The EMBO Journal Vol.18 No.19 pp.5310–5320, 1999

Cyclins D1 and D2 mediate Myc-induced proliferation via sequestration of p27^{Kip1} and p21^{Cip1}

Ignacio Perez-Roger[1], Soo-Hyun Kim, Beatrice Griffiths, Andreas Sewing[2] and Hartmut Land[3,4]

Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, UK

[1]Present address: Instituto de Biomedicina de Valencia, CSIC, Jaime Roig 11, 46010-Valencia, Spain
[2]Present address: Smithkline Beecham Pharmaceuticals, New Frontiers Science Park (North), Third Avenue, Harlow CM19 5AW, UK
[3]Present address: Center for Cancer Biology, University of Rochester Medical Center, 601 Elmwood Avenue, Box 633, Rochester, NY 14642, USA

[4]Corresponding author
e-mail: land@urmc.rochester.edu

Cyclin E–Cdk2 kinase activation is an essential step in Myc-induced proliferation. It is presumed that this requires sequestration of G_1 cell cycle inhibitors p27^{Kip1} and p21^{Cip1} (Ckis) via a Myc-induced protein. We provide biochemical and genetic evidence to show that this sequestration is mediated via induction of cyclin D1 and/or cyclin D2 protein synthesis rates. Consistent with this conclusion, primary cells from *cyclin D1*$^{-/-}$ and *cyclin D2*$^{-/-}$ mouse embryos, unlike wild-type controls, do not respond to Myc with increased proliferation, although they undergo accelerated cell death in the absence of serum. Myc sensitivity of *cyclin D1*$^{-/-}$ cells can be restored by retroviruses expressing either cyclins D1, D2 or a cyclin D1 mutant forming kinase-defective, Cki-binding cyclin–cdk complexes. The sequestration function of D cyclins thus appears essential for Myc-induced cell cycle progression but dispensable for apoptosis.

Keywords: cyclin D/cyclin E/myc/p21^{Cip1}/p27^{Kip1}

Introduction

The proto-oncogene c-*myc* is a key regulator of cell proliferation and apoptosis (reviewed in Henriksson and Lüscher, 1996; Amati *et al.*, 1998; Dang, 1999). c-*myc* encodes a basic-helix–loop–helix–leucine zipper transcription factor (Myc) that dimerizes with Max (Blackwood and Eisenman, 1991; Prendergast *et al.*, 1991) and binds to DNA in a sequence-specific manner (Blackwell *et al.*, 1990; Prendergast and Ziff, 1991). Myc–Max heterodimers activate transcription (Amati *et al.*, 1992; Kretzner *et al.*, 1992) and are required for Myc-induced cell transformation, cell cycle progression and apoptosis (Amati *et al.*, 1993a,b). Recently, a co-factor (TRRAP) that binds to the functionally essential N-terminal domain of Myc and is required for Myc function has been identified (McMahon *et al.*, 1998). TRRAP and its highly related yeast homologue Tra1 are members of large protein complexes involved in the regulation of transcription (Saleh *et al.*, 1998) as well as histone acetylation.

Myc is rapidly induced by growth factors (reviewed in Kelly and Siebenlist, 1986) and is required for mitogenic signalling by the colony-stimulating factor (CSF) (Roussel *et al.*, 1991) and platelet-derived growth factor (PDGF) receptors (Barone and Courtneidge, 1995). Constitutive expression of Myc prevents exit from the cell cycle as well as differentiation (Marcu *et al.*, 1992; Henriksson and Lüscher, 1996) and induces apoptosis in the absence of survival cytokines (Evan *et al.*, 1992; Harrington *et al.*, 1994). Moreover, Myc activity is sufficient to drive resting cells into the cell cycle (Eilers *et al.*, 1991) and homozygous inactivation of c-*myc* in rat fibroblasts caused a marked prolongation of cell doubling time (Mateyak *et al.*, 1997). Although several genes have been identified that are regulated by Myc (Bush *et al.*, 1998; Xiao *et al.*, 1998; reviewed in Dang, 1999), none of these genes has been clearly implicated in Myc-induced progression from G_1 into the S phase of the cell cycle.

G_1 progression is controlled by the activities of the cyclin-dependent kinase complexes cyclin D–Cdk4 (or Cdk6) and cyclin E–Cdk2 (for reviews see Draetta, 1994; Sherr, 1994). Cyclins D and E are essential for G_1–S progression of higher eukaryotic cells (Baldin *et al.*, 1993; Quelle *et al.*, 1993; Knoblich *et al.*, 1994; Ohtsubo *et al.*, 1995) and when overexpressed are able to shorten the G_1 interval (Ohtsubo and Roberts, 1993; Quelle *et al.*, 1993; Resnitzky *et al.*, 1994; Wimmel *et al.*, 1994), indicating that both cyclins are rate-limiting for G_1–S transition. Regulation of cyclin E–Cdk2 activity occurs at multiple levels, involving the synthesis of the subunits, assembly, phosphorylation–dephosphorylation and the association of inhibitory proteins such as p21^{Cip1} and p27^{Kip1} (reviewed in Morgan, 1995; Sherr and Roberts, 1995).

Activation of cyclin E–Cdk2 is regarded as an essential event in Myc-induced G_1–S progression. Triggering a c-Myc–estrogen receptor fusion protein (MycER) induces cyclin E–Cdk2 activity early in the G_1 phase of the cell cycle (Steiner *et al.*, 1995). This activation is required for the subsequent induction of cyclin A expression (Rudolph *et al.*, 1996) and DNA synthesis (I.Perez-Roger and H.Land, unpublished data), as both events are blocked by the Cdk2 inhibitor roscovitine. Moreover, dominant-negative forms of Myc inhibit cyclin E–Cdk2 kinase activity when expressed in proliferating cell cultures (Berns *et al.*, 1997).

An important clue as to how Myc activates cyclin E–Cdk2 came from experiments showing that Myc can rescue cell cycle arrest induced by over-expression of the cell cycle inhibitor p27^{Kip1} through interfering with the binding of p27^{Kip1} to cyclin E–Cdk2 complexes (Vlach *et al.*, 1996). Similarly, in Rat1 cells arrested at confluency in the absence of serum, MycER-dependent activation of

cyclin E–Cdk2 is preceded by an inhibition of $p27^{Kip1}$ binding to newly formed cyclin E–Cdk2 complexes and a protein-synthesis independent induction of *cyclin E* gene transcription (Perez-Roger *et al.*, 1997). In addition, MycER also can override a $p21^{Cip1}$-dependent cell cycle arrest imposed by a strong Raf signal in NIH 3T3 cells via blocking $p21^{Cip1}$ binding to cyclin E–Cdk2 and increasing cyclin E synthesis rates (Sewing *et al.*, 1997; A.Sewing, I.Perez-Roger and H.Land, in preparation). In these systems activation of cyclin E–Cdk2 occurs without apparent changes in $p27^{Kip1}$ or $p21^{Cip1}$ levels, although a decrease in $p27^{Kip1}$ expression becomes detectable 3–5 h after cyclin E–Cdk2 activation (I.Perez-Roger and H.Land, unpublished data). This is consistent with the notion that degradation of $p27^{Kip1}$ requires Cdk2-dependent phosphorylation (Sheaff *et al.*, 1997; Vlach *et al.*, 1997). Since Myc is able to rescue the cell cycle arrest imposed by a non-phosphorylatable, degradation-resistant $p27^{Kip1}$ mutant (Thr/Ala 187) (Vlach *et al.*, 1996), it is likely that phosphorylation-dependent degradation and release of $p27^{Kip1}$ from cyclin E–Cdk2 complexes in response to Myc (Muller *et al.*, 1997) is a consequence rather than the cause of cyclin E–Cdk2 activation.

Cyclin E–Cdk2 activation by Myc is likely to require the induction of a factor that competes with the cyclin E–Cdk2 complex for the binding of cell cycle-dependent kinase inhibitors. Mutations in Myc that disable DNA binding or the N-terminal domain required for gene regulation are unable to rescue the $p27^{Kip1}$-induced arrest (Vlach *et al.*, 1996). We have also shown that increasing the synthesis rate of cyclin E alone is not sufficient to induce cyclin E–Cdk2 activity, as $p27^{Kip1}$ binds to and inhibits the newly formed complexes. In the presence of active Myc, however, binding of $p27^{Kip1}$ to cyclin E–Cdk2 is prevented, and the increased rate of cyclin E synthesis is able to feed a small pool of inhibitor-free cyclin E–Cdk2 complexes that are accessible to phosphorylation by cyclin activating kinase (CAK) and concomittantly become active (Perez-Roger *et al.*, 1997). These findings suggest that Myc regulation of cyclin E–Cdk2 activity involves at least two distinct mechanisms: stimulation of cyclin E synthesis rates and a reduction of $p27^{Kip1}$ and $p21^{Cip1}$ binding to cyclin E–Cdk2, presumably via induction of a competitive binding partner for the inhibitors. It was our aim in this study to identify such a competitor and to test its function in Myc-induced activation of cyclin E–Cdk2.

Results

In order to search for direct evidence of Myc-dependent $p27^{Kip1}$- and $p21^{Cip1}$-binding proteins we investigated two previously characterized cell systems in which cyclin E–Cdk2 kinase activity is inhibited by either $p27^{Kip1}$ or $p21^{Cip1}$. First, we used Rat1-derived cells, termed R1MER, which when contact-inhibited in low serum express high levels of $p27^{Kip1}$ and can be induced to enter the cell cycle upon activation of a Myc-estrogen receptor chimera (MycER) by 4OH-tamoxifen (TMX). Expression of $p21^{Cip1}$ is not detectable in these cells (Perez-Roger *et al.*, 1997). Secondly, we chose NIH 3T3-derived cells, termed NIHRM, in which a $p21^{Cip1}$-dependent cell cycle arrest can be induced by activation of a Raf-androgen receptor chimera (Sewing *et al.*, 1997). Activation of a 4OH-tamoxifen-restricted mutant Myc-estrogen receptor chimera ($MycER^{TM}$) (Littlewood *et al.*, 1995) causes rapid re-entry into the cell cycle in these cells (A.Sewing, I.Perez-Roger and H.Land, in preparation). To test the possibility that Myc may induce the expression of proteins binding to the inhibitors, Myc was activated by addition of 4OH-tamoxifen for 5 h and the cells were pulse-labelled with [^{35}S]methionine for the last 30 min. $p27^{Kip1}$ and $p21^{Cip1}$ antibodies were used to precipitate protein complexes containing $p27^{Kip1}$ and $p21^{Cip1}$ from R1MER and NIHRM cell lysates, respectively. The precipitated proteins were then separated by gel electrophoresis.

Myc-induced cyclin D1 and D2 synthesis rates promote associaton of $p27^{Kip1}$ and $p21^{Cip1}$ with cyclin D–Cdk complexes

In R1MER cells, Myc activation leads to increased co-precipitation of a single protein band with an apparent molecular weight of 36 kDa with $p27^{Kip1}$ (Figure 1A). Immunodepletion of the lysates with cyclin D1 antibodies prior to immunoprecipitation with $p27^{Kip1}$ antibodies shows that the 36 kDa band corresponds to cyclin D1 (Figure 1B). Consistent with this observation, Myc activation induces an increase in cyclin D1 expression, which precedes activation of cyclin E-dependent kinase activity; during the same time, $p27^{Kip1}$ levels remain unaltered (Figure 1C, left top panel; see also Perez-Roger *et al.*, 1997), and the increase in cyclin D1–$p27^{Kip1}$ binding is proportional to the increase in cyclin D1 expression (Figure 1C, left bottom panel).

In NIHRM cells containing active Raf, immunoprecipitation with $p21^{Cip1}$ antibodies following Myc activation reveals increased co-precipitation of a protein band with a mobility corresponding to 35 kDa (Figure 2A). Immunodepletion of the lysates with cyclin D2 antibodies prior to immunoprecipitation with $p21^{Cip1}$ antibodies shows that the 35 kDa band corresponds to cyclin D2 (Figure 2B). Cyclin D2 expression is rapidly induced by Myc and precedes cyclin E–Cdk2 activation; during this time $p21^{Cip1}$ levels stay constant (Figure 2C, left panel), and the increase in cyclin D2-$p21^{Cip1}$ binding is proportional to the increase in cyclin D2 expression (Figure 2C, right panel).

Direct immunoprecipitation of cyclins D1 and D2 from the pulse-labelled lysates described above shows that Myc induces significant increases in cyclin D1 protein synthesis rates in R1MER and cyclin D1 and/or D2 sythesis in NIH 3T3-derived cell lines (Figure 3). Expression of cyclin D2 in Rat1 cells is not detectable, although the antibodies used recognize rat cyclin D2 in primary rat embryo cells. Similar to its murine counterpart, rat cyclin D2 has an increased electrophoretic mobility relative to cyclin D1 (not shown). Moreover, the lack of cyclin D2 expression in R1MER cells is in agreement with the observation of Pusch *et al.* (1997) that *cyclin D2* mRNA cannot be detected in the same cell type. In NIHMTR cells, containing $MycER^{TM}$ only, Myc activation induces cyclin D1 and cyclin D2 synthesis (Figure 3). Similarly, activation of $MycER^{TM}$ in secondary mouse embryo fibroblasts induces expression of cyclins D1 and D2 (not shown). In NIHRM cells, expressing RafAR and $MycER^{TM}$, cyclin D1 synthesis rates are highly induced by active RafAR and do not show a further increase

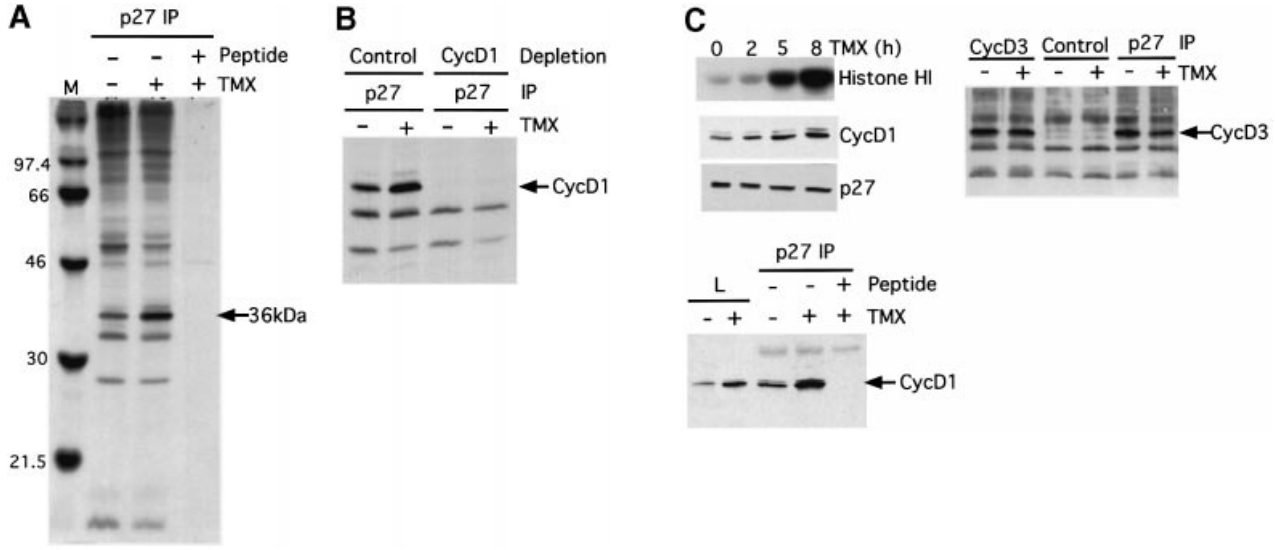

Fig. 1. Myc induces cyclin D1 association with p27^{Kip1} in R1MER cells. (**A**) Quiescent R1MER cells treated with TMX for 5 h (+) and untreated controls (–) were pulse-labelled with [^{35}S]methionine for 30 min as described in Materials and methods. Cell lysates were incubated with anti-p27^{Kip1} antibodies or peptide-blocked antibodies. Immunoprecipitates were run on SDS gels and analysed by fluorography. M: molecular weight markers. (**B**) Aliquots of the same lysates shown in (A) were depleted with normal rabbit serum agarose beads (Control) or with anti-cyclin D1 antibodies (CycD1) prior to immunoprecipitation with anti-p27^{Kip1} antibodies. (**C**) Left top panel: quiescent R1MER cells were treated with TMX and lysates were prepared 0, 2, 5 and 8 h later. Cyclin E dependent kinase activity was measured using Histone HI as substrate. The levels of cyclin D1 and p27^{Kip1} were analysed by Western blotting. Left bottom panel: lysates from untreated cells (–) or cells treated with TMX for 5 h (+) were immunoprecipitated with anti-p27^{Kip1} or peptide-blocked anti-p27^{Kip1} antibodies and the levels of cyclin D1 in the immunoprecipitates were analysed by Western blotting. L, lysate. Right panel: lysates from untreated cells (–) or cells treated with TMX for 5 h (+) were immunoprecipitated with anti-cyclin D3, anti-p27^{Kip1} or control antibodies and the levels of cyclin D3 in the immunoprecipitates were analysed by Western blotting.

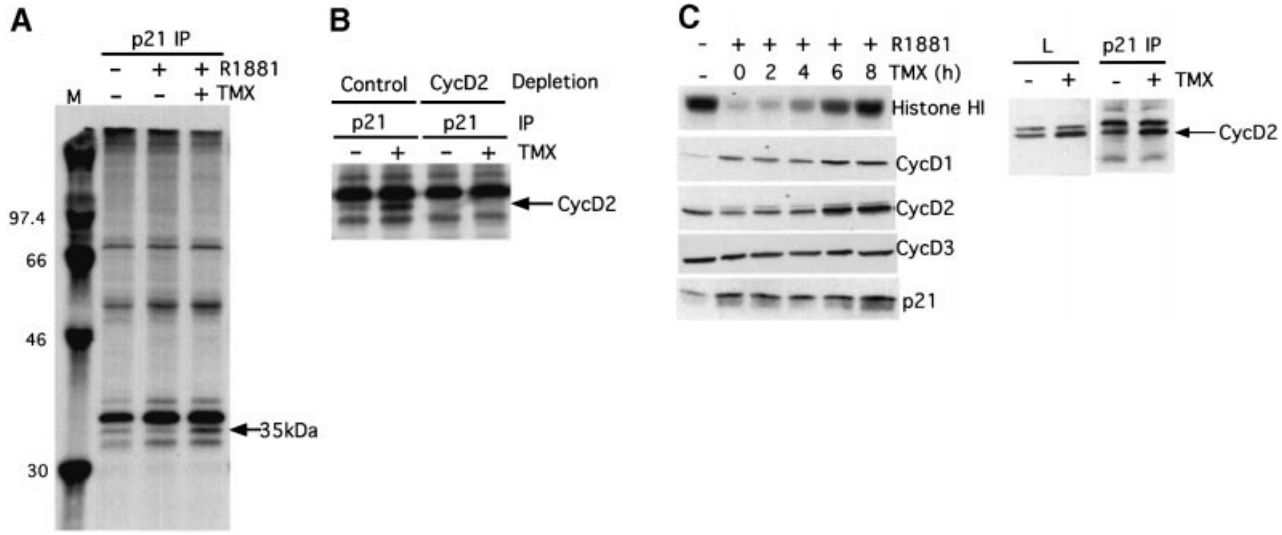

Fig. 2. Myc induces cyclin D2 association with p21^{Cip1} in NIHRM cells. (**A**) Growing NIHRM cells (– R1881, – TMX), Raf-arrested NIHRM cells (+ R1881, – TMX) and Raf-arrested NIHRM cells treated with TMX for 5 h (+ R1881, + TMX) were pulse-labelled with [^{35}S]methionine for 30 min as described in Materials and methods. Cell lysates were incubated with anti-p21^{Cip1} antibodies. Immunoprecipitates were run on SDS gels and analysed by fluorography. M: molecular weight markers. (**B**) Aliquots of the lysates from Raf-arrested NIHRM cells (–) or from Raf-arrested cells treated with TMX for 5 h (+) were depleted with normal rabbit serum agarose beads (Control) or with anti-cyclin D2 antibodies (CycD2) prior to immunoprecipitation with anti-p21^{Cip1} antibodies. (**C**) Left panel: Raf-arrested NIHRM cells were treated with TMX and cell lysates were prepared after 0, 2, 4, 6 and 8 h. Growing cells (– R1881, – TMX) were also included in the experiment. Cyclin E dependent kinase activity was measured using Histone HI as substrate; the levels of cyclin D1, cyclin D2, cyclin D3 and p21^{Cip1} were analysed by Western blotting. Right panel: lysates from Raf-arrested NIHRM cells (–) or from Raf-arrested cells treated with TMX for 5 h (+) were immunoprecipitated with anti-p21^{Cip1} antibodies and the levels of cyclin D2 in the immunoprecipitates were analysed by Western blotting. L, lysate.

following Myc activation (Figure 3). This is also reflected in the lack of increased binding of cyclin D1 to p21^{Cip1} after Myc activation in these cells (not shown).

Cyclin D3 synthesis rates, expression levels and p27^{Kip1} binding are unaffected by Myc (Figures 1C, right panel, 2C and 3). Similarly, Cdk4 and p21^{Cip1} synthesis rates also do not respond to Myc activation (Figure 3). While pulse-labelled p21^{Cip1} can be detected, as its synthesis rate is induced by Raf, p27^{Kip1} cannot be identified in the pulse-label experiments, as its expected position in the gel is obscured by an unidentified band (not shown).

Our data indicate that cyclins D1 and D2 behave like

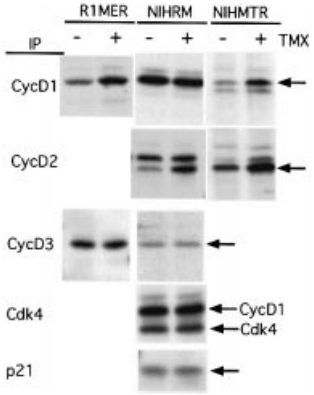

Fig. 3. Myc induces cyclin D1 and D2 synthesis. Quiescent R1MER cells, Raf-arrested NIHRM cells and quiescent NIHMTR cells were treated with TMX for 5 h (+) or untreated (–) and pulse-labelled with [^{35}S]methionine for 30 min as described in Materials and methods. Cell lysates were incubated with the antibodies indicated on the left. Immunoprecipitates were run on SDS gels and analysed by fluorography. The arrows on the right indicate the position of the immunoprecipitated proteins.

the predicted Myc-dependent p27^{Kip1}- and p21^{Cip1}-binding proteins. With regard to cyclin D1 this is surprising, as we have previously shown that *cyclin D1* mRNA synthesis is not stimulated by activation of MycER with 4OH-tamoxifen in Rat1 cells (Solomon *et al.*, 1995). This observation was confirmed independently following our results described above (not shown). Thus we suggest that the increased rate of protein synthesis of cyclin D1 in response to Myc activation is likely to be due to post-transcriptional control. In contrast, expression of *cyclin D2* mRNA in NIHRM cells is rapidly induced by Myc and precedes the increase in protein sythesis, indicating control of cyclin D2 by Myc at the level of gene expression (Figure 4).

Sequestration of p27^{Kip1} by cyclin D–Cdk

To estimate the extent to which the Myc-dependent increase in cyclin D1 protein synthesis in R1MER cells can affect the distribution of p27^{Kip1}, we explored whether cyclin D1 accumulation is able to significantly reduce the pool of p27^{Kip1} resistant to immuno-depletion with a combination of cyclin D1, D2, D3 and cyclin E antibodies. Indeed, 5 h after activation of Myc the pool of p27^{Kip1} resistant to immuno-depletion is markedly reduced (Figure 5). Given that the steady-state levels of cyclin D3 (Figure 2A) and cyclin E (Steiner *et al.*, 1995; Perez-Roger *et al.*, 1997) are not significantly altered by Myc, the changes in cyclin D1 synthesis and/or accumulation can contribute to a significant sequestration of p27^{Kip1}.

Cyclin D synthesis drives activation of cyclin E–Cdk2 by affecting p27^{Kip1}–cyclin–Cdk binding dynamics

Taken together, our findings suggest that activation of cyclin E–Cdk2 kinase by Myc is mediated via increased cyclin D1 and D2 synthesis rates followed by sequestration of the cell cycle inhibitors p27^{Kip1} and p21^{Cip1} through cyclin D–Cdk complexes. The validity of this idea can be scrutinized further by testing three key predictions raised by this model. First, increased cyclin D synthesis should be able to activate cyclin E–Cdk2. Secondly, this should

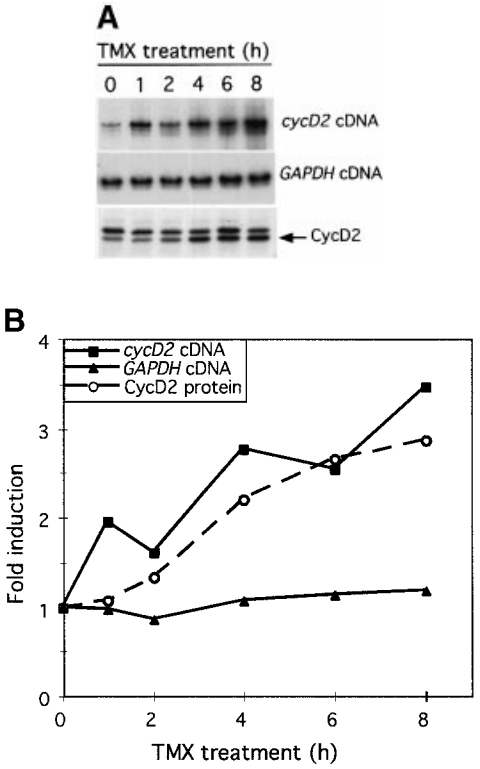

Fig. 4. Myc induces accumulation of *cyclin D2* mRNA. (**A**) Total RNA was prepared from Raf-arrested NIHRM cells at the times indicated after TMX addition and analysed by RT–PCR using primers specific for *cyclin D2* and *GAPDH*. Cells treated in parallel were pulse-labelled with [^{35}S]methionine for 30 min as described in Materials and methods and cyclin D2 protein was immunoprecipitated from cell lysates (bottom). (**B**) Phosphorimager quantitation of the experiment shown in (A).

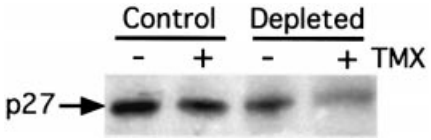

Fig. 5. Myc reduces the pool of p27^{Kip1} resistant to immunodepletion with cyclin D and cyclin E-specific antibodies. Lysates containing 200 μg of protein from R1MER cells, untreated (–) or treated with TMX for 5 h (+), were incubated with 30 μl of normal rabbit serum beads (Control) or with 1 μg of cyclin D1, cyclin D2, cyclin D3 and cyclin E antibodies and 30 μl of protein A beads (Depleted), in a final volume of 500 μl for 2 h at 4°C. The beads were removed by centrifugation and the supernatant was subjected to another round of immunodepletion. The supernatant (40 μl, equivalent to 16 μg of protein) was analysed by immunoblotting with anti-p27^{Kip1} monoclonal antibodies.

occur by affecting p27^{Kip1} binding to cyclin D–Cdk complexes. Thirdly, there should be a net gain of newly synthesized inhibitor-free cyclin E–Cdk2 as evidence for a shift in the Cip/Kip-cyclin–Cdk binding equilibrium. In order to test these predictions we used Rat1 cells in which the expression of human cyclin E is under the control of a Gal4–ER–VP16 chimera rendering cyclin E expression 4OH-tamoxifen-sensitive (E6 cells) (Perez-Roger *et al.*, 1997).

First, in the absence of serum, induction of the human cyclin E protein does not lead to kinase activation (Perez-Roger *et al.*, 1997; Figure 6A, E6P, top panel), as it is in a ternary complex with Cdk2 (Figure 6A) and p27^{Kip1}

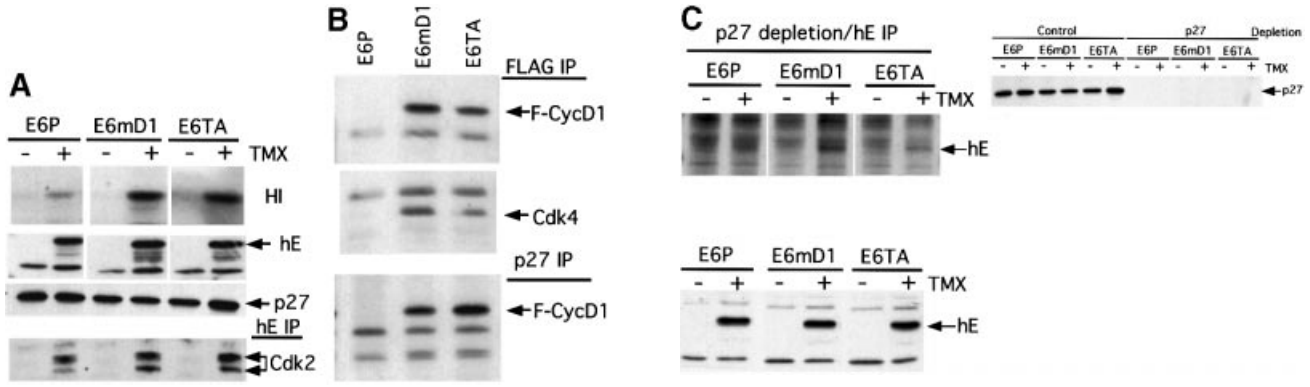

Fig. 6. Cyclin D1 synthesis rates control cyclin E–Cdk2 activation independent of cyclin D-dependent kinase activity. (**A**) Quiescent E6 cells infected with the empty pBabe-puro retrovirus (E6P), the vector containing the FLAG-tagged wild-type mouse *cyclin D1* cDNA (E6mD1) or the FLAG-tagged T156A mutant of the mouse *cyclin D1* cDNA (E6TA) were treated with 10 nM TMX (+) or the solvent control (ethanol) (–) to induce expression of human cyclin E; cell lysates were prepared after 8 h and analysed for human cyclin E-dependent kinase activity in immunoprecipitates using Histone HI as substrate (HI). The expression of human cyclin E (hE) and the levels of $p27^{Kip1}$ were analysed by Western blotting. Human cyclin E complexes were immunoprecipitated and analysed for the presence of Cdk2 by Western blotting (Cdk2). (**B**) Expression of FLAG-tagged wild-type and T156A mutant mouse cyclin D1 in E6 cells was analysed by immunoprecipitation with anti-FLAG antibodies and Western blotting using anti-cyclin D1 antibodies (top panel). Reprobing the same blot with anti-Cdk4 antibodies demonstrated binding of Cdk4 to wild-type and mutant FLAG-cyclin D1 (middle panel). $p27^{Kip1}$ binding to wild-type and mutant FLAG-cyclin D1 was analysed by immunoprecipitating [^{35}S]methionine pulse-labelled cell lysates with anti-$p27^{Kip1}$ antibodies (bottom panel). (**C**) Quiescent E6P, E6mD1 and E6TA cells were treated with TMX (+) or the solvent control (ethanol) (–) for 4 h and pulse-labeled with [^{35}S]methionine for 30 min as described in Materials and methods. Following immunodepletion with anti-$p27^{Kip1}$ antibodies cell extracts were incubated with anti-human cyclin E-specific antibodies. Immunoprecipitates were run on SDS gels and analysed by fluorography (left top panel). Expression of human cyclin E (hE) was monitored by Western blotting (left bottom panel). Before and after depletion, aliquots of the different samples were taken and analysed by Western blotting to confirm successful depletion of $p27^{Kip1}$ from the extracts (right panel).

(Perez-Roger *et al.*, 1997). However, when induced in the presence of retrovirally expressed flag-tagged murine cyclin D1 (Figure 6B, top panel), human cyclin E–Cdk2 activity is highly induced (Figure 6A, E6mD1, top panel). During this activation, human cyclin E expression and $p27^{Kip1}$ levels remain high (Figure 6A), yet a relative increase in the CAK-phosphorylated faster migrating form of Cdk2 can be found associated with human cyclin E (Figure 6A, bottom panel). This demonstrates that an increase in cyclin D synthesis can stimulate activation of cyclin E–Cdk2.

Secondly, does this link between cyclin D synthesis and cyclin E kinase activation require the kinase activity of cyclin D–Cdk complexes or are the altered kinetics in $p27^{Kip1}$ binding to the various cyclin–Cdk complexes sufficient for cyclin E–Cdk2 activation? In order to test this question, we introduced a flag-tagged mutant cyclin D1 (T156A) (Diehl and Sherr, 1997) into the E6 cells. This mutant is able to form complexes with CDK4 and with $p27^{Kip1}$ to an extent similar to its wild-type counterpart (Figure 6B, middle and bottom panel, respectively), but is unable to stimulate kinase activity (Diehl and Sherr, 1997). As shown in Figure 6A (E6TA), the synthesis of this cyclin D1 mutant was able to stimulate cyclin E–Cdk2 activity to the same degree as wild-type cyclin D1. This indicates that increased binding of $p27^{Kip1}$ by cyclin D–Cdk4 complexes can be sufficient to cause activation of cyclin E–Cdk2.

Thirdly, the activation of cyclin E–Cdk2 activity is indeed associated with an increase in inhibitor-free cyclin E–Cdk2 complexes. As shown in Figure 6A, induction of human cyclin E synthesis alone does not efficiently stimulate human cylin E–Cdk2 kinase activity in E6 cells. Consistent with this finding, we do not detect pulse-labelled human cyclin E in cell lysates after depletion with $p27^{Kip1}$ antibodies (Figure 6C, left top panel). Only when exogenous murine wild-type cyclin D1 (in E6mD1 cells) or the corresponding T156A mutant (in E6TA cells) are coexpressed, and human cyclin E–Cdk2 becomes active (Figure 6A), can newly synthesized human cyclin E be detected in $p27^{Kip1}$-depleted lysates, although equivalent amounts of human cyclin E are expressed under all conditions (Figure 6C, left bottom panel). Thus an increase in the synthesis rate of cyclin D–Cdk complexes causes redistribution of newly synthesized cyclin E into a $p27^{Kip1}$-free pool.

An increase in inhibitor-free cyclin E–Cdk2 complexes can also be found after Myc activation in R1MER and NIHRM cells. As mentioned above, activation of Myc not only induces the synthesis of cyclins D1 or D2 but also the synthesis of cyclin E, as demonstrated by immunoprecipitation of cyclin E after pulse-labelling cells with [^{35}S]methionine (Figure 7A and B). At least in R1MER cells this regulation occurs at the transcriptional level (Perez-Roger *et al.*, 1997). Analogous to E6mD1 and E6TA cells, the increase in cyclin E synthesis is followed by a significant increase of newly synthesized cyclin E in $p27^{Kip1}$ or $p21^{Cip1}$-depleted cell lysates (Figure 7A and B, respectively). In contrast, there is no accumulation of newly synthesized cyclin E in the $p21^{Cip1}$-bound fraction in NIHRM cells (Figure 7B), indicating that the newly synthesized cyclin E preferentially accumulates in the inhibitor-free fraction. Due to an adverse signal noise ratio, this experiment is not informative in R1MER cells. Nevertheless, virtually all of the detectable cyclin E-dependent kinase activity is associated with the $p27^{Kip1}$ or $p21^{Cip1}$-free fraction in R1MER or NIHRM cells (Figure 7A and B, respectively). This suggests that the newly synthesized cyclin E is utilized to produce a significant proportion of the active kinase complexes.

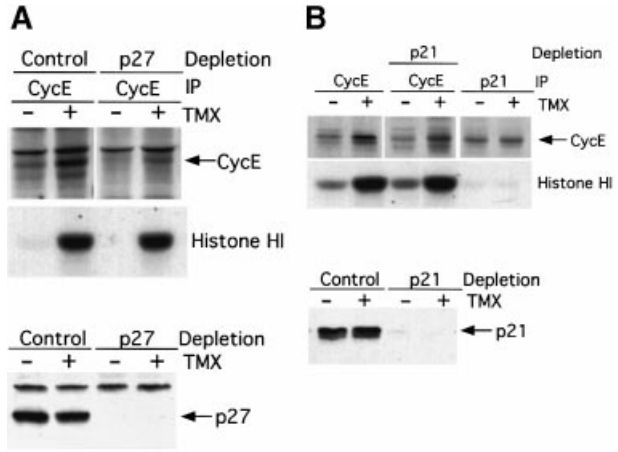

Fig. 7. Myc induces synthesis of cyclin E protein, which is Cki-free and associated with kinase activity. (**A**) Quiescent R1MER cells treated with TMX for 5 h (+) and untreated controls (–) were pulse-labelled with [^{35}S]methionine for 30 min as described in Materials and methods. Control and p27^{Kip1}-immunodepleted extracts were incubated with anti-cyclin E antibodies. Immunoprecipitates were run on SDS gels and analysed by fluorography. Cyclin E-dependent kinase activity was analysed on parallel samples using Histone HI as substrate. The extent of the p27^{Kip1} depletion was monitored by Western blotting (bottom panel). (**B**) Untreated Raf-arrested NIHRM cells (–) and cells treated with TMX for 5 h (+) were pulse-labelled with [^{35}S]methionine for 30 min as described in Materials and methods. Extracts were incubated with anti-cyclin E or anti-p21^{Cip1} antibodies or were immunodepleted with anti-p21^{Cip1} antibodies and then incubated with anti-cyclin E antibodies. Immunoprecipitates were run on SDS gels and analysed by fluorography. Kinase activity in the immunoprecipitates was analysed on parallel samples using Histone HI as substrate. The extent of the p21^{Cip1} depletion was monitored by Western blotting (bottom panel).

Cyclins D1 and D2 required for Myc-induced proliferation but dispensable for apoptosis

To explore the role of cyclin D1 or cyclin D2 synthesis rates in Myc-induced cell proliferation, we tested the effect of wild-type Myc over-expression on primary mouse embryo cells (MEF) from *cyclin D1*$^{-/-}$ (Fantl *et al.*, 1995) and *cyclin D2*$^{-/-}$ (Sicinski *et al.*, 1996) embryos and their respective wild-type litter-mate controls. The cells were infected with the recombinant retrovirus pBabe-puro (Morgenstern and Land, 1990) containing the human c-*myc* cDNA or no cDNA insert as a control. After 3 days of selection in puromycin containing medium (mock-infected cultures showed no surviving cells at this time), virally infected cells were pooled. Western blot analysis shows equivalent levels of human c-Myc expression in the wild-type as well as in the cultures from the knock-out embryos (Figure 8A). Moreover, there are no compensatory changes in gene expression of cyclins D1, D2 and D3, although we detect an increase in cyclin D1 levels in Myc-overexpressing cells. p27^{Kip1} and p21^{Cip1} levels also remain virtually unaltered (Figure 8A).

To test the proliferative behaviour of the infected cells, we monitored their ability to form colonies at low cell density (Figure 8B) and measured their proliferation rates in mass culture (Figure 8C). In wild-type littermate control cells, Myc strongly induces the formation of macroscopically visible colonies, as reported previously (Mougneau *et al.*, 1984). In contrast, *cyclin D1*$^{-/-}$ and *cyclin D2*$^{-/-}$ cells are unable to do so, although there is no significant difference in the behaviour of null and wild-type cells when infected with a control virus (Figure 8B, top and bottom panels, respectively). Similarly, Myc-expressing wild-type cells show an increased rate of proliferation, while Myc-expressing cells from *cyclin D1*$^{-/-}$ embryos fail to respond and proliferate at the same rate as control infected wild-type and *cyclin D1*$^{-/-}$ cells (Figure 8C, top panel). Myc-expressing *cyclin D2*$^{-/-}$ cells show an intermediate phenotype when compared with their *cyclin D1*$^{-/-}$ and wild-type counterparts in this assay (Figure 8C, bottom panel).

In order to test whether the pronounced deficiency in the response to Myc is specific for the lack of D cyclins, we wondered whether the phenotype could be rescued by exogenous expression of either wild-type cyclins D1 or D2. Based on our observation that *cyclin D1*$^{-/-}$ and *cyclin D2*$^{-/-}$ cells are deficient in inducing DNA synthesis when exposed to Myc in 0.5% serum (Figure 9A), we tested the response of *cyclin D1*$^{-/-}$ cells to co-expression of Myc and cyclins D1 or D2. While neither of the D cyclins had an effect on their own, both cyclins D1 and D2 rescued the ability of Myc to stimulate DNA synthesis (Figure 9B) to an extent comparable to the Myc response in wild-type cells (compare Figure 9A and B). Importantly, when Myc is co-expressed with the kinase-defective cyclin D1 mutant T156A, which is able to sequester Cip/Kip-type cell cycle inhibitors, a phenotypic rescue of similar quality was obtained (Figure 9B). Taken together these experiments demonstrate that expression of both cyclin D1 and D2 is essential for over-expressed Myc to stimulate cell cycle progression and establish a clear genetic link between Myc and cyclins D1 and D2. It is particularly interesting, however, that kinase activity or specificity does not appear to become limiting for the Myc response in *cyclin D1*$^{-/-}$ cells, but that instead the Cip/Kip sequestration function of the D cyclins appears to play a key role in this link.

Recently, Alevizopoulos *et al.* (1998) reported that in contrast to Myc, adenovirus E1A can prevent p27^{Kip1}-dependent growth arrest via a mechanism independent of Cdk2 activation. Based on the results reported here we predicted that, different from Myc, the ability of E1A to induce DNA synthesis should not be compromised in *cyclin D1*$^{-/-}$ and *cyclin D2*$^{-/-}$ cells. Indeed, in both knock-out cell types, adenovirus 12S E1A stimulates DNA synthesis to levels similar to those in wild-type cells (Figure 9A), indicating that the genetic interactions described here are truly Myc-specific. This is also reflected by the fact that until now no obvious proliferation defect of *cyclin D1*$^{-/-}$ embryo cells in tissue culture has been reported (Fantl *et al.*, 1995). This is consistent with the behaviour of wild-type, *cyclin D1*$^{-/-}$ and *cyclin D2*$^{-/-}$ control cells in our cell proliferation assays.

As Myc can trigger apoptosis (Evan *et al.*, 1992) as well as proliferation, we also wanted to test whether the lack of cyclin D1 or D2 would compromise Myc-induced cell death in the absence of survival factors (Harrington *et al.*, 1994). Wild-type, *cyclin D1*$^{-/-}$ and *cyclin D2*$^{-/-}$ cells infected with the Myc-expressing or a control retrovirus were plated and grown to confluency in 10% serum. Subsequently, the serum concentration in the medium was reduced to 0.05% and the cultures were monitored by microscopic inspection. Within 3 days, cell death was evident in all cultures in which Myc was over-expressed,

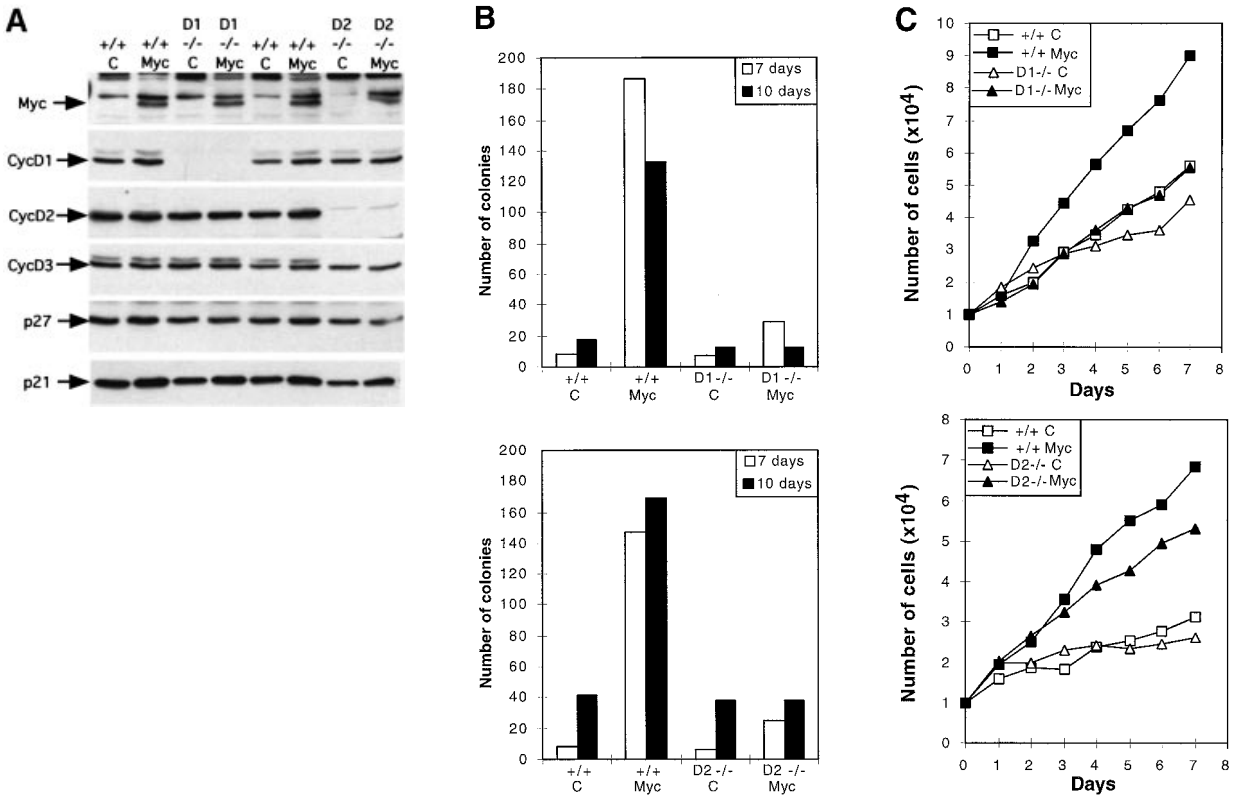

Fig. 8. Myc-induced proliferation is impaired in primary cells from *cyclin D1*$^{-/-}$ and *cyclin D2*$^{-/-}$ mouse embryos. (**A**) Expression levels of D-type cyclins and Ckis in Myc-expressing and control cells from *cyclin D1*$^{-/-}$, *cyclin D2*$^{-/-}$ and wild-type litter mate control mouse embryos. Primary cells were infected with retoviruses, selected and seeded as described in Materials and methods. Cell lysates were prepared and analysed by Western blotting for the expression of the proteins indicated. (**B**) Induction of colony formation by Myc: control and Myc-infected cells from *cyclin D1*$^{-/-}$ (top) and *cyclin D2*$^{-/-}$ (bottom) mouse embryos and their respective wild-type litter mate controls were seeded in duplicate at a density of 1×10^4 cells on 10 cm dishes. Macroscopically visible colonies were stained with crystal violet and counted 7 and 10 days after seeding. (**C**) Myc-induced proliferation rates: control and Myc-infected cells from *cyclin D1*$^{-/-}$ (top) and *cyclin D2*$^{-/-}$ (bottom) mouse embryos and their respective wild-type litter mate controls were seeded at 1×10^4 cells/well on 12-well dishes and cultured in 10% FBS. Triplicate cell cultures were trypsinized and counted every day for 7 days. Mean values are shown, with standard deviations mostly not exceeding 10%.

including the *cyclin D1*$^{-/-}$ and *cyclin D2*$^{-/-}$ cells (Figure 10). We therefore not only show that Myc is active in all the cultures, but also demonstrate that Myc-dependent cell cycle control and apoptosis are genetically distinct and independent programmes.

Discussion

Myc activation of cyclin E–Cdk2 kinase activity is an essential early step in the cascade of events that lead to Myc-induced proliferation. An important aspect of this activation is the fact that Myc disables the binding of the Ckis p27^{Kip1} and p21^{Cip1} to newly formed cyclin E–Cdk2 complexes (Vlach *et al.*, 1996; Perez-Roger *et al.*, 1997; A.Sewing, I.Perez-Roger and H.Land, in preparation). We now show biochemical and genetic evidence that the inhibition of Cki binding to cyclin E–Cdk2 by Myc is mediated via an induction of cyclin D1 and/or cyclin D2 protein synthesis rates. This leads to the preferential association of p27^{Kip1} and p21^{Cip1} with cyclin D–Cdk complexes. At the same time Myc also induces cyclin E protein synthesis rate helping to promote a net gain of newly formed Cki-free cyclin E–Cdk2 complexes. These become active concomitantly with phosphorylation of the kinase subunit by CAK (see also Perez-Roger *et al.*, 1997). Consistent with our model of this dynamic equilibrium (Figure 11), cyclin E–Cdk2 kinase activity can be controlled by changes in cyclin D synthesis rates. Moreover, as shown with a cyclin D mutant forming kinase-defective Cki-binding cyclin D–Cdk complexes, this link between cyclins D–Cdk and cyclin E–Cdk2 is independent of cyclin D–Cdk activity, but correlates with the ability of cyclin D–Cdk complexes to bind or sequester Ckis. This is strongly supported by the fact that the deficiency of *cyclin D1*$^{-/-}$ mouse embryo cells to respond to Myc with increased proliferation is restored by expression of the same cyclin D mutant. Consistent with our findings, transient over-expression of catalytically inactive cyclin D–Cdk, or cyclin E–Cdk2 complexes can rescue the cell cycle inhibitory effect of a dominant-negative Mad–Myc chimera (Berns *et al.*, 1997).

Due to the nature of physical interactions between cyclin D–Cdks and the cell cycle inhibitors p27^{Kip1} and p21^{Cip1}, cyclin D–Cdk complexes can fulfil a dual function as cell cycle kinases and as buffers for sequestration or release of cell cycle inhibitors. The release of p27^{Kip1} from cyclin D–Cdk plays an important role during the cell cycle arrest induced by the INK4-type cell cycle inhibitors p15^{INK4b} and p16^{INK4a}. p15^{INK4b}, induced in response to the anti-mitogenic cytokine TGF-β, disrupts cyclin D–Cdk4 complexes via binding to Cdk4 and leads to a redistribution of p27^{Kip1} to cyclin E–Cdk2 complexes.

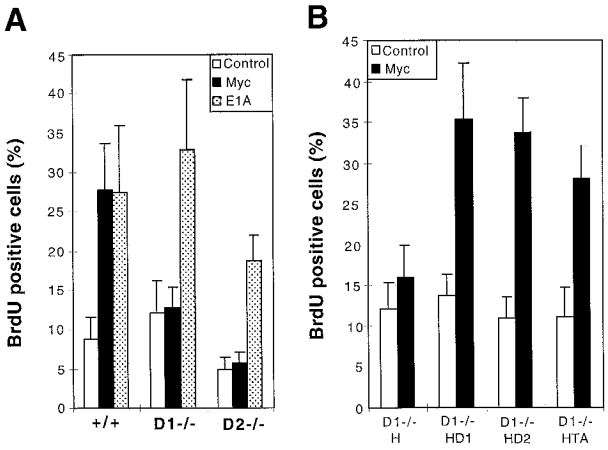

Fig. 9. The Myc-specific proliferation defect in *cyclin D1*$^{-/-}$ cells can be rescued by exogenous expression of cyclin D1 or cyclin D2. (**A**) Control, Myc-infected and E1A-infected cells from wild-type, *cyclin D1*$^{-/-}$ and *cyclin D2*$^{-/-}$ mouse embryos were seeded onto coverslips on 12-well dishes, at a density of 1×10^5/coverslip. After the cells had attached the serum concentration was lowered to 0.5% for 16 h. BrdU was added to the medium and 1 h later the cells were fixed and analysed by immunofluorescence as described in Materials and methods. Shown is the percentage of BrdU positive cells from a total of >600 cells in at least five different fields. (**B**) Control and Myc-infected cells from *cyclin D1*$^{-/-}$ mouse embryos were re-infected with the empty pBabe-hygro retrovirus (H), the vector containing the wild-type mouse *cyclin D1* cDNA (HD1), the wild-type mouse *cyclin D2* cDNA (HD2) or the T156A mutant of the mouse *cyclin D1* cDNA (HTA). The cells were selected and seeded onto coverslips on 12-well dishes and treated and analysed as in (A).

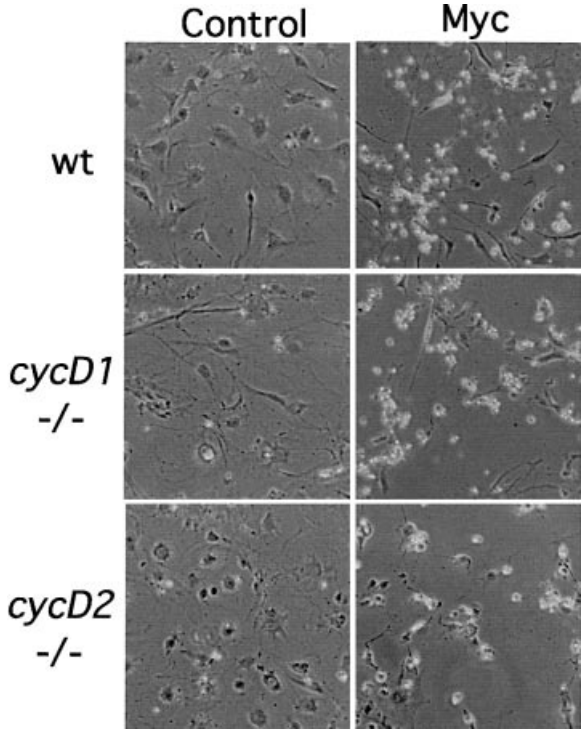

Fig. 10. Myc induces cell death in *cyclin D1*$^{-/-}$ and *cyclin D2*$^{-/-}$ cells. Control and Myc-infected cells from wild-type, *cyclin D1*$^{-/-}$ and *cyclin D2*$^{-/-}$ mouse embryos were plated at a density of 2×10^4 cells on 12-well dishes and cultured in 10% FBS. When the cultures were confluent, the serum concentration was reduced to 0.05%. Microphotographs were taken 3 days later.

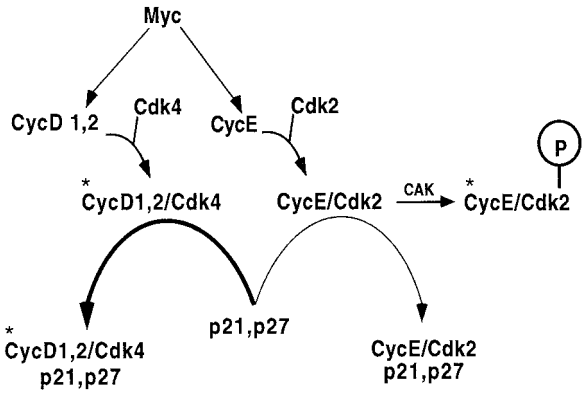

Fig. 11. Myc induced cyclin E–Cdk2 activation involves two steps, the inhibition of Cki (p27^{Kip1} and p21^{Cip1}) binding to cyclin E–Cdk2 and the induction of cyclin E protein synthesis rate. Sequestration of the Ckis is mediated via an induction of cyclin D1 and D2 protein synthesis rates. This causes the preferential association of Ckis with cyclinD–Cdk complexes. Concomitantly increased cyclin E protein synthesis feeds the pool of newly formed Cki-free cyclin E–Cdk2 complexes which become CAK-phosphorylated and kinase-active. The asterisks denote active complexes.

This results in the inhibition of both cyclin D and cyclin E-dependent kinase activities (Hannon and Beach, 1994; Reynisdottir *et al.*, 1995; Reynisdottir and Massague, 1997). A similar mechanism has been observed in response to increased p16^{INK4} expression (Jiang *et al.*, 1998; McConnell *et al.*, 1999). We conclude from our experiments that the sequestration of p27^{Kip1} and p21^{Cip1} by cyclin D–Cdk is also a necessary step in Myc-induced cyclin E–Cdk2 activation and proliferation. This is supported by a variety of independent observations. Cyclin D–Cdk complexes have a high affinity for p27^{Kip1} and p21^{Cip1} (LaBaer *et al.*, 1997), and at the same time the inhibitors can be found in kinase-active complexes (Florenes *et al.*, 1996; Soos *et al.*, 1996; LaBaer *et al.*, 1997). In proliferating cells p27^{Kip1} is predominantly in complexes with cyclin D–Cdk (Toyoshima and Hunter, 1994; Poon *et al.*, 1995; Soos *et al.*, 1996). Moreover, when catalytically inactive cyclin D–Cdk complexes were added together with cyclin E to extracts from G_0 or TGF-β-arrested cells, activation of cyclin E-dependent kinase occurred concomittantly with the binding of p27^{Kip1} to cyclin D–Cdk (Polyak *et al.*, 1994). In this context it is particularly intriguing that p21^{Cip1} and p27^{Kip1} have recently been shown to be essential for cyclin D–Cdk complex formation and therefore instrumental in promoting kinase activiy (Cheng *et al.*, 1999). It is thus possible that these assembly factor-like qualities are at least in part responsible for the potent Cki sequestration capacity of cyclin D–Cdk complexes. Consistent with this view, activation of cyclin E–Cdk2 kinase activation in response to estrogens in breast cancer cells coincided with a shift of p21^{Cip1} from its association with cyclin E–Cdk2 to the increasing numbers of cyclin D–Cdk4 complexes (Planas-Silva and Weinberg, 1997).

Our data demonstrate that cyclins D1 and D2 have all the hallmarks of the Myc-dependent p27^{Kip1} and p21^{Cip1} binding proteins predicted earlier (Vlach *et al.*, 1996; Perez-Roger *et al.*, 1997). With regard to cyclin D1, however, this is surprising, as we have previously shown that *cyclin D1* mRNA synthesis is not stimulated by activation of MycER with 4OH-tamoxifen in Rat1 cells

(Solomon *et al.*, 1995). This observation was confirmed independently following our results described above. Thus we suggest that the increased rate of protein synthesis of cyclin D1 in response to Myc activation is likely to be due to post-transcriptional control. This mechanism requires further study. In contrast, we show that *cyclin D2* mRNA expression is rapidly induced by Myc, presumably via a mechanism involving the reversal of Mad–Max-dependent repression (Bouchard *et al*., 1999). Vlach *et al.* (1996) did not expect cyclins D1 and D2 to be likely candidates for the Myc-induced sequestration activity. In their experiments with constitutively over-expressed Myc and $p27^{Kip1}$, they found no evidence for elevated cyclin D levels several days after retroviral infection. In our experimental system, in which we can follow the events in the first few hours after Myc activation, the increase in cyclin D1 and D2 accumulation is transient and begins to decline coincidental with the decrease in endogenous $p27^{Kip1}$ levels between 8 and 10 h after Myc activation. $p27^{Kip1}$ levels then remain low in proliferating cells (not shown). It thus appears that we have revealed a kick-start mechanism that enables Myc to drive the exit from G_0. Importantly, however, we have also shown that cyclins D1 and D2 are essential for Myc-induced long-term proliferation, indicating that cyclin D1 and D2 are permanent functional targets of Myc, at least when overexpressed. In these experiments we detect elevated cyclin D1 levels in Myc-expressing wild-type MEFs. Nevertheless, the constitutive and regulated Myc systems may detect distinct sequestration activities and it is possible that in addition to cyclins D1 and D2 other factors may also be involved in promoting Myc-dependent $p27^{Kip1}$ and $p21^{Cip1}$ resistance. In the absence of further detectable Myc-induced $p27^{Kip1}$ and $p21^{Cip1}$ binding proteins, such factors may operate upstream of D cyclins possibly through affecting the rate of cyclin D–Cdk/Kip,Cip–Cki association.

The clear dependency of Myc-induced proliferation on cyclin D1 and D2 in primary MEFs is not conserved in immortalized cell lines from *cyclin D1*$^{-/-}$ and *cyclin D2*$^{-/-}$ mouse embryos (I.Perez-Roger and H.Land, unpublished data). However, this is not too surprising, given that Myc-induced proliferation is the consequence of increases in cyclin synthesis rates designed to overcome the restrictive effects of Ckis. Spontaneous immortalization involves loss of either p53 or $p19^{ARF}$ with high frequency (Zindy *et al.*, 1998), an event that significantly lowers the Cki threshold. In fact, activation of MycER^{TM} in *cyclin D1*$^{-/-}$ and *cyclin D2*$^{-/-}$ cell lines is sufficient to trigger DNA synthesis. Either increased cyclin D1 or D2 synthesis alone appears sufficient to mediate Myc-dependent $p27^{Kip1}$ sequestration. We suspect that cell lines from *cyclin D1/D2* double knock-out mice will be required to reveal the cyclin D dependency of Myc in immortalized cells.

An unsuspected but very significant byproduct of this study is the observation that D cyclins are required for Myc-induced cell cycle progression but dispensable for Myc-dependent apoptosis. We thus provide clear evidence that these two major Myc-induced cell response programmes are genetically distinct and can be engaged independently. Such a separation has not been possible through analysis of a series of Myc and Max mutants (Amati *et al.*, 1993b). Together with our current data, this indicates the existence of programme-specific Myc targets. Their identification will be an important step in learning how to manipulate specific aspects of Myc function.

Materials and methods

Cell culture

Rat1 fibroblasts expressing the Myc-estrogen receptor hormone binding domain chimera, MycER (R1MER cells; Perez-Roger *et al.*, 1997), Rat1 cells expressing the chimeric transcription factor GalER-VP16 (Braselmann *et al.*, 1993) and a Gal4 promoter-dependent human *cyclin E* cDNA (E6 cells; Perez-Roger *et al.*, 1997), NIH 3T3 cells expressing the 4OH-tamoxifen-restricted mutant Myc-estrogen receptor fusion protein (MycER™; Littlewood *et al.*, 1995) (NIHMTR cells) or NIH 3T3 cells expressing a Raf-androgen receptor hormone binding domain (RafAR) (Sewing *et al.*, 1997) together with MycER^{TM} (NIHRM cells; A.Sewing, I.Perez-Roger and H.Land, in preparation) were cultivated in phenol red-free Dulbecco's modified Eagle's medium (DMEM) suplemented with either 10% charcoal-stripped fetal bovine serum (FBS, for Rat1 derived cells) or 10% charcoal-stripped newborn bovine serum (NBS, for NIH 3T3 derived cells).

To obtain NIHMTR cells, NIH 3T3 fibroblasts were infected with the recombinant retrovirus pBabe-puro containing MycER^{TM} and selected in 2.5 μg/ml of puromycin (Sigma). Various clones were isolated and clone number 6 was used in this study.

R1MER and NIHMTR cells were grown to confluency and kept in either serum-free medium in the presence of 5 μg/ml insulin (R1MER) or in 0.1% NBS (NIHMTR) for 48 h before activation of the chimeric Myc proteins with 200 nM 4OH-tamoxifen (TMX). NIHRM cells were treated with 500 nM R1881 (methyltrienolone, NLP-005; Dupont) to activate RafAR; after establishing a $p21^{Cip1}$-dependent cell cycle arrest (16 h), the MycER^{TM} fusion protein was activated by addition of 200 nM TMX.

E6 cells were infected with the recombinant retrovirus pBabe-puro containing the FLAG-tagged wild-type mouse *cyclin D1* cDNA, the FLAG-tagged T156A mutant of the mouse *cyclin D1* cDNA (Diehl and Sherr, 1997) or the empty vector and selected in 2.5 μg/ml of puromycin. In each infection, puromycin-resistant cells were pooled and termed E6mD1, E6TA and E6P, respectively. Confluent cultures were kept in serum-free medium for 48 h and human cyclin E expression was induced by adding 10 nM TMX.

Primary fibroblasts from wild-type, *cyclin D1*$^{-/-}$ and *cyclin D2*$^{-/-}$ mouse embryos were prepared according to Land (1995). Cells (1.5×10^6) from wild-type or knock out mice were seeded on 10 cm Petri dishes and 24 h later were infected in the presence of 8 μg/ml polybrene (Sigma) with retroviruses produced in BOSC cells (Pear *et al.*, 1993). Cells infected with the empty pBabe-puro retrovirus or the vector expressing the human c-Myc or the adenovirus-5 12S E1A proteins were selected in 2.5 μg/ml of puromycin and resistant cells were pooled after 3 days. Cells (1×10^6) were seeded on 10 cm Petri dishes and after 4 days cell lysates were prepared for Western blot analysis. Cells re-infected with pBabe-hygro or the vector containing the wild-type mouse *cyclin D1* cDNA, the wild-type mouse *cyclin D2* cDNA or the T156A mutant of the mouse *cyclin D1* cDNA were selected in 100 μg/ml of hygromycin B (Calbiochem) for 4 days, by which time uninfected cultures showed no surviving cells. Resistant cells were pooled and seeded onto coverslips on 12-well dishes.

Immunoprecipitation of [^{35}S]methionine pulse-labelled proteins

Quiescent R1MER and NIHMTR cells and Raf-arrested NIHRM cells were exposed to TMX for 5 h, while control cells were left untreated. For the last hour the cells were in methionine-free DMEM and [^{35}S]methionine (400 μCi/ml, Pro-Mix, Amersham) was added for the last 30 min. Cells were lysed in Triton lysis buffer (TLB: 50 mM Tris–HCl pH 8, 150 mM NaCl, 1 % Triton, 1 mM dithiothreitol, 10 mM NaF, 0.5 mM Na_3VO_4, 18 μg/ml aprotinin and 100 μg/ml phenylmethylsulfonyl fluoride). Cellular debris was removed by centrifugation. Lysates were precleared by incubation with 50 μl of normal rabbit serum agarose beads (Sigma) for 1 h at 4°C. The amount of incorporated radioactivity in the precleared lysates was determined by TCA precipitation. $5–10 \times 10^6$ c.p.m. were mixed with 1 μg of antibody and 30 μl of protein A beads (4 Fast Flow, Amersham Pharmacia Biotech), which had been previously blocked in 3% BSA for 1 h. The incubation was at 4°C for 16 h on a rotating wheel in a final volume of 500 μl. Immunodepletions were carried out by incubating the extracts with 10 μl

of normal rabbit serum beads or 1 μg of the relevant antibody and 10 μl of protein A beads for 2 h at 4°C. The supernatant was collected and subjected to two more rounds of immunodepletion prior to the immunoprecipitation. Immunoprecipitated complexes were washed four times with TLB and resolved by SDS–PAGE. Gels were fixed in 25% isopropanol, 10% acetic acid for 15 min, washed with water and soaked in 1 M salicylic acid for 15 min before being dried and exposed to X-Omat film (Kodak).

Immunofluorescence

Cells grown on coverslips on 12-well dishes were incubated with 50 μM BrdU for 1 h, then washed twice with PBS, fixed in 3.7% formaldehyde for 10 min, permeabilized in a solution containing 0.5% Triton X-100 and 2 M HCl for 10 min, and then incubated in blocking solution (5% powder skimmed milk, 10% FBS, 0.5% BSA, 0.1% Triton X-100 in PBS) for 30 min. Two washes with PBS were included between each step. Cells were then incubated with 30 μl of undiluted anti-BrdU mouse monoclonal antibody (Amersham Pharmacia Biotech) for 1 h, washed thoroughly with 0.1% Tween 20 in PBS and incubated with a Cy3-conjugated anti-mouse antibody (Jackson Immuno Research Laboratories) diluted 1:1000 in 3% BSA and 1 μg/ml Hoechst 33342 dye for 1 h. After extensive washes with 0.1% Tween 20 in PBS, the coverslips were mounted on slides. All the incubations were carried out at room temperature in the dark. Total number of cells and BrdU positive cells were counted using a fluorescence microscope. In each experiment, >600 cells were counted in at least five different fields.

Immunoblotting, immunoprecipitation and kinase activity

Cell lysate preparation, immunoblotting, immunoprecipitation and kinase assays were performed as described previously (Perez-Roger *et al.*, 1997). The following antibodies were obtained from Santa Cruz: α-CycE (sc-481, mouse and rat CycE, IP and kinase activity), α-CycE (sc-198, human CycE, IP, kinase activity and Western), α-CycD1 (sc-450, Western), α-CycD2 (sc-593, IP and Western), α-CycD3 (sc-182, IP and Western), α-Cdk2 (sc-163, Western), α-Cdk4 (sc-260, IP), α-p27 (sc-528, IP), α-p27 (sc-1641, Western), α-p21 (sc-397, IP), α-p21 (sc-6246, Western). For FLAG IP and Western, we used the M2 monoclonal antibody from Sigma. The α-CycD1 rabbit polyclonal antibody 287.3 used for immunoprecipitation was a kind gift of Gordon Peters. Myc was detected with the 9E10 monoclonal antibody (Evan *et al.*, 1985).

RT–PCR

Total RNA was extracted from cells using the RNeasy Mini Kit from Qiagen. RT and PCR were performed as described previously (Perez-Roger *et al.*, 1997). Denaturation, annealing and extension steps were at 94, 52 and 72°C, repectively, for 1 min each. The number of cycles was 18, 22 and 26 to ensure that the reaction had not reached saturation. Shown is the result of the reaction after 22 cycles. The sequence of the primers used is:

CycD2-5′:	GCTCGCCCACCTTCCACTCTTCTC
CycD2-3′:	GTCAGCGGGATGGTCTCTTTCAGC
GAPDH-5′:	CGTCTTCACCACCATGGAGA
GAPDH-3′:	CGGCCATCACGCCACAGTTT

Acknowledgements

We would like to thank Dr M.Eilers for communicating data prior to publication, Drs C.Dickson and C.Sherr for cDNAs, C.Dickson, P.Sicinski and E.Lam for making available transgenic mice, G.Peters for cyclin D1 antibodies, and Mme Salin-Drouin at ISOVESCO for the gift of 4OH-tamoxifen. I.P.-R. was the recipient of an EC host institution fellowship. This work was supported by the EC project grant: BMH4-CT98-3532.

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Received March 29, 1999; revised August 3, 1999; accepted August 31, 1999