cyclin D2^{-/-} cells after infection with Myc. Cyclin D2^{-/-} primary cells underwent accelerated senescence in culture, suggesting that failure to up-regulate Cyclin D2 can account, at least in part, for the loss-of-function phenotype of Myc in such cells. It remains to be tested whether cyclin D2 is required for tumorigenesis by Myc *in vivo*; such experiments are under way.

Similarly, antibodies against cyclin D1 ablate cell cycle progression in RAT1 cells and mouse fibroblasts downstream of Myc (Roussel *et al.*, 1995; Steiner *et al.*, 1995), and cyclin D1^{-/-} fibroblasts fail to be immortalized by Myc. Therefore, parallel pathways may exist that lead to up-regulation of cyclin D1 protein expression by Myc. This has been suggested from the finding that the eukaryotic translation factor, eIF4, is up-regulated by Myc via an E-box in the promoter of the gene, and from the observation that enhanced levels of eIF-4 may up-regulate translation of D-type cyclins (e.g. Johnston *et al.*, 1998). Alternatively, induction of cyclin D1 (or cyclin D3) expression by Myc-independent signalling pathways contributes to deregulation of cell cycle progression by Myc. This might explain observations that induction of Myc is not sufficient to induce proliferation in a number of cell types. For example, activation of both Myc and Ras signalling is required for efficient cell cycle entry of REF52 cells (Leone *et al.*, 1997).

Loss of cyclin D2 did not significantly affect the ability of Myc to induce apoptosis in primary mouse fibroblasts, demonstrating that Myc has other functions in these cells in addition to up-regulation of cyclin D2. At least some of these functions also affect cell proliferation in fibroblasts. This can be concluded from a number of experiments in which the growth-promoting properties of Myc and cyclin D proteins have been analysed using a variety of proliferation assays. The results are often very similar for both proteins, for example, ectopic expression of either D-type cyclins or Myc rescues mitogenic signalling by a defective CSF-1 receptor and complements transformation-deficient alleles of bcr-abl (Roussel *et al.*, 1991, 1995; Afar *et al.*, 1994, 1995). Also, cyclin D1 mimics the effect of Myc on activation of cyclin E-Cdk2 kinase in breast cancer cells (Prall *et al.*, 1998). However, Myc and D-type cyclins do not always act in an identical fashion; for example, Myc-transformed cells show a partial, but not complete resistance against elevated levels of

p27 and p16 (Vlach *et al.*, 1996; Alevizopoulos *et al.*, 1997), and ectopic expression of cyclin D2 does not substitute for Myc in this function (Figure 10; Alevizopoulos *et al.*, 1997). Similarly, ectopic expression of cyclin D does not rescue the growth defect of fibroblasts carrying a null mutation of c-myc (Mateyak *et al.*, 1999). Clearly therefore, Myc controls cell proliferation and also p27 metabolism by pathways other than up-regulation of cyclin D2. Also, accumulation of cyclin D2, in contrast to Myc, can occur in stably arrested cells (Meyyappan *et al.*, 1998).

Finally, loss of cyclin D2 expression does not account for the loss-of-function phenotype of Myc during embryogenesis, as the phenotype of cyclin D2-deficient mice is less severe than that of c-Myc-deficient mice (Davis *et al.*, 1993; Sicinski *et al.*, 1996). Again, these findings point to a function of Myc unrelated to its ability to up-regulate cyclin D2.

Materials and methods

Tissue culture

RAT1 cells, primary mouse embryo fibroblasts and NIH 3T3 cells were grown in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Culture of RAT1-MycERTM cells has been described previously (Littlewood *et al.*, 1995). The detailed description of mouse embryo fibroblasts expressing MycERTM proteins will be published elsewhere. Staining for senescent cells was carried out as described (Dimri *et al.*, 1995).

Transient transfections into NIH 3T3 cells were performed as described previously for HeLa cells (Desbarats *et al.*, 1996). The expression vectors used, CMV-Myc, CMV-Max, CMV-Mad, CMV-MadΔN, CMV-MadΔLZ and CMV-USF, have been described previously (Desbarats *et al.*, 1996; Sommer *et al.*, 1997). The cyclin D2 reporter constructs were generated by inserting a 2.3 kb fragment from the murine cyclin D2 promoter into the *SacI* site of pGL2 (Promega). Mutants were generated using the ExSite kit (Stratagene) and verified by sequencing. In each transfection, 10 ng of CMV-lacZ were transfected to normalize different transfection efficiencies. The total amount of DNA was kept constant in each transfection by adding equal amounts of expression plasmids.

Retroviral supernatants were generated by transient transfections of BOSC23 and Phoenix cells and used to infect RAT1 cells and primary mouse embryo fibroblasts as published (Pear *et al.*, 1993; Grignani *et al.*, 1998). Infected cells were selected with 5 µg/ml puromycin or 150 µg/ml hygromycin as appropriate.

To generate a retrovirus expressing cyclin D2, rat cyclin D2 cDNA was amplified with the following primers: 5'-primer, CGGGATCCACCATGGAGCTGCTGTGCTGTGAGG; and 3'-primer, CGGAATTCTAGAGAGAGAGAGAGAAGGGGCTAGC and cloned into pbabe-puro and -hygro (Morgenstern and Land, 1990). To generate a virus expressing both cyclin D2 and Cdk4, a cDNA encoding human cyclin D2 was fused to an internal ribosomal entry site and cloned downstream of human Cdk4 into pbabe-puro. Viruses expressing p27 and p16 have been described (Müller *et al.*, 1997). To generate pbabe-H2BGFP, the puromycin gene was excised from pbabe-puro and replaced with an open reading frame encoding an H2B-GFP chimeric protein (Kanda *et al.*, 1998).

RNA extraction and Northern blotting

Total RNA was isolated with the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Twenty micrograms per lane of denatured RNA were size fractionated in 1% agarose-6% formaldehyde gel, hydrolyzed in 50 mM NaOH for 20 min then transferred onto nylon membranes (Zeta-Probe, Bio-Rad). Blots were submitted to UV light (GS Gene Linker, Bio-Rad) and pre-hybridized in 50% deionized formamide, 5× standard saline citrate (SSC), 5× Denhardt's, 50 mM NaPO₄ pH 6.5, 0.1% SDS containing 100 µg/ml denatured salmon sperm DNA for 2–8 h at 42°C. Hybridization was performed at 42°C for 18 h in 50% deionized formamide, 5× SSC, 1× Denhardt's, 50 mM NaPO₄ pH 6.5, 0.1% SDS, 10% dextran sulfate containing 100 µg/ml

denaturated salmon sperm DNA and 2×10^6 c.p.m./ml of ^{32}P -labelled mouse cyclin D2 or rat GAPDH cDNAs as probes. Membranes were washed, dried and then exposed to Biomax MR films (Kodak).

Immunoblotting and immunoprecipitation

Immunoblotting, immunoprecipitation and gel filtration conditions have been described previously (Steiner *et al.*, 1995). For depletion experiments, three sequential immunoprecipitations were performed. The α -D-type cyclins and α -Cdk2 antibodies were chemically cross-linked to protein A-Sepharose beads using dimethylpimelimidate (Harlow and Lane, 1988), since they showed a high rate of leakage from the protein A beads in the absence of cross-linking (data not shown).

The following antibodies were used. Cyclin D1: DCS-6, DCS-11; cyclin D2: DCS-3, DCS-5 (Lukas *et al.*, 1995), M-20 (Santa Cruz); cyclin D3: DCS-22, DCS-28 (Bartkova *et al.*, 1998); p27: K25020 (Transduction Laboratories), C-19 (Santa Cruz); cyclin A: C-19 (Western blotting), H-432 (immunofluorescence); cyclin E: M-20; Cdk2: M2; Cdk4: C-22 (all Santa Cruz Biotechnology). Where indicated, linear exposures of the blots were scanned using an Arcus II scanner (Agfa) and quantitated using NIH Image software.

Microinjections

MEF-MycER cells were plated at a density of 60% on coverslips in 6-well plates. After overnight incubation, cells were serum-starved for 2 days in DMEM containing 0.1% FCS. Cells were microinjected into the nucleus with protein A affinity-purified mouse monoclonal anti-cyclin D2 DCS-3 at a concentration of 3.4 mg/ml in phosphate-buffered saline using a Zeiss AIS microinjection system and an Axiovert inverted microscope. As control, protein A affinity-purified isotypic control IgG2a antibodies (Ascites, M-7769, Sigma; 8 mg/ml) were injected. Cells were induced by the addition of 200 nM of 4-OHT for 22 h, washed once with PBS, fixed in methanol/acetone (1:1) for 5 min and then subjected to indirect immunofluorescence as described previously (Rudolph *et al.*, 1996). Microinjected cells were detected by CyTM3-conjugated anti-mouse secondary antibodies.

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