Oncogene (1997) 15, 1347–1356

Repression of c-Myc responsive genes in cycling cells causes G_1 arrest through reduction of cyclin E/CDK2 kinase activity

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The c-*myc* gene encodes a sequence-specific DNA binding protein involved in proliferation and oncogenesis. Activation of c-*myc* expression in quiescent cells is sufficient to mediate cell cycle entry, whereas inhibition of c-*myc* expression causes cycling cells to withdraw from the cell cycle. To search for components of the cell cycle machinery that are targets of c-Myc, we have made a mutant c-Myc protein, named MadMyc, that actively represses c-*myc* target genes. Expression of MadMyc in cycling NIH3T3 cells causes a significant accumulation of cells in G_1. The MadMyc-induced G_1 arrest is rescued by ectopic expression of cyclin E/CDK2 and cyclin D1/CDK4, but not by Cdc25A, a known cell cycle target of c-Myc. The MadMyc G_1 arrest does not require the presence of a functional retinoblastoma protein and is associated with a strong reduction in cyclin E/CDK2 kinase activity in arrested cells. MadMyc does not cause alterations in the expression levels of cyclin E, CDK2, $p27^{kip1}$, cyclin D1 or CDK4 in G_1-arrested cells. These data indicate that inhibition of c-Myc activity in exponentially growing cells leads to G_1 arrest through loss of cyclin E-associated kinase activity.

Keywords: c-Myc; cell cycle; cyclin; CDK

Introduction

The c-*myc* proto-oncogene belongs to a family of related genes implicated in the control of normal proliferation and the induction of neoplasia (Henriksson and Luscher, 1996). *Myc* expression is activated by mitogenic signals (Kelly *et al*., 1983) and is suppressed by growth-inhibitory signals (Einat *et al*., 1985; Pietenpol *et al*., 1990; Alexandrow *et al*., 1995), but is invariant in exponentially growing cells (Hann *et al*., 1985; Thompson *et al*., 1986). Activation of c-*myc* is sufficient to induce cell cycle entry in quiescent cells (Eilers *et al*., 1991). Moreover, constitutive expression of c-*myc* inhibits differentiation and prevents cells from leaving the cell cycle (Coppola and Cole, 1986; Freytag, 1988). Conversely, inhibition of c-*myc* expression using an antisense approach leads to growth arrest and induction of differentiation (Heikkila *et al*., 1987; Prochownik *et al*., 1988; Biro *et al*., 1993).

The c-*myc* gene encodes a transcription factor of the helix–loop–helix-zipper class, which must dimerize with its partner Max, in order to bind DNA and transactivate from specific Myc sites (Blackwood and Eisenman, 1991; Kretzner *et al*., 1992). The growth-stimulatory activity of c-Myc depends on its ability to act as a transcription factor (Stone *et al*., 1987; Goruppi *et al*., 1994). This suggests that Myc regulates the activation of genes involved in cell proliferation. Several genes have been identified as direct c-Myc target genes, including ornithine decarboxylase, α-prothymosin, *cad*, eIF-4E, eIF-2α, plasminogen activator inhibitor 1 and *cdc25A* (Prendergast *et al*., 1990; Eilers *et al*., 1991; Bello-Fernandez *et al*., 1993; Rosenwald *et al*., 1993; Miltenberger *et al*., 1995; Galaktionov *et al*., 1996). However, with the possible exception of Cdc25A (Galaktionov *et al*., 1996), none of these genes provide a good explanation how c-Myc exerts its strong effect on the cell cycle.

A frequent event during the process of cellular differentiation is a reduction in c-Myc protein levels and a concomitant increase in Mad or Mxi proteins (Ayer and Eisenman, 1993; Larsson *et al*., 1994; Hurlin *et al*., 1995a,b). Mad and Mxi proteins also interact with Max and Mad:Max and Mxi:Max heterodimers bind to the same CACGTG Myc:Max recognition sites. However, Mad:Max and Mxi:Max heterodimers repress transcription by ternary complex formation with the mammalian homologue of the yeast repressor SIN 3 (Ayer and Eisenman, 1993; Ayer *et al*., 1993, 1995; Zervos *et al*., 1993; Schreiber-Agus *et al*., 1995), reviewed by Bernards (1995). Indeed, when Mad is ectopically expressed in exponentially growing cells, an inhibition of cell proliferation is observed (Chen *et al*., 1995; Västrik *et al*., 1995) and cells accumulate in the G_1 phase of the cell cycle (Roussel *et al*., 1996). Consistent with opposing activities of Myc and Mad family proteins, expression of Mad inhibits Myc-mediated transformation (Lahoz *et al*., 1994; Cerni *et al*., 1995; Koskinen *et al*., 1995; Schreiber-Agus *et al*., 1995) and mediates repression of c-Myc target genes (Wu *et al*., 1996).

Progression through the cell cycle depends on the activation of cyclin dependent kinases (CDKs) and their regulatory subunits, the cyclins (reviewed by Sherr, 1996). To pass the restriction point in late G_1, which commits the cells to complete a mitotic division cycle, the activities of cyclin D/CDK4 (or CDK6) and cyclin E/CDK2 are required to hyperphosphorylate and thereby inactivate the retinoblastoma protein, pRb (see Beijersbergen and Bernards, 1996 for a review). The pRb family proteins p107 and p130 are also CDK substrates (Beijersbergen *et al*., 1995; Mayol *et al*., 1996). Hypophosphorylated pRb family proteins bind to and inactivate members of the E2F/DP family of transcription factors. The E2F/DP family members positively activate genes whose products are required for cell cycle progression (Beijersbergen and Bernards, 1996).

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Received 13 February 1997; revised 14 May 1997; accepted 14 May 1997

The activity of CDK complexes depend of their expression levels, association with cyclins, phosphorylation status and the association with specific CDK-inhibitors (CKIs) (Sherr, 1993; Sherr and Roberts, 1995). The critical role of cyclin/CDK complexes in G_1-S progression make them plausible candidates to be regulated by c-Myc. Previous studies on the role of c-Myc in the transcriptional regulation of cyclins appear sometimes inconsistent. For example, A-type cyclin and E-type cyclin transcription is enhanced (strongly *versus* moderately) in response to c-Myc (Jansen-Durr *et al*., 1993), whereas no significant changes were observed in the amounts of cyclin E and D1 in other reports (Hermeking *et al*., 1995; Steiner *et al*., 1995; Vlach *et al*., 1996). Furthermore, it seems that the c-Myc effects on D-type cyclins are cell type-dependent (Jansen-Durr *et al*., 1993; Solomon *et al*., 1995; Marhin *et al*., 1996). Nevertheless, several recent studies indicate that activation of c-*myc* causes a rapid induction of G_1 cyclin/CDK kinase activity (Steiner *et al*., 1995; Vlach *et al*., 1996), indicating that c-Myc indeed targets the G_1 cyclin/CDK complexes. However, the precise mechanism by which Myc acts to control cyclin/CDK complex activity is still unclear.

In this work, we have further studied cell cycle targets of c-Myc. We show that inactivation of Myc activity in exponentially growing cells causes cells to arrest in G_1 as a result of a defect in cyclin E/CDK2 kinase activity.

Results

MadMyc expression causes exponentially growing NIH3T3 cells to arrest in G_1

To ask which components of the cell cycle machinery are targets of c-Myc, we constructed a strong antagonist of c-Myc activity. Even though it is well-established that Mad:Max and Myc:Max complexes can bind to the same CACGTG motif in transient transfection experiments, it is not clear whether under physiological conditions Myc:Max and Mad:Max heterodimers also bind to exactly the same sites. Therefore, to create a strong repressor of c-Myc-responsive transcription, we removed the amino terminal transactivation domain of c-Myc and replaced it with the amino terminal transcriptional repression domain of Mad, thus generating MadMyc (Figure 1a). This chimeric protein contains the complete DNA binding and dimerization domains of c-Myc. It is therefore likely that MadMyc will bind to the same sites in the genome that are normally occupied by wild type c-Myc, thereby causing active repression of c-Myc-responsive genes.

To study the effect of MadMyc on cell cycle, we co-transfected exponentially growing NIH3T3 cells with the MadMyc expression vector and a CD20 expression vector. After 48 h, transfected cells were detected by flow cytometry with anti CD20 antibody. Cell cycle distribution was measured by propidium iodide staining of DNA as described (Beijersbergen *et al*., 1995).

Figure 1b shows that transfection of MadMyc caused a significant accumulation of cells in G_1 (22% increase, compared with a 44% increase seen with p27). Because cycling NIH3T3 cells have a large G_1 population, a

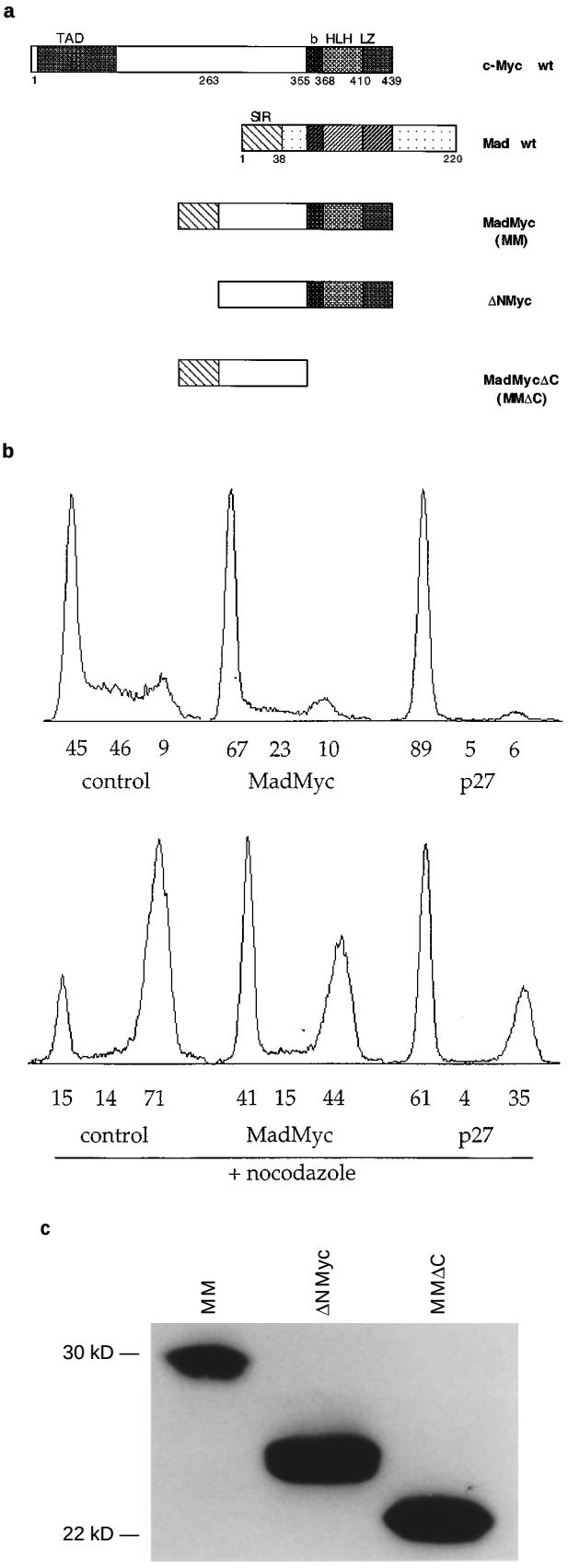

Figure 1 Cell cycle arrest by MadMyc proteins. (**a**) Schematic representation of Mad-Myc fusions proteins. MadMyc was generated as a potent repressor of c-Myc function. For MadMyc, amino acids 1–263 of wild type c-Myc was replaced by amino acids 1–38 (the Sin 3-interaction region) of Mad. ΔNMyc is c-Myc wild type deleted for amino acids 1–263 and MadMycΔC is MadMyc deleted for amino acids 355–439. Abbreviations: TAD, transcription activation domain; b, basic

specific G_1 arrest is more readily visualized by treating transfected cells with nocodazole 16 h prior to analysis (ven den Heuvel and Harlow, 1993). Nocodazole treatment causes cells to arrest in the G_2/M phase of the cell cycle, unless a specific block prevents transfected cells from reaching G_2/M. Cells transfected with CD20 alone and treated with nocodazole arrested efficiently in G_2/M, whereas co-transfection with either MadMyc or p27 caused a specific G_1 increase of 26 and 46%, respectively (Figure 1b). In multiple independent experiments, we observed that expression of MadMyc reproducibly caused an absolute increase of between 20 and 30% in the G_1 population. Consistent with this result obtained in transiently-transfected cells, MadMyc suppressed growth of stably transfected NIH3T3 cells efficiently. An eightfold reduction in the amount of puromycin-resistant colonies was observed when MadMyc expression vector was co-transfected when compared to puromycin vector alone (Table 1a).

To test whether the growth-inhibitory function of MadMyc depended on specific DNA binding and on the presence of the Mad repression domain, two additional mutants were generated; ΔNMyc lacks the Mad repression domain, MadMycΔC lacks the DNA-binding/heterodimerization domain (Figure 1a). Indeed, both mutants (both of which were expressed at high levels, Figure 1c) failed to induce a significant G_1 arrest and did not suppress colony outgrowth as efficiently as MadMyc (Table 1a,b). These observations indicate that sequence-specific DNA binding and transcriptional repression by MadMyc are required to cause a G_1 cell cycle arrest.

Table 1 Growth-inhibitory activity of MadMyc proteins

A. *Colony formation assay NIH3T3*

Experiment:	*No of colonies* I	II
Control	204/160	172/164
MadMyc	26/27	18/20
ΔNMyc	100/80	128/148
MMΔC	100/96	102/108

B. *Cell cycle profile MadMyc mutants*

	Phase G_0/G_1	S	G_2/M
Control	35	8	57
MadMyc	63	14	23
ΔNMyc	42	9	49
MMΔC	33	8	59

A. Colony formation assay with NIH3T3 cells. NIH3T3 cells seeded in 100 mm dishes were transfected with 20 μg of the indicated expression plasmids together with 1 μg of the pCMVBabe-Puro vector. After 16 h the cells were washed twice with DMEM/10% newborn calf serum. Twenty-four hours after transfection, medium was replaced by medium containing 8 μg/ml puromycin. After 2 weeks, cells were fixed and stained and the number of puromycin resistant colonies was counted. The experiments were performed in duplicate. B. Cell cycle profile of MadMyc mutant-transfected cells. NIH3T3 cells were transfected with pCMV-CD20 (4 μg) in combination with pRc-CMV or MadMyc, ΔNMyc and MMΔC expression vectors (15 μg). Cells were washed and blocked with Nocodazole as described in Materials and methods. Transfected cells were analysed by FACS and the cell cycle profile of CD20 positive cells was determined. The percentage of cells in G_0/G_1 phase, S phase and G_2/M phase of the cell cycle were determined with the computer program Modfit, and the average of three experiments is depicted

MadMyc antagonizes c-Myc

To test whether MadMyc causes repression of a *bona fide* c-*myc* target gene, ornithine decarboxylase (Bello-Fernandez *et al*., 1993), we used a reporter plasmid that carries the ornithine decarboxylase promoter and first intron (that harbors the c-Myc binding sites) linked in frame in exon 2 to chloramphenicol acetyl transferase (CAT). Figure 2 shows, as expected, that this ODC-CAT reporter gene is activated significantly by expression of c-Myc (Bello-Fernandez *et al*., 1993). Co-expression of c-Myc and increasing amounts of MadMyc caused a concentration-dependent inhibition of the reporter gene construct, whereas expression of MadMyc alone repressed ODC-CAT levels below basal activity (Figure 2). These data support the notion that MadMyc antagonizes c-Myc function by repressing the expression of c-Myc target genes.

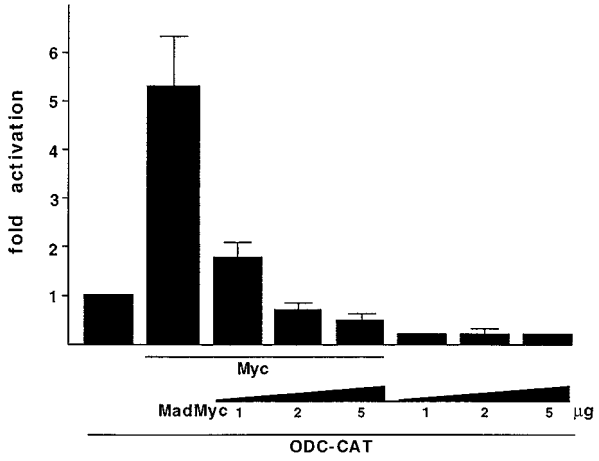

Figure 2 Transcription-repression by MadMyc of the ODC-CAT promoter, NIH3T3 cells were transfected with the ODC-CAT reporter alone or co-transfected with reporter plasmid and pJ3-c-*myc* (5 μg) together with increasing amounts of the MadMyc expression vector. Cells were harvested 48 h after transfection, and cells extracts were analysed for CAT activity as described. pCMV-luciferase was included as an internal control. CAT activities were corrected with the internal control and the basal activity of the reporter was arbitrarily set at one

To further investigate whether MadMyc acts by antagonizing c-Myc function, we asked whether the MadMyc-induced G_1 arrest could be overruled by expression of elevated levels of wild type c-Myc. Figure 3a shows that co-transfection of MadMyc and c-Myc expression vectors completely reversed the MadMyc-induced G_1 increase. Rescue of the MadMyc G_1 cell cycle arrest was only seen with certain concentrations

region; HLH, helix–loop–helix domain; LZ, leucine zipper motif; SIR, Sin 3-interaction region. (**b**) Effect of MadMyc on cell cycle progression. NIH3T3 cells were transfected with pCMV-CD20 (4 μg) in combination with pRc-CMV, MadMyc or p27 expression vectors (15 μg). Cells were treated with nocodazole (lower panel) as described in Materials and methods. Transfected cells were analysed by FACS and the cell cycle profile of CD20-positive cells was determined. The percentage of cells in G_0/G_1 phase, S phase and G_2/M phase of the cell cycle was determined with the computer program Modfit, and the average of three experiments is depicted below the DNA histograms. (**c**) Expression of MadMyc mutants. NIH3T3 cells were transfected with MadMyc, ΔNMyc and MadMycΔC expression vectors. Cell lysates were separated on a 12% SDS-polyacrylamide gel. The separated proteins were transferred to nitrocellulose and were detected by Western analysis with monoclonal antibody 9E10

of c-Myc expression vectors as too high a level of c-Myc induces apoptosis (Evan *et al*., 1992). Note that the c-Myc expression vector used in this experiment (pCMV-Myc) differs from the vector used in the ODC-CAT experiment (pJ3-Myc, driven by the SV40 early promoter). These data support the notion that MadMyc inhibits cell cycle progression through inhibition of wild type c-Myc function.

MadMyc induces G_1 arrest by targeting G_1 cyclin/kinase complexes

It has recently been shown that c-Myc activates the expression of Cdc25A, a gene that plays a role in the G_1 phase of the cell cycle (Galaktionov *et al*., 1996). To test whether suppression of Cdc25A is responsible for the MadMyc-induced G_1 arrest, we co-transfected MadMyc and Cdc25A expression vectors and analysed cell cycle distribution. Figure 3b shows that Cdc25A (which was expressed at elevated levels in transfected cells, Figure 3d) did not rescue the MadMyc-induced G_1 increase, indicating that MadMyc does not inhibit cell cycle progression primarily by suppression of Cdc25A expression.

Previous studies have shown that induction of c-Myc in quiescent cells activates G_1 specific cyclin/kinase complexes by as yet unknown mechanism(s) (Steiner *et al*., 1995; Rudolph *et al*., 1996). To investigate whether the MadMyc-induced G_1 arrest involves repression of the activity of G_1 cyclin/CDK complexes, we tested the ability of MadMyc to cause G_1 arrest in the presence of ectopically expressed cyclin E/CDK2 or cyclin D1/CDK4 complexes. Figure 3c shows that co-expression of both cyclin E/CDK2 and cyclin D1/CDK4

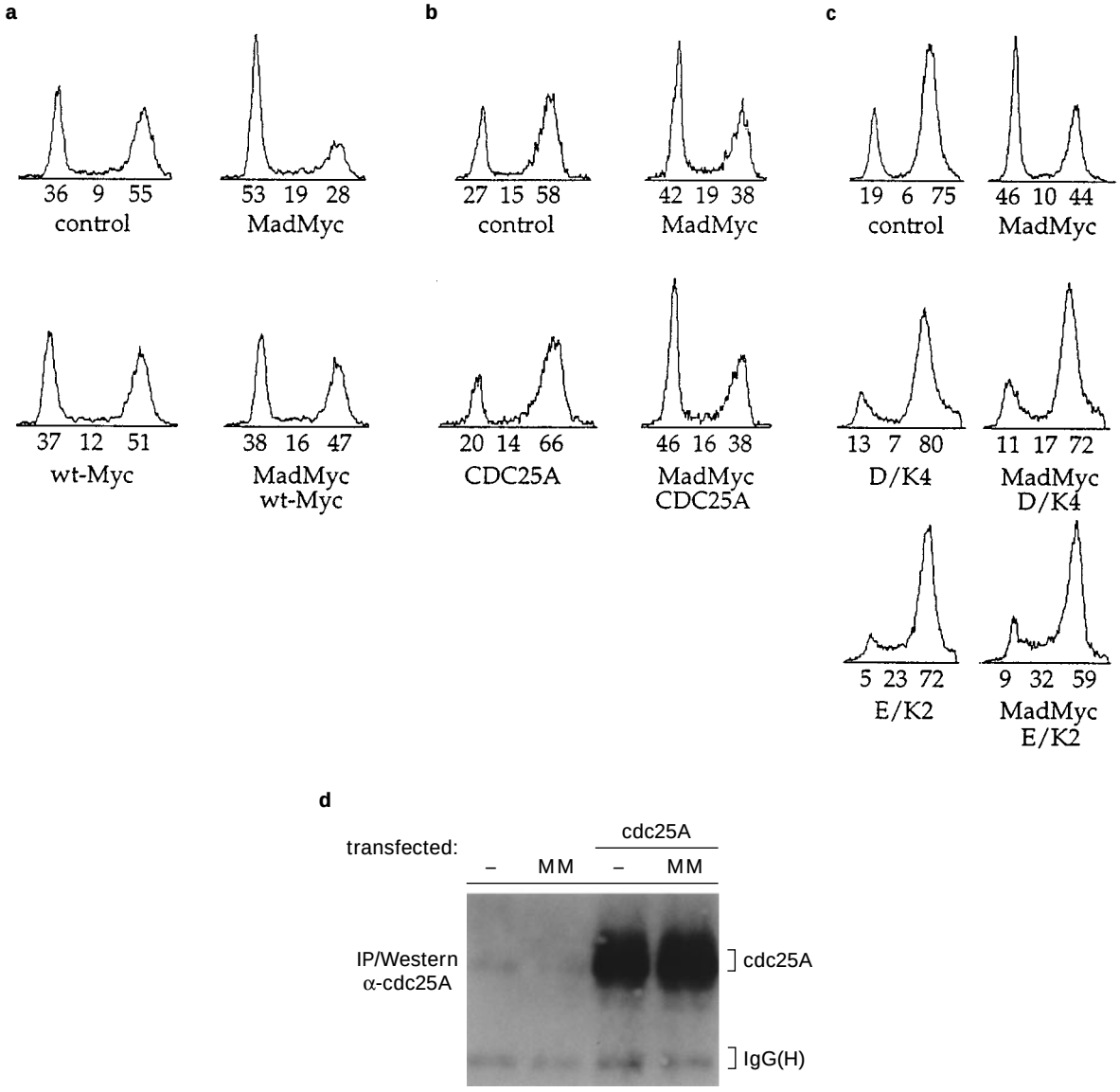

Figure 3 Rescue of the MadMyc G_1 arrest. NIH3T3 cells were transfected with 4 μg of pCMV-CD20 in combination with either 15 μg of control vector or 15 μg of pCMV-MadMyc together with (**a**) 1.5 μg pCMV-c-*myc* (**b**) 10 μg pCMV-Cdc25A (**c**) cyclin D1/CDK4 (6 and 3 μg each) or cyclin E/CDK2 (6 and 3 μg each) expression plasmids. Cells were washed and blocked with nocodazole as described. The cell cycle profile of CD20 positive cells and the percentage of cells in each stage were determined by FACS and the computer program Modfit. The average percentages of three experiments is depicted below the histograms. (**d**) Expression of Cdc25A in transfected cells. NIH3T3 cells were transfected with MadMyc expression vector or with pCMV-MadMyc (15 μg) and pCMV-Cdc25A (10 μg) expression vectors as indicated. Cdc25A protein levels were measured in an immunoprecipitation/Western blotting experiment

complexes completely abolished the MadMyc G_1 arrest. Expression of cyclin E/CDK2 did not affect the repression of the ODC-CAT reporter gene by MadMyc (Data not shown), indicating that cyclin E/CDK2 acts downstream of the MadMyc transcriptional repression.

To study further the effect of MadMyc on G_1 cyclin/kinase complexes, we measured the phosphorylation status of the G_1 cyclin/CDK substrates pRb and p107 in MadMyc-arrested cells. We co-transfected pRb or p107 with MadMyc and analysed the phosphorylation status of the pocket proteins by Western analysis. We observed a significant accumulation of both hypophosphorylated pRb and p107 when MadMyc was co-expressed (Figure 4a). Furthermore, when either cyclin E/CDK2 or cyclin D1/CDK4 was co-expressed with MadMyc, a complete reversal of the MadMyc-induced pRb hypophosphorylation was seen (Figure 4b). We conclude from these data that the lack of pRb phosphorylation in MadMyc-transfected cells is the result of a reduced G_1 cyclin/CDK kinase activity in these cells. Taken together, these data provide strong evidence that MadMyc-induced G_1 arrest is the result of a specific reduction in G_1 cyclin/CDK complex activity.

MadMyc -induced cell cycle G_1 arrest is independent of pRb

Next we asked whether the accumulation of hypophosphorylated pRb is responsible for the observed G_1 arrest in MadMyc-transfected cells. To test this, we transfected $pRb^{-/-}$ 3T3 cells with MadMyc expression vector and measured its effect on cell cycle distribution. Table 2a shows that MadMyc also induced a significant increase in G_1 cells in the $pRb^{-/-}$ cells. Similarly, stable expression of MadMyc suppressed colony outgrowth in cells lacking functional pRb (Table 2b). These data show that accumulation of hypophosphorylated pRb is not primarily responsible for the observed MadMyc G_1 arrest, which suggests the involvement of other cell cycle targets in the G_1 arrest.

Rescue of MadMyc cell cycle arrest by kinase-inactive G_1 cyclin complexes

It is well-established that $pRb^{-/-}$ cells do not require cyclin D1/CDK4 kinase activity to progress through a cell cycle (Koh *et al*., 1995; Lukas *et al*., 1995; Medema *et al*., 1995). Since the $pRb^{-/-}$3T3 cells were still sensitive to a MadMyc-induced G_1 arrest (Table 2a,b) it is likely that cyclin D-associated kinase activity is not a crucial target of MadMyc. Nevertheless, we did observe a complete reversal of the MadMyc induced G_1 increase upon ectopic expression of cyclin D1/CDK4 (Figure 3c). To study this apparent contradiction in more detail, we co-transfected MadMyc and cyclin D1 in the presence of increasing concentrations of a dominant negative mutant of CDK4 (CDK4-DN). We measured the activity of cyclin D-associated kinase activity in transfected cells by monitoring the phosphorylation status of p107 in transfected cells. Since p107 is phosphorylated only by cyclin D/CDK4 and not by other G_1 cyclin/kinase complexes (Beijersbergen *et al*., 1995), phosphorylation of p107 is a good indicator of cyclin D1/CDK4 kinase activity in transfected cells.

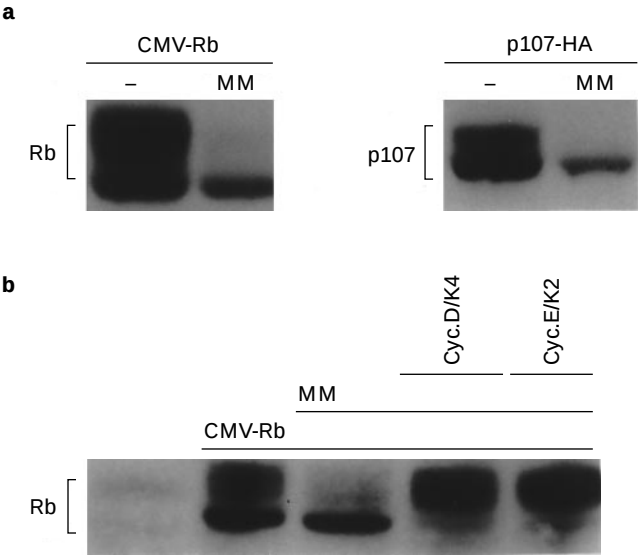

Figure 4 Effect of MadMyc on the phosphorylation of pRb and p107. (**a**) NIH3T3 cells were transfected with 3 μg pCMV-Rb or 5 μg pCMV-p107-HA in the absence (−) or presence (MM) of 15 μg of pCMV-MadMyc. Cells were lysed and cell extracts were subjected to low percentage SDS–PAGE. The separated proteins were transferred to nitrocellulose and were detected by Western analyses. (**b**) Co-transfected cyclin E/CDK2 and cyclin D/CDK4 complexes phosphorylate Rb in the presence of MadMyc

Table 2 MadMyc induces G_1 arrest in $Rb^{-/-}$ 3T3 cells and reduces colony outgrowth

A. *MadMyc induces G_1 arrest in $Rb^{-/-}$ 3T3 cells*

	G_0/G_1			Phase S			G_2/M		
Experiment:	*I*	*II*	*III*	*I*	*II*	*II*	*I*	*II*	*III*
Control	32.5	18.1	25.8	50.4	56.4	51.1	17.1	25.5	23.1
MadMyc (2 μg)	nd	23.3	33.2	nd	45.7	49.1	nd	31.0	17.7
MadMyc (5 μg)	nd	27.1	36.1	nd	44.2	46.4	nd	28.7	17.5
MadMyc (15 μg)	52.7	35.7	45.0	36.4	33.8	42.7	11.0	30.5	12.4

B. *Colony formation assay $Rb^{-/-}$ 3T3*

	No. of colonies		
Experiment:	*I*	*II*	*III*
Control	150	120	250
MadMyc	40	62	40

A. $Rb^{-/-}$ 3T3 cells were transfected with 4 μg pCMV-CD20 together with control vector or 2, 5 or 15 μg of pCMV-MadMyc. The cell cycle profile of CD20-positive cells and the percentage of cells in each stage were determined by FACS and the computer program Modfit. The percentages of cells in each stage were obtained from three independent experiments and are depicted in panel A. B. A colony formation assay was performed as described in Table 1A

Figure 5b shows that at increasing concentrations of co-transfected CDK4-DN, the phosphorylation of p107 decreased. At the highest concentration of CDK4-DN, p107 was almost completely hypophosphorylated, indicating that in these cells CDK4-DN inactivated most if not all the ectopically expressed cyclin D1. Nevertheless, in these cells, cyclin D1/CDK4-DN expression completely reversed the cell cycle-inhibitory effect of MadMyc (Figure 5a). These data indicate that the cyclin D1/CDK4 complex does not require kinase activity to rescue the MadMyc G_1 arrest.

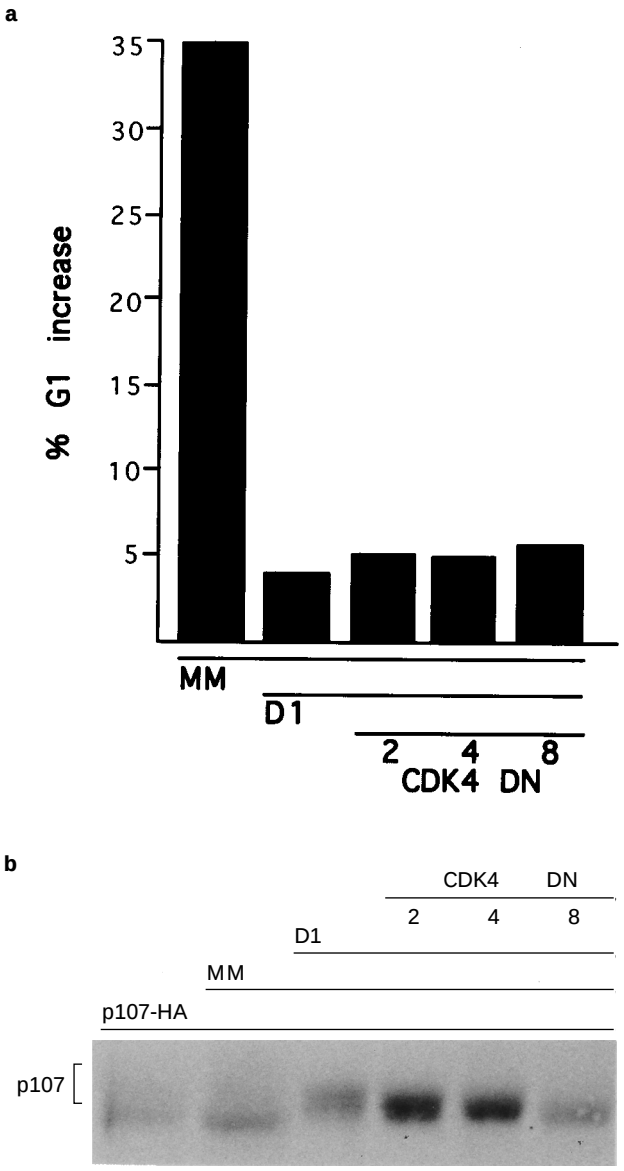

Figure 5 MadMyc G_1 arrest is rescued by inactive cyclin D1/CDK4 complexes. (**a**) NIH3T3 cells were transfected with 4 μg pCMV-CD20, cyclin D1 (2 μg) and an increasing amount of a CDK4-Dominant Negative (DN) expression vector in the presence or absence of pCMV-MadMyc (15 μg). The cells profile of the CD20 positive cells was determined as described. The increase in G_1 phase is calculated by subtracting % G_1 phase in the presence of MadMyc from the % of G_1 phase in the absence of MadMyc. The data are represenatative for at least three independent experiments. (**b**) In a parallel experiment, similar transfections were performed in the presence of pCMV-p107-HA. Cells were lysed and cell extracts were subjected to low percentage SDS–PAGE. The separated proteins were transferred to nitrocellulose and were detected by Western analysis with monoclonal antibody 12CA5

MadMyc-arrested cells have reduced E-associated kinase activity

The data shown above are compatible with a model in which MadMyc acts primarily to inhibit the activity of the cyclin E/CDK2 kinase complex. To test this, we co-transfected CD20 and MadMyc expression vectors in NIH3T3 cells, or with CD20 vector alone as a control. After 2 days, cells were stained with FITC-labeled CD20 antibody and transfected cells were collected using a Fluorescent Activated Cell Sorter (FACS). Flow sorted cells were analysed for the expression levels of G_1-cyclins, CDKs and CKIs by Western analysis. Figure 6a shows that MadMyc expression has no effect on expression levels of CDK4, CDK2, cyclin D1, cyclin E or $p27^{kip1}$, five cell cycle proteins involved in G_1 regulation. To test whether under these experimental conditions MadMyc affected the activity of cyclin E/CDK2 complexes, we performed a cyclin E-associated kinase assay on FACS-sorted cells. Figure 6b shows that expression of MadMyc resulted in a significant decrease in cyclin E-associated kinase activity (on average fivefold in three independent experiments). We conclude from this that MadMyc causes an arrest in G_1 cell cycle progression by inhibiting cyclin E-associated kinase activity without altering the expression levels or cyclin E, CDK2 or $p27^{kip1}$.

Discussion

MadMyc induces growth arrest

We have used a chimeric MadMyc protein to investigate which components of the cell cycle machinery are targets of c-Myc. The MadMyc protein used here has the DNA binding and Max dimerization domain of c-Myc but has the transcriptional repression domain of Mad instead of the Myc transactivation domain. Expression of MadMyc should therefore turn the c-Myc binding elements in Myc-responsive promoters from positive elements into negative elements, thereby causing active repression of c-Myc target genes. Indeed, when we tested MadMyc for its ability to modulate the activity of a *bona fide* c-Myc target, ornithine decarboxylase (ODC) (Bello-Fernandez *et al*., 1993), we found that MadMyc both caused repression of basal levels of ODC promoter activity and repressed the c-Myc-activated expression of the ODC promoter (Figure 2), thus confirming that MadMyc can mediate active repression of c-Myc target genes.

When transiently expressed in exponentially growing NIH3T3 cells, MadMyc caused a significant increase in the G_1 population (Figure 1b). In addition, stable expression of MadMyc prevented colony outgrowth of transfected cells (Table 1). Two lines of evidence indicate that this growth-inhibitory activity of MadMyc is the result of its ability to antagonize c-Myc function. First, mutants of MadMyc that lacked either the C-terminal DNA binding domain or the N-terminal Mad repression domain failed to cause an efficient G_1 arrest (Table 1). Second, inhibition of cell cycle progression by MadMyc was overruled by co-expression of wild type c-Myc, suggesting that MadMyc

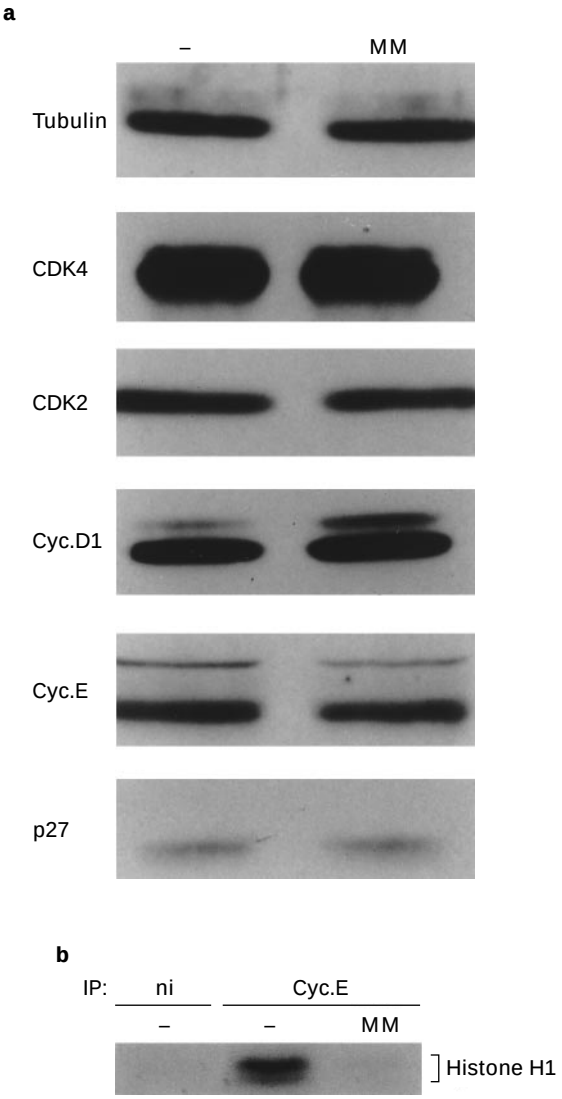

Figure 6 Western analysis and cyclin E kinase assay on FACS-sorted cells. (**a**) NIH3T3 cells were transfected with 4 μg pCMV-CD20 together with 15 μg control vector (−) or 15 μg pCMV-MadMyc (MM). Transfected cells were selected on a FACS sorter, lysed, and cell extracts were subjected to SDS–PAGE. The separated proteins were transferred to nitrocellulose and were detected by Western analysis. (**b**) The same cell lysates that were analysed by Western were used in a cyclin E-associated kinase assay in which Histone H1 was used as a substrate.

arrests cell cycle by limiting c-Myc activity in tranfected cells.

Mechanism of MadMyc growth arrest

MadMyc growth-arrested cells have hypophosphorylated pRb and p107, suggesting that the growth arrest is the consequence of a defect in G_1 cyclin/CDK kinase activity. Indeed, when we co-transfected MadMyc together with either cyclin E/CDK2 or cyclin D1/CDK4, we observed a complete reversal of the MadMyc G_1 arrest (Figure 3c). The transient transfection procedure used here did not allow the direct determination of the activity of cyclin D/CDK kinase complexes, as not enough transiently transfected cells can be selected by flow sorting to allow detection of cyclin D-associated kinase activity. Nevertheless, several lines of evidence indicate that although cyclin D/CDK4 activity may be affected by MadMyc, it is not the primary target of MadMyc. First, the MadMyc-induced cell cycle arrest was independent of the presence of a functional pRb protein, as $pRb^{-/-}$ 3T3 cells were also arrested by MadMyc (Table 2). Since $pRb^{-/-}$ cells do not require cyclin D/CDK4 activity (Koh *et al*., 1995; Lukas *et al*., 1995; Medema *et al*., 1995), it is unlikely that cyclin D-associated kinase activity is the primary target of MadMyc. That agents that do target cyclin D-associated kinase activity directly cause a pRb-dependent G_1 arrest was recently shown. A dominant-negative allele of Ha-*ras*, which specifically inhibits cyclin D1-associated kinase activity, can cause G_1 arrest in asynchronously growing 3T3 cells, but not $pRb^{-/-}$3T3 cells (Peeper *et al*., 1997). Second, expression of a kinase-inactive cyclin D1/CDK4 complex also rescued the MadMyc-induced growth arrest, indicating that the kinase activity of the complex is irrelevant for the rescue of the MadMyc G_1 arrest (Figure 5). Third, experiments which investigated signal transduction through the CSF-1 receptor suggested that c-Myc and cyclin D cross-regulate each other's expression, and do not function in a linear signaling pathway (Roussel *et al*., 1995). Furthermore, cyclin D can cooperate with c-Myc to induce B cell lymphomas in transgenic mice (Bodrug *et al*., 1994). Taken together, these data favor a model in which c-Myc and cyclin D cooperate in parallel pathways rather than in a linear pathway.

The rescue of the MadMyc G_1 arrest by cyclin E/CDK2 complexes raised the possibility that MadMyc affects the activity of cyclin E/CDK2 kinase. When we measured cyclin E-associated kinase activity we could indeed show a significant reduction in cyclin E-associated kinase activity in MadMyc-transfected cells (Figure 6). Interestingly, MadMyc did not affect the expression levels of cyclin E, CDK2 or the CKI p27, suggesting an indirect effect of MadMyc on the cyclin E/CDK2 kinase complex. Recent evidence indicate that the CDK-activating enzyme Cdc25A is an important target of Myc during cell cycle entry (Galaktionov *et al*., 1996). Reduction of Cdc25A expression by MadMyc and subsequent decrease in CDK2 activating activity may therefore account for the observed reduction in cyclin E/CDK2 kinase activity. However, we could not show a rescue of the MadMyc G_1 arrest by co-expression of Cdc25A expression vector that directs the synthesis of high levels of Cdc25A protein (Galaktionov *et al*., 1995) (Figure 3b,d). Furthermore, ectopically expressed cyclin E/CDK2 kinase is active in MadMyc-expressing cells, suggesting that Cdc25A is not limiting for activation of cyclin E/CDK2 in these cells (Figure 4b). Taken together, these data indicate that in cycling cells repression of c-Myc activity does not cause G_1 arrest primarily by depleting cells of Cdc25A. Consistent with this, activation of c-Myc in quiescent cells *in vivo* leads to only a partial activation of cyclin E/CDK2 complexes, which could be further activated by incubation with Cdc25A *in vitro*. (Steiner *et al*., 1995). Thus, activation of c-Myc alone is not sufficient to give full activation of Cdc25A, whereas inhibition of c-Myc does not appear to lead to a complete shut-off of Cdc25A activity.

Even though cyclin E/CDK2 efficiently rescued a MadMyc-induced G_1 arrest in transient transfection

experiments, our recent data indicate that MadMyc mediated inhibition of colony outgrowth (Tables 1 and 2) is not rescued by co-expression of cyclin E/CDK2 (K Berns and R Bernards, unpublished data). This indicates that, even though the immediate effects of MadMyc on cell cycle are mediated through cyclin E/CDK2, the long-term effects of MadMyc may involve multiple cellular targets, which do not become limiting in a short term transient transfection assay. Such additional targets that are affected by MadMyc in long term assays may include established c-Myc targets such as ornithine decarboxylase and Cdc25A.

Several other studies have recently provided independent evidence that c-Myc regulates the activity of the cyclin E/CDK2 kinase complex. Steiner *et al*., showed that activation of c-Myc in quiescent cells induced the activation of cyclin E-dependent kinases without significant changes in the amount of cyclin E/CDK2 complex. Activation of c-Myc resulted in a release of inactive cyclin E/CDK2 from a high molecular weight complex in lower molecular weight active complex (Steiner *et al*., 1995). More recent data indicate that c-Myc liberates cyclin E/CDK2 from inactive p27-containing complexes and it has been suggested that c-Myc acts by inducing sequestration of p27 away from cyclin E/CDK2 by a non-covalent interaction (Vlach *et al*., 1996). Similarly, it has been suggested that c-Myc overcomes a p53 G_1 arrest by sequestering p21 (Hermeking *et al*., 1995). In view of these data we speculate that the reduced cyclin E/CDK2 activity observed in MadMyc-arrested cells may be due to a decreased expression of an inhibitor of CKIs. Our data are consistent with the 'seqestration-of-CDK-inhibitor' model in that we show that kinase-inactive cyclin D1/CDK4 complexes, which have retained the ability to sequester CKIs, can also release cells from a MadMyc induced G_1 arrest. That cyclin D/CDK4 complexes can indeed act to sequester CKIs such as p27 away from cyclin E/CDK2 has recently been demonstrated (Polyak *et al*., 1994). The elucidation of the precise defect in cyclin E/CDK2 kinase activity described here requires biochemical experiments involving rather large numbers of cells which cannot be obtained from transiently transfected cells selected by flow sorting. Therefore, future research will focus on the study of MadMyc targets in other systems.

Materials and methods

Cell lines

NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% newborn calf serum. $pRb^{-/-}$ 3T3 cells (Zalvide and DeCaprio, 1995) were cultured in DMEM in the presence of 10% fetal calf serum. Transfections were performed using the calcium phosphate precipitation technique (Van der Eb and Graham, 1980).

Plasmids

The sequence coding for c-Myc was excised from pJ3-myc (Beijersbergen *et al*., 1994a) and cloned in pRc/CMV. For pCMVMadMyc, the N-terminal c-Myc coding region (amino acids 1–263) in pCMVc-*myc* was removed via a HindIII/ClaI digestion and replaced by a cDNA fragment encoding amino acids 1–38 of human Mad. The amino acid 1–38 Mad fragment was made by PCR using pJ3-Mad as a template.

pCMVΔNMyc was made by PCR using pCMVc-*myc* as a template, it encodes c-*myc* amino acids 263–440. pCMVMadMycΔC was constructed by PCR with a 5′mad primer and a 3′c-*myc* primer (at the position of amino acid 355) using pCMVMadMyc as a template. The mad(1–38)myc(263–355) fusion was cloned into a pcDNA3 vector containing a myc monoclonal antibody 9E10-tag at the 3′end, enabling detection of MadMycΔC protein with the 9E10 antibody. The plasmids pCMV-Rb, pCMV-107HA, pCMVCD20, pCMV-p27, pCMV-cyclin E, cyclin D, CDK2 and CDK4 were described previously (Beijersbergen *et al*., 1994b. 1995). pODC-CAT (kindly donated by Dr H van Steeg, RIVM, Bilthoven, The Netherlands) is a reporter plasmid containing 787 base pairs of the 5′ regulatory region of the human ODC gene, linked to exon 1 and intron 1 and part of exon 2 (78 bp), fused to CAT. pCMV-cdc25A was generated by cloning the coding sequence from human cdc25A (kind gift of Dr B Amati) in pRc/CMV.

Antibodies

The following antibodies for detection in Western were used: against human pRb: C15 (Santa Cruz, sc); p107HA: 12CA5 (directed against HA epitope); MadMyc, ΔNMyc, MadMycΔC: 9E10 (Evan and Hancock, 1985); cyclin D: anti-human D1 (UBI); cyclin E; M20 (sc); p27: C19 (sc) or K25020 (Transduction Laboratories); CDK2: M2 (sc); CDK4: C22 (sc). Ccd25A was immunoprecipitated with a c-terminal peptide antibody. Western blot analysis was done using antibodies against the Gst-Cdc25A fusion protein (kind gifts of Dr K Galaktionov). For the IP-kinase assays described here we used M20 (sc) for the cyclin E immunoprecipitations.

Cell cycle analysis

NIH3T3 cells were transfected using the calcium phosphate precipitation technique. After 16 h the cells were washed twice with DMEM/10% NCS. Twenty-four hours after transfection, nocodazole (50 ng/ml) was added and the cells were incubated for another 16 h at 37°C. The cells were harvested, washed with PHB (PBS, 20 mM HEPES, 0.1% BSA) and stained with 20 μl of FITC conjugated anti CD20 monoclonal antibody (Becton-Dickinson) for 1 h on ice. The cells were washed and fixed in 70% ethanol at 4°C. Before FACS analysis, the cells were washed with PHN (PBS, 20 mM HEPES, 0.5% NP-40), and incubated with 10 μg/ml propidium iodide and 250 μg/ml RNAse A. The FACS analysis was performed on a Becton-Dickinson FACScan flow cytometer. DNA content histograms of cells expressing transfected genes were obtained by selecting CD20-positive cells with a fluorescent intensity greater than the background level. The percentages of cells within the different phases of the cell cycle were determined with the computer program ModFit.

CAT assays

NIH3T3 cells were transfected with the expression vectors as indicated together with 4 μg pODC-CAT and 1.0 μg pCMV-luciferase. pRc/CMV was added to a total of 20 μg DNA per 100 mm dish. Cells were harvested 40 h after transfection and resuspended in 100 μl 0.1 M Tris-HCL. Cells were freeze/thawed three times and centrifuged at 4000 g for 10 min. Supernatants were assayed for CAT activity using the phase extraction method and luciferase activity (Promega, Luciferase system). CAT activity was normalised to luciferase activity.

Western blot analysis

Cells were lysed in SDS-containing sample buffer. The cell extracts were separated on SDS-polyacrylamide gels and

transferred to nitrocellulose and blocked in PBS/0.05% Tween-20/ 5% dried milk for 2 h at room temperature. The membrane was incubated with antibody diluted in PBS/ 0.05% Tween- 20/ 1% dried milk for 16 h at 4°C. After washing the membrane, the antibody was detected using horseradish peroxidase-linked goat or mouse IgG and enhanced chemiluminescence (ECL, Amersham). MadMyc, ΔNMyc and MadMycΔC were detected with 9E10 at 1:10 dilution, cyclin E with M20 in 1:500 dilution, cyclin D with anti-human D in 1:2000 dilution, human Rb with C15 in 1:4000 dilution, p107HA with 12CA5 in 1:10 dilution, p27 with C19 or K25020 in 1:500 dilution, CDK2 with M2 in 1:2000 dilution, CDK4 with C22 in 1:2000 dilution.

Cdc25A was detected in an immunoprecipitation/Western blotting experiment as described (Galaktionov *et al*., 1996).

Immune-complex kinase assays

Empty vector or pCMV-MadMyc together with pCMV-CD20 were transfected into NIH3T3 cells. After 16 h, the cells were washed and 40 h after transfection the cells were harvested, washed and stained with FITC conjugated anti CD20 monoclonal antibody. CD20-positive cells were selected on a FACS sorter. Selected cells were used to perform cyclin E immunoprecipitations and analysis of cyclin E-dependent kinase according to (Dulic *et al*., 1992) using the anti-cyclin E (M20) antibody and histone H1 as a substrate. Parallel cell lysates were used for Western blot analysis.

Acknowledgements

We thank Drs B Amati, M Eilers, K Galaktionov M, Pagano and H van Steeg for the gift of plasmids and antisera, Dr J DeCaprio for the gift of the $pRb^{-/-}$ cells, Dr R Beijersbergen for helpful discussions and E Noteboom for assistance with FACS sorting. This work was supported by a grant from The Netherlands Organization for Scientific research (NWO).

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