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p27^{kip1}-independent cell cycle regulation by MYC

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MYC transcription factors are potent stimulators of cell proliferation. It has been suggested that the CDK-inhibitor p27^{kip1} is a critical G1 phase cell cycle target of c-MYC. We show here that mouse embryo fibroblasts deficient for both p27^{kip1} and the related p21^{cip1} are still responsive to stimulation by c-MYC and can be arrested in G1 by a dominant negative mutant of c-MYC. This growth arrest can be overruled by ectopic expression of E2F or adenovirus E1A, but not by a mutant of E1A defective for binding to retinoblastoma family proteins. We show that fibroblasts with a genetic disruption of all three retinoblastoma family members (pRb, p107 and p130) are unresponsive to a dominant negative c-MYC mutant. These data indicate that p27^{kip1} is not the only rate limiting cell cycle target of c-MYC and suggest that regulation of E2F is also essential for c-MYC's mitogenic activity. *Oncogene* (2000) **19,** 4822–4827.

Keywords: CDK-inhibitors; cell cycle; retinoblastoma protein; c-*Myc* oncogene

Introduction

The relevance of MYC transcription factors in regulation of cell proliferation is underscored by the fact that the c-*Myc* and N-*Myc* genes are frequently deregulated in human cancer, either by gene amplification or by chromosomal translocation (Nesbit *et al*., 1999). Consistent with this, over-expression of *Myc* proto-oncogenes in transgenic mice predisposes to tumorigenesis (reviewed by Berns *et al*., 1994). The oncogenic activity of *Myc* genes can also be recapitulated in cell culture experiments. Expression of c-*Myc* alone causes immortalization of primary rodent fibroblasts and in combination with a *Ras* oncogene, full oncogenic transformation (Land *et al*., 1983).

Biochemically, MYC proteins act as transcription factors that can both stimulate and repress a number of genes. Among the cell cycle targets of c-MYC are *Cdc25A*, *CyclinD2*, *E2f2* and possibly also *Cdk4* (Bouchard *et al*., 1999; Galaktionov *et al*., 1996; Hermeking *et al*., 2000; Perez-Roger *et al*., 1999; Sears *et al*., 1997). Activation of c-MYC alone in quiescent cells is sufficient to induce S phase entry (Eilers *et al*., 1991). One of the first consequences of MYC induction in quiescent cells is activation of cyclin E/cdk2 kinase activity, resulting from loss of p27^{kip1} from cdk2 complexes (Steiner *et al*., 1995; Vlach *et al*., 1996). Conversely, a dominant negative mutant of c-MYC, named MadMyc, arrests cycling cells in G1 with concomitant loss of cyclin E/cdk2 kinase activity (Berns *et al*., 1997). Activation of MYC causes sequestration of p27^{kip1} in cyclin D/cdk4 complexes, probably as a result of transcriptional activation of cyclin D2 by c-MYC (Bouchard *et al*., 1999; Perez-Roger *et al*., 1999). In support of the view that p27^{kip1} is a critical target of c-MYC during cell cycle entry is the finding that c-MYC overrules a G1 arrest induced by p27^{kip1} (Vlach *et al*., 1996). Besides activation of cyclin E/cdk2 kinase activity, MYC has been linked to the induction of E2F transcription activity (Jansen-Durr *et al*., 1993; Leone *et al*., 1997; Sears *et al*., 1997), which appears to be independent of regulation of cyclin E/cdk2 activity by MYC (M. Eilers, submitted for publication).

We show here that, apart from p27^{kip1}, c-MYC has other critical cell cycle targets.

Results

MadMyc-induced cell cycle G1 arrest is independent of p27^{kip1} and p21^{cip1}

To evaluate the contribution of p27^{kip1} and p21^{cip1} to the cell cycle effects of c-MYC, we expressed a dominant negative mutant of c-MYC, named MadMyc (Berns *et al*., 1997) in p27^{kip1}; p21^{cip1} double knockout mouse embryo fibroblasts (DKO MEFs). For this, MadMyc expression vectors were electroporated into low passage MEFs together with an H2B-GFP construct to allow selection of transfected cells by flow cytometry. To better visualize the cell cycle effects, we treated electroporated cells with nocodazole, which arrests cells in M phase, unless they are arrested in G1 as a result of expression of the introduced gene. Figure 1 shows that MadMyc expression induced a G1 arrest that was comparable to that induced by a dominant negative mutant of CDK2 (CDK2-DN), both in p27/p21 heterozygous and in DKO MEFs. A DNA binding domain mutant of MadMyc (MadMycDC) was unable to induce G1 arrest in MEFs of either genotype (Figure 1a). The intercrossings performed to generate p27/p21 heterozygous and homozygous knockout embryos did not allow the generation of littermate control wild-type embryos. Therefore, we used MEFs derived from *p21$^{+/-}$*; *p27$^{+/-}$* embryos as littermate controls. Multiple experiments in wild-type MEF

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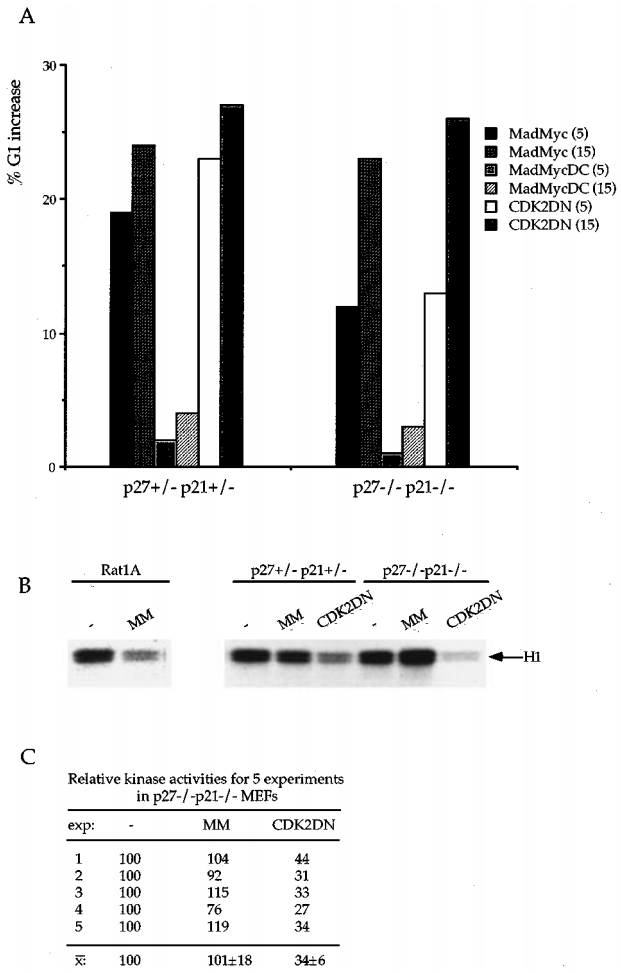

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Relative kinase activities for 5 experiments in p27-/-p21-/- MEFs

exp:	-	MM	CDK2DN
1	100	104	44
2	100	92	31
3	100	115	33
4	100	76	27
5	100	119	34
$\bar{x}$:	100	101±18	34±6

Figure 1 CIP/KIP independent G1 arrest by MadMyc. (**a**) Early passage p27/p21 heterozygous and DKO MEFs were electroporated with 1.5 μg H2B-GFP in combination with increasing amounts (5 or 15 μg) of pCMVMadMyc, pCMVMadMycDC or pCMV-CDK2DN. Cells were blocked with nocodazole. The cell cycle profile of GFP-positive cells was determined 48 h after electroporation and depicted is the increase in the G_1 population compared to control electroporations. The data shown are representative for multiple experiments performed in several independently established MEF cultures. (**b**) Rat1A cells, p27/p21 heterozygous and DKO MEFs were electroporated with 1.5 μg H2B-GFP in combination with 10 μg Rc/CMV (−), pCMVMadMyc (MM) or pCMVCDK2DN. Forty-eight hours after electroporation GFP-positive cells were isolated using a FACS sorter, lysed and per sample 25 μg of cell extract was used in a cyclin E-associated kinase assay in which Histone H1 was used as a substrate. (**c**) Quantification of cyclin E-associated kinase activity determined as described under (**b**) in five independent experiments in DKO MEFs. Cyclin E-associated kinase activity was quantitated using a PhosphorImager

cultures derived from other crossings indicated that MadMyc arrests wild-type MEFs as efficiently as the *p21*$^{+/-}$; *p27*$^{+/-}$ or DKO MEFs depicted in Figure 1 (data not shown and Figure 3). As activation of MYC was shown previously to lead to an increase in cyclin E/cdk2 kinase activity through sequestration of p27^{kip1}, we asked whether MadMyc affected cyclin E-associated kinase activity in DKO MEFs. For this, transfected cells had to be selected by sorting on a FACS as the transfection efficiencies with the use of the electroporation technique were very low. This procedure yielded only very low numbers of cells, which precluded extensive analyses of the transfected primary cultures. We therefore limited our biochemical analysis of MadMyc-transfected cells to the determination of the cyclin E-associated kinase activity, using histone H1 as a substrate. Figure 1b shows that in DKO MEFs, expression of MadMyc does not lead to inhibition of cyclin E-associated kinase activity, in spite of the fact that these cells had entered into G1 arrest in response to MadMyc expression (Figure 1a). Surprisingly, in p27/p21 heterozygous MEFs and in wild-type MEFs, MadMyc did not affect cyclin E-associated kinase activity either (Figure 1b and data not shown). In five independent experiments, we observed that MadMyc reproducibly failed to inhibit cyclin E/cdk2 kinase activity in the DKO MEFs, whereas CDK2DN efficiently inhibited cyclin E-associated kinase activity (Figure 1c). We have shown earlier that in NIH3T3 cells expression of MadMyc does lead to inhibition of cyclin E-associated kinase activity (Berns *et al*., 1997). Consistent with this earlier finding, expression of MadMyc in the Rat1A fibroblast cell line also caused a significant reduction in cyclin E kinase activity (Figure 1b). Apparently, primary fibroblasts differ from established fibroblast cell lines in this respect, even though both undergo a similar G1 arrest in response to MadMyc. Possibly, the reduction in cyclin E kinase activity in established cell lines is a consequence of the arrest, rather than the causal event. We conclude that for the MadMyc-induced G1 arrest in primary MEFs the presence of both p27^{kip1} and p21^{cip1} and inhibition of cyclin E/cdk2 kinase activity are not necessary.

p27^{kip1} and p21^{cip1} are dispensable to MYC-induced S phase entry

Next, we asked whether expression of wild-type c-MYC in DKO MEFs affected their cell cycle distribution. To this end, DKO MEFs were infected with a retroviral vector that directs the synthesis of the tamoxifen-inducible MYCER protein (Eilers *et al*., 1989). The sensitivity of these cells to MYC-induced apoptosis made it impossible to study MYC-induced cell cycle entry in the absence of serum. Therefore we activated MYCER by tamoxifen addition to cells that were exponentially growing in the presence of 10% serum and studied effects on cell cycle distribution 24 or 48 h later by measuring BrdU incorporation. Table 1 shows that tamoxifen addition caused a significant S phase increase in both p27/p21 heterozygous and DKO MEFs that expressed MYCER, but not in control virus-infected cells. Again, similar data were obtained in non-littermate wild-type control MEFs (data not shown). Together, these data indicate that c-MYC can stimulate S phase entry in the absence of both p27^{kip1} and p21^{cip1}, and suggest that c-MYC has additional cell cycle targets that contribute to S phase induction by c-MYC.

The retinoblastoma family proteins are required for MadMyc G1 arrest

The finding that MadMyc caused a G1 arrest in p27/p21 DKO fibroblasts suggested that other components of the cell cycle machinery became rate limiting in these cells upon expression of MadMyc. To investigate this further, we co-expressed several potent cell cycle regulators together with MadMyc in DKO

MEFs and studied effects on cell cycle. In agreement with our previous studies, we found that wild-type c-MYC releases the MadMyc induced G1 arrest in DKO MEFs, suggesting that also in these cells MadMyc arrests by reducing c-MYC activity (Figure 2a). Significantly, expression of E1A, but not a mutant of E1A that is unable to bind to retinoblastoma family proteins (collectively known as pocket proteins), also rescued the MadMyc G1 arrest. As E2F transcription factors are probably the most relevant downstream targets of pocket proteins, these data suggested that E2F activity might be rate limiting in MadMyc-arrested DKO MEFs. As FACS sorting of transiently transfected cells yields only small numbers of cells, we used a replication-deficient adenovirus vector expressing MadMyc to address this issue. Figure 2b shows that adenovirus-directed MadMyc expression in primary MEFs reduced the expression of the E2F target gene *p107* (Hurford *et al*., 1997; Zhu *et al*., 1995), but not of *cyclin D2*. Moreover, expression of E2F1 resulted in a complete override of the MadMyc G1 arrest in DKO MEFs (Figure 2a).

To provide more evidence that regulation of E2F activity is important for MadMyc-induced G1 arrest, we turned to the use of MEFs in which one or more of the retinoblastoma family proteins had been inactivated. Consistent with our earlier data (Berns *et al*., 1997), $Rb^{-/-}$ MEFs arrested in response to MadMyc as efficiently as wild-type MEFs (approximately 22% G1 increase, Figure 3). However, in $Rb^{-/-}$; $p107^{-/-}$ double knockout MEFs, MadMyc was less efficient (14% G1 increase). Finally, in $Rb^{-/-}$; $p107^{-/-}$; $p130^{-/-}$ triple knockout (TKO) MEFs, MadMyc became very ineffective in inducing G1 arrest (8% increase, Figure 3). It is unlikely that the reduced sensitivity of the TKO MEFs to MadMyc is caused by a general failure of cell cycle control mechanisms in these cells, as the TKO MEFs were still efficiently arrested by CDK2-DN (Figure 3a) or pRb (data not shown). Therefore it can be concluded that MadMyc requires the presence of all three pocket proteins for its cell cycle-inhibitory

Table 1 Induction of S phase by c-MYC in p27/p21 knockout MEFs

	LZRS-iresGFP				*LZRS-MYCER-iresGFP*			
	p27+/−p21+/−		*p27−/−p21−/−*		*p27+/−p21+/−*		*p27−/−p21−/−*	
	ΔS (%)	GFP^+ (%)	ΔS (%)	GFP^+ (%)	ΔS (%)	GFP^+ (%)	ΔS (%)	GFP^+ (%)
Experiment 1[a]	0	100	0	100	35	100	29	100
Experiment 2	0.5	87	0	84	21	65	21	70
Experiment 3	0	84	0.5	80	23.5	76	19.5	73
Experiment 4	1	62	2	58	12	53	16	49

[a]GFP-positive MEFs were sorted after infection. p27/p21 heterozygous and DKO MEFs were infected either with control LSRS-iresGFP or LZRS-MYCER-iresGFP retrovirus. The difference in the percentage of cells in S phase in the absence or presence of tamoxifen (ΔS) was determined by BrdU incorporation 24 (exp. 2) or 48 (exp. 1, 3, 4) h after induction. The percentage of GFP-positive cells (GFP^+) in the presence of tamoxifen is indicated. Depicted are the average values between duplicates and the error is in all cases less than 3%

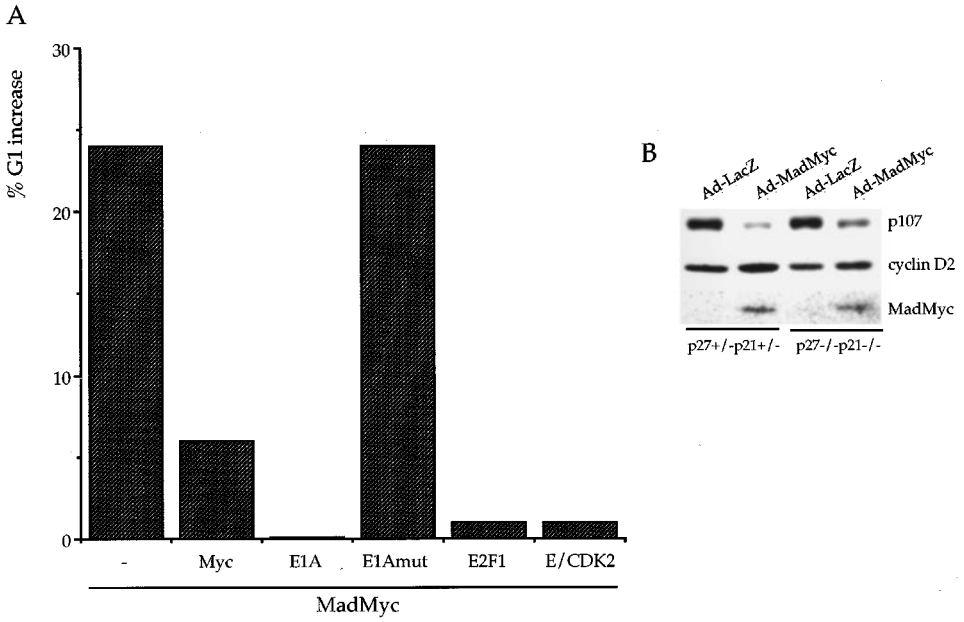

Figure 2 Rescue of the MadMyc G_1 arrest. (**a**) Early passage p27/p21 DKO MEFs were electroporated with 1.5 μg H2B-GFP together with 10 μg Rc/CMV (−), 4 μg pCMV-MYCHA, 6 μg pCMV-12SE1A, 6 μg pCMV-12SE1A(mut928/961), pCMV-E2F1/DP1 (4 and 1 μg each) or pCMV-cyclinE/cdk2 (3 μg each), in the absence or presence of 10 μg pCMVMadMyc. The cells were blocked with nocodazole. Forty-eight hours after electroporation the cell cycle profiles of GFP-positive cells was determined by FACS analysis. The increase in G_1 phase is calculated by subtracting per cent G_1 phase in the presence of MadMyc from the per cent of G_1 phase in the absence of MadMyc. The data are representative for multiple independent experiments. (**b**) p27/p21 heterozygous and DKO MEFs were infected with Ad-LacZ or Ad-MadMyc at a multiplicity of infection of 1000. Twenty-four hours after infection cells were lysed and equal amounts of protein lysates were subjected to Western analysis

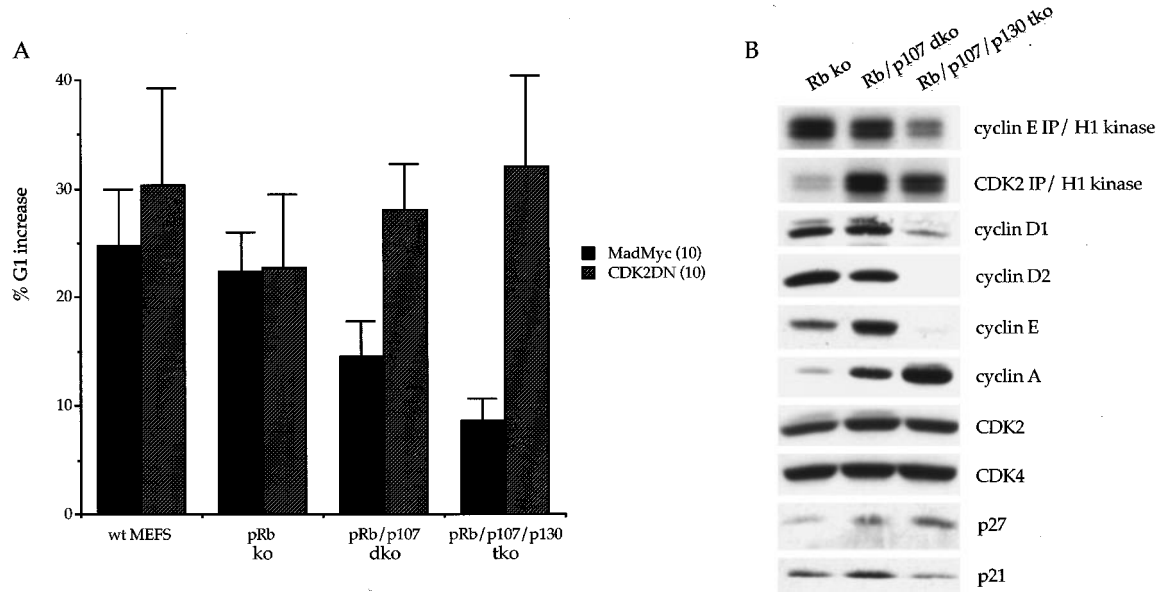

Figure 3 Pocket protein-dependency of MadMyc-induced G1 arrest. (**a**) Low passage wild-type MEFs (wt) or MEFs deficient for either pRb; pRb+p107 or pRb+p107+p130 were electroporated with 1.5 μg H2B-GFP together with 10 μg Rc/CMV, 10 μg pCMV/MadMyc or 10 μg pCMV-CDK2DN. Cells were blocked with nocodazole. Forty-eight hours after electroporation the cell cycle profile of the GFP-positive cells was determined and depicted is the increase in G_1 population compared to the control transfections. The data shown are an average of four experiments performed with different MEF isolates. (**b**) MEFs deficient for either pRb, pRb+p107 or pRb+p107+p130 were lysed at comparable passage numbers and equal amounts of protein were subjected to Western analysis. The same lysates were used in both cyclin E as cdk2-associated kinase assays in which histone H1 was used as a substrate

activity. As all three pocket proteins interact with E2Fs, it is likely that progressive loss of pocket proteins leads to an incremental increase in E2F activity. Indeed, the TKO MEFs have a larger percentage of cells in S phase accompanied by higher cyclin A protein levels, which is indicative for increased E2F activity (Figure 3b). It is somewhat surprising that, judging from the protein levels, another E2F target, *cyclin E*, appears to be expressed at a low level in these cells. Possibly, as a result of high E2F activity in the TKO cells, the cyclin E protein requirement is reduced. It is also possible that cyclin E protein turnover is increased in TKO cells, resulting in low protein levels in the presence of high mRNA levels. A more detailed analysis of these cells will be required to address this issue. Nevertheless, it is likely that the TKO MEFs have higher E2F activity, which reduces their sensitivity to MadMyc. Furthermore, the observation that TKO MEFs with low cyclin E-associated kinase activity are resistant to MadMyc-induced G1 arrest provides additional support for the notion that cyclin E-associated kinase activity is not a primary target of c-MYC (Figure 3b).

Discussion

Unraveling the mechanism of MYC-induced G1 progression has received much attention and has led to the prevailing view that MYC regulates cyclin E-associated kinase activity via sequestration of $p27^{kip1}$. We have used a genetic approach to investigate whether $p27^{kip1}$ is a critical target in both MYC-induced proliferation and MadMyc-mediated cell cycle arrest. Introduction of MYC or MadMyc in p27/p21 double knockout (DKO) MEFs have clearly demonstrated that p27 and p21 are both dispensable for MYC and MadMyc function. Surprisingly, our data also showed that reduction of cyclin E-associated kinase activity is not an essential step in a MadMyc-induced G1 arrest in primary cells. Together, these findings strongly suggest that MYC has other critical targets besides $p27^{kip1}$ and cyclin E-associated kinase activity. At first glance, this conclusion seems to be at odds with our earlier observation that expression of cyclin E/cdk2 could override a MadMyc-induced G1 arrest in NIH3T3 cells (Berns *et al*., 1997). However, also in p27/p21 DKO MEFs, in which MadMyc does not cause a reduction in cyclin E-associated kinase activity, ectopic expression of cyclinE/cdk2 could override the MadMyc G1 arrest (Figure 2). Thus, even though cyclin E/cdc2 is not a primary target of MadMyc in these cells, ectopic expression rescued the cell cycle arrest. These data highlight that override-of-growth-arrest experiments must be interpreted with caution: that a gene can override a G1 arrest is no formal proof that this gene is a critical target of the growth arrest-inducing agent, as it is expressed at non-physiological levels. This notion is underscored by the observation that overexpression of either cyclin E or E2F1, which act in part through independent pathways, can compensate for the absence of the other in a short-term assay (Leone *et al*., 1999). Possibly, also in our experiment, overexpression of cyclin E complexes compensated for another activity that became rate-limiting as a result of MadMyc expression.

The present data do not unambiguously elucidate the nature of the rate-limiting activity that causes MadMyc G1 arrest in the presence of high cyclin E-associated kinase activity. Nevertheless, several lines of evidence suggest that E2F activity is targeted by MadMyc. First, ectopic E2F1 expression overruled a MadMyc G1 arrest (Figure 2a). Second, adenovirus-directed MadMyc expression in MEFs reduced the transcription of the

E2F target gene *p107* (Figure 2b). Third, MEFs deficient for all three members of the retinoblastoma protein family, having high 'physiological' E2F activity, were reduced in their sensitivity to MadMyc G1 arrest (Figure 3a). This latter experiment only indicates that the pocket proteins are required to establish a MadMyc G1 arrest, but does not reveal whether MadMyc acts upstream or downstream of the pocket proteins to establish cell cycle arrest. Our finding that MadMyc expressing cells arrest in G1 with high cyclin E-associated kinase activity argues against the notion that MadMyc acts upstream of the pocket proteins. We therefore favor the hypothesis that MadMyc directly acts to inhibit expression of one or more E2Fs. That Myc indeed can regulate expression of specific E2F genes was recently demonstrated (Sears *et al*., 1997). In contrast to this model, recent data have shown that MadMyc has no effect on a $6\times$E2F-luciferase reporter in U2-OS cells, suggesting that MadMyc is not directly involved in E2F regulation (Santoni-Rugiu *et al*., 2000). This apparent discrepancy may reflect a difference between U2-OS cells and MEFs. However, we feel it is more likely that MadMyc regulates a specific subset of E2Fs rather than reducing overall E2F activity, which may have been missed in their analyses. That small changes in overall E2F activity can have dramatic effects on cell proliferation was recently demonstrated: whereas E2F3 only represents some 5–10% of total cellular E2F activity, E2f3 knockout MEFs have a severe defect in cell proliferation (Humbert *et al*., 2000). Finally, our conclusion that MadMyc targets E2F activity is supported by experiments performed in established p27 knockout cell lines, which indicated that MYC can induce E2F activity in the absence of cyclin E regulation (M. Eilers, submitted for publication).

In conclusion, our data indicate that regulation by c-MYC of cyclin E/cdk2 kinase activity through $p27^{kip1}$ is not the rate-limiting step in G1–S phase transition. Presumably, regulation of E2F activity is involved through an as yet unresolved mechanism. Our override experiments suggest that in short-term assays over-expressed cyclin E/Cdk2 complexes can compensate for reduced E2F activity. From these observations and those of others a picture begins to emerge in which c-MYC regulates several independent pathways that together regulate G1–S phase progression. In favor of this multiple target model is our recent finding that in a long-term growth assay, besides c-*Myc* and N-*Myc*, no single gene can rescue the growth defect of c-*myc* null fibroblasts (Berns *et al*., 2000).

Materials and methods

MEFs

Embryos with targeted disruptions of both p27 and p21 genes were produced by intercrossing the individual knockout strains (Deng *et al*., 1995; Fero *et al*., 1996). MEFs were prepared from day 13.5 mouse embryos and frozen at passage 1. For the generation of MYCER expressing clones, passage 1 MEFs were plated at a density of 5×10^5 cells per 10 cm dish. Infection with LZRS-iresGFP or LZRS-MYCER-iresGFP was carried in two rounds with fresh retroviral supernatant produced in Phoenix cells. The generation of MEFs deficient for combinations of pocket proteins will be described elsewhere (J.H. Dannenberg and H.P.J. te Riele, in preparation). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) in the presence of 10% fetal calf serum supplemented with penicillin/streptomycin.

Plasmids and antibodies

The plasmids pCMVMadMyc, pCMVMadMycDC, pCMVMycHA, pCMV-cyclinE, pCMV-CDK2, pCMV-CDK2DN, pCMV-E2F1, pCMV-Dp1, pCMV-12SE1A, pCMV-12SE1A (928/961), H2B-GFP, LZRS-iresGFP and LZRS-MYCER-iresGFP were described previously (Berns *et al*., 1997; Hateboer *et al*., 1996; Jacobs *et al*., 1999; Kanda *et al*., 1998). The following antibodies for detection in Western were used: against p107: C18 (Santa Cruz: sc); MadMyc: 9E10 (sc); cyclin D1: 72-13G (sc); cyclin D2: M20 (sc); cyclin E: M20 (sc); cyclin A: C19 (sc); CDK2: M2 (sc); CDK4: C22 (sc); p27: K25020 (Transduction Laboratories) and p21: C19 (sc). For the IP-kinase assays we used M20 (sc) and M2 (sc) for cyclin E and CDK2 immunoprecipitations respectively.

Electroporation of MEFs

1×10^6 MEFs were resuspended in 100 μl electroporation buffer containing 2 mM HEPES pH 7.2, 15 mM K_2HPO_4/KH_2PO_4, 250 mM Mannitol and 1 mM $MgCl_2$. In total, 15 μg of DNA was added to the cell suspension which was transferred to a 0.1 cm gene pulser cuvette (BIO-RAD) and pulsed with Gene Pulser II apparatus and Gene Pulser II RF module (BIO-RAD) for 15 bursts at 140 V. Five minutes after electroporation, cells were seeded in a 10 cm dish.

Cell cycle analysis

Passage 2–3 MEFs were transfected using electroporation. To select for transfected cells, H2B-GFP was co-transfected. Thirty-two hours after transfection, nocodazole (1 μg/ml) was added and the cells were incubated for another 16 h at 37°C. For FACS analysis, cells were trypsinized, washed and resuspended in 250 μl solution containing 0.1% Triton X-100 (Sigma) and 50 μg/ml propidium iodide (Sigma) in PBS and incubated for 1 h at 4°C. The FACS analysis was performed on a Becton-Dickinson FACScan flow cytometer and percentages of GFP-positive cells within the different phases of the cell cycle were determined with the computer program ModFit. For the first BrdU labeling experiment with MEFs infected with LZRS-iresGFP or LZRS-MYCER-iresGFP, the GFP positive cells were sorted on a MoFlo FACS-sorter to ensure that all cells expressed MYCER. Since infection efficiencies ranged between 80–95% (without induction) the sorting step was omitted in subsequent experiments. For the labeling, cells were seeded in duplicate at a density of $1-2\times10^5$ cells per 6 well. The next day, the medium was replaced by fresh DMEM with or without 250 nM tamoxifen. MEFs were labeled with 10 μM BrdU (Sigma) 24 or 48 h later for 1 h. After harvest, cells were fixed and stained with FITC-conjugated Anti-BrdU monoclonal antibody (Becton Dickinson), according to a standard protocol (Boehringer Mannheim) and resuspended in PBS containing 20 μg/ml of propidium iodide. The percentage of cells in S phase was determined by FACS with the computer program Cell Quest.

Western analysis and kinase assays

Cells were lysed in ELB buffer supplemented with protease inhibitors for 30 min on ice and were centrifuged at 14 000 r.p.m. for 15 min at 4°C. Protein concentrations were determined with BIO-RAD protein-assay, and equal amounts of proteins were separated by SDS–PAGE and blotted onto nitrocellulose. Immunodetection was performed as described (Berns *et al*., 1997). For the kinase-assays on transfected cells, GFP-positive cells were selected on a FACS-sorter 48 h after electroporation. Immuno-complex kinase assays on sorted or

non-sorted cells were performed according to Dulic *et al*. (1992) using the anti-cyclin E (M20) or the anti-CDK2 (M2) antibodies and histone H1 (Boehringer Mannheim) as a substrate.

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