The EMBO Journal vol.13 no.17 pp.4080–4086, 1994

Interaction of c-Myc with the pRb-related protein p107 results in inhibition of c-Myc-mediated transactivation

Roderick L.Beijersbergen[1], E.Marielle Hijmans[1], Liang Zhu[2] and René Bernards[1]

[1]Division of Molecular Carcinogenesis, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands and [2]Division of Molecular Oncology, The Cancer Center of the Massachusetts General Hospital and Harvard Medical School, 149 13th Street, Charlestown, MA 02129, USA

Communicated by W.Moolenaar

The product of the c-*myc* proto-oncogene, c-Myc, is a sequence-specific DNA binding protein with an N-terminal transactivation domain and a C-terminal DNA binding domain. Several lines of evidence indicate that c-Myc activity is essential for normal cell cycle progression. Since the abundance of c-Myc during the cell cycle is constant, c-Myc's activity may be regulated at a post-translational level. We have shown previously that the N-terminus of c-Myc can form a specific complex with the product of the retinoblastoma gene, pRb, *in vitro*. These data suggested a model in which pRb, or pRb-related proteins, regulate c-Myc activity through direct binding. We show here that the pRb-related protein p107, but not pRb itself, forms a specific complex with the N-terminal transactivation domain of c-Myc *in vivo*. Binding of p107 to c-Myc causes a significant inhibition of c-Myc transactivation. Expression of c-Myc releases cells from a p107-induced growth arrest, but not from pRb-induced growth arrest. Our data suggest that p107 can control c-Myc activity through direct binding to the transactivation domain and that c-Myc is a target for p107-mediated growth suppression.

Key words: c-Myc/p107/pRb/transactivation

Introduction

The product of the c-*myc* proto-oncogene, c-Myc, is a nuclear phosphoprotein that binds to DNA in a sequence-specific fashion (Blackwood and Eisenman, 1991). Binding of c-Myc to DNA requires dimerization to a second protein, named Max, through a helix–loop–helix/leucine zipper motif that is present in both c-Myc and Max proteins. The N-terminus of c-Myc contains a conserved domain with strong transcription activation ability (Kato *et al.*, 1990). It is generally believed that c-Myc functions as a transcription factor that controls the activity of cellular genes that are involved in growth control. Consistent with the notion that c-Myc is a transcription factor with two functionally important domains is the demonstration by Stone *et al.* (1987) that mutations in both the N-terminal transactivation domain and the C-terminal DNA binding domain severely inhibit c-Myc's transforming activity. Further evidence that transformation by c-Myc closely parallels its transcriptional activity derives from a comparative study of c-Myc and L-Myc. The higher transformation activity of c-Myc was shown to correlate with the higher transactivation ability of c-Myc (Barrett *et al.*, 1992).

The activity of c-Myc is regulated at several levels. When quiescent cells are stimulated to enter the cell cycle by growth factor addition, the c-*myc* gene is transcriptionally activated (Kelly *et al.*, 1983). In contrast, when exponentially growing cells progress through a cell cycle, c-*myc* mRNA and protein levels appear to be invariant (Hann *et al.*, 1985; Thompson *et al.*, 1986). Several lines of evidence indicate that c-Myc activity is required for cell cycle progression (Einat *et al.*, 1985; Heikkila *et al.*, 1987; Eilers *et al.*, 1991). This may indicate that the activity of c-Myc during a normal cell cycle is regulated at a post-translational level. Evidence for post-translational regulation of c-Myc was obtained by several groups. For example, phosphorylation of a conserved serine residue in the c-Myc transactivation domain enhances the ability of c-Myc to activate transcription (Seth *et al.*, 1991; Gupta *et al.*, 1993). In addition, binding of the c-Myc–Max heterodimer to DNA can be modulated by other proteins that compete with c-Myc for its dimerization partner, Max (Ayer *et al.*, 1993).

We have shown that the transactivation domain of c-Myc or N-Myc can form a specific complex with the product of the retinoblastoma gene, pRb, *in vitro* (Rustgi *et al.*, 1991). These data raised the possibility that yet another level of regulation of c-Myc activity may exist: pRb, or pRb-related proteins, could modulate c-Myc activity through direct binding. We show here that not pRb itself, but the pRb-related protein p107 (Ewen *et al.*, 1991; Zhu *et al.*, 1993), can form a specific complex with the c-Myc transactivation domain *in vivo*. We show that c-Myc transactivation can be regulated through direct association with p107. Furthermore, we provide evidence that the p107-mediated growth suppression is mediated, at least in part, through the suppression of c-Myc transactivation.

Results

c-Myc interacts with p107 in vivo

To test whether pRb or the pRb-related protein p107 could interact with c-Myc *in vivo*, we transiently transfected the osteosarcoma cell line U-2 OS with either pRb and c-Myc expression vectors or with p107 and c-Myc expression vectors. Forty-eight hours after transfection the cells were labeled with [^{32}P]orthophosphate, lysed by sonication in non-ionic detergent and immunoprecipitated with antibodies against c-Myc, pRb or p107. Figure 1A (lanes 3

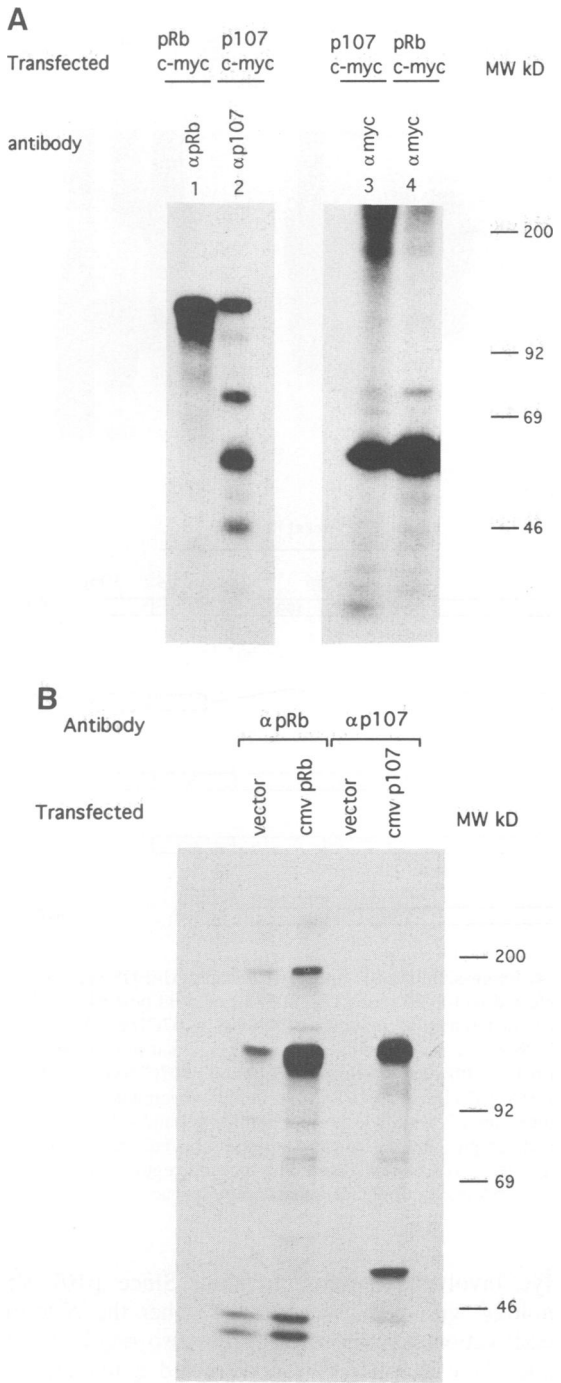

Fig. 1. A 60 kDa protein co-immunoprecipitates with p107 and not with pRb. (**A**) U-2 OS osteosarcoma cells were transiently transfected with either pJ3Ω c-Myc and pCMVpRb expression vectors or with c-Myc and pCMVp107 expression vectors. Transfected cells were labeled with [^{32}P]orthophosphate and cell lysates were immunoprecipitated with c-Myc-, pRb- or p107-specific antibodies. From each lysate 90% was immunoprecipitated with pRb antibodies (lane 1) or with p107 antibodies (lane 2). 10% of each lysate was immunoprecipitated with pan Myc antibody (lanes 3 and 4). Immune complexes were separated on a 7.5% SDS−polyacrylamide gel. (**B**) Expression of pRb and p107 after transient transfection. U-2 OS cells were transfected with pCMVpRb or pCMVp107 expression vectors. Transfected and untransfected cells were metabolically labeled with [^{35}S]methionine for 4 h and cell lysates were immunoprecipitated with pRb-specific (lanes 1 and 2) or p107-specific (lanes 3 and 4) antibodies as described in panel A. c-Myc is not detected in the p107 immunoprecipitate because it is labeled very inefficiently in a 4 h [^{35}S]methionine labeling.

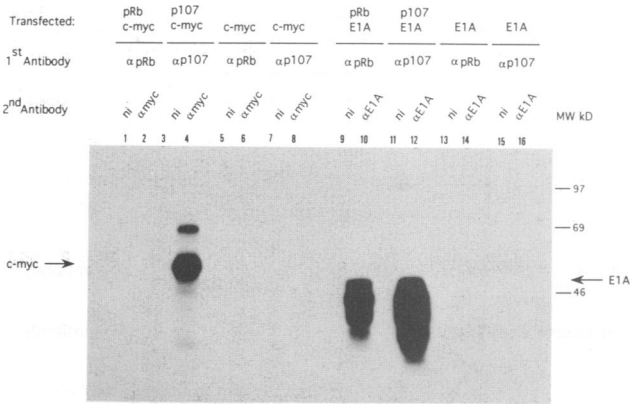

Fig. 2. Binding of c-Myc to the pRb-related protein p107 *in vivo*. U-2 OS cells were transfected with c-Myc together with pCMVpRb or pCMVp107 expression vectors as indicated or with c-Myc together with pCMVp107 antisense (lanes 5–8). As a control, cells were transfected with pRSVE1A together with pCMVpRB or pCMVp107 and pRSV-E1A with pCMV p107 antisense (lanes 13–16). Transfected cells were labeled with [^{32}P]orthophosphate and cell lysates were subjected to sequential immunoprecipitation. In a first immunoprecipitation, lysates were incubated with pRb antibodies or p107 antibodies as indicated (1st antibody). pRb- and p107-associated proteins were released as described in Materials and methods and re-immunoprecipitated with pan Myc antibody (lanes 2, 4, 6 and 8), E1A-specific antibody M73 (lanes 10, 12, 14 and 16) or control antibody (lanes 1, 3, 5, 7, 9, 11, 13 and 15). Immune complexes were separated on 7.5% SDS−polyacrylamide gels, the gels were dried and proteins were detected by autoradiography.

and 4) shows that c-Myc is expressed both when co-transfected with p107 and with pRb. The p107 monoclonal antibody precipitated p107 and a few additional proteins, one of which was of 60 kDa molecular weight and co-migrated with c-Myc (Figure 1A, lanes 2, 3 and 4). The 60 kDa protein was not co-immunoprecipitated with the pRb antibody (Figure 1A, lane 1), even though pRb and p107 were expressed at equal levels (compare Figure 1B, lanes 2 and 4). As can be seen in Figure 1A and B, only a few additional proteins are co-immunoprecipitated with p107 in U-2 OS cells.

To investigate whether the p107-associated protein of 60 kDa was indeed c-Myc, a sequential immunoprecipitation was performed. p107 and pRb immunoprecipitates, identical to those shown in Figure 1A, lanes 1 and 2, were boiled in SDS-containing buffer. Proteins released from the immunoprecipitates were diluted and re-immunoprecipitated with either a c-Myc-specific antiserum or a non-immune serum. Figure 2 (lane 4) shows that the 60 kDa p107-associated protein could be re-immunoprecipitated with c-Myc antibody, but not with control antibody. No detectable c-Myc protein was co-immunoprecipitated with pRb (Figure 2, lane 2). Furthermore, no c-Myc could be precipitated with p107 antibody when cells were transfected with c-Myc expression vector alone, indicating that c-Myc was immunoprecipitated in association with p107 and not recognized directly by the p107 antibody (Figure 2, lane 8). Note that endogenous c-Myc was not immunoprecipitated in association with transfected p107. This is most likely caused by the fact that the sequential immunoprecipitation is relatively insensitive: only 10% of the c-Myc protein that is present in the p107 immunoprecipitate can be re-immunoprecipitated

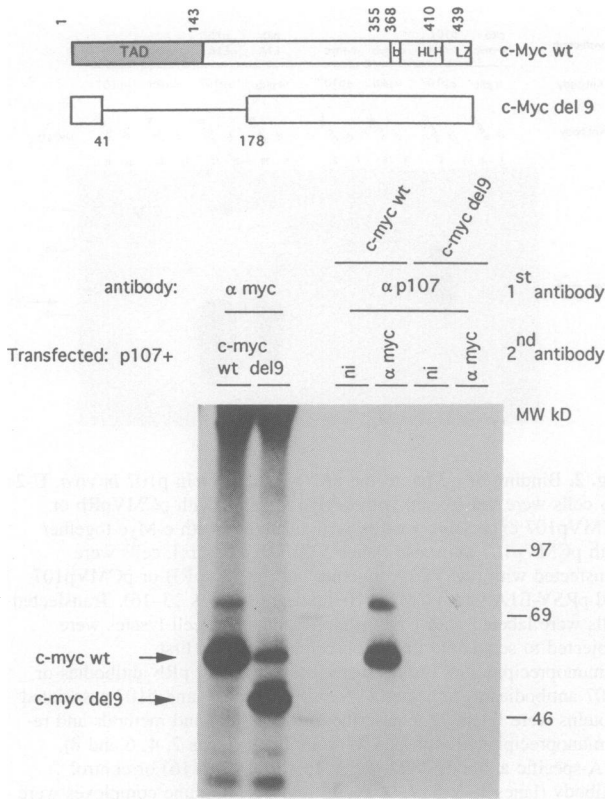

Fig. 3. p107 binds to the transactivation domain of c-Myc. The c-Myc mutant del9 has an N-terminal deletion spanning amino acids 41–178. U-2 OS cells were transfected with pCMVp107 together with either pJ3Ω c-Myc or pJ3Ω c-Myc del9, labeled with [^{32}P]orthophosphate. 10% of the lysate was immunoprecipitated with c-Myc monoclonal antibodies 2 and 3 (lanes 1 and 2) and 90% with p107 antibodies (lanes 3–6). The p107-associated proteins were released as described in Materials and methods and re-immunoprecipitated with c-Myc monoclonal antibodies 2 and 3 or control antibody. The immune complexes were separated on 7.5% SDS–polyacrylamide gels and proteins were detected by autoradiography.

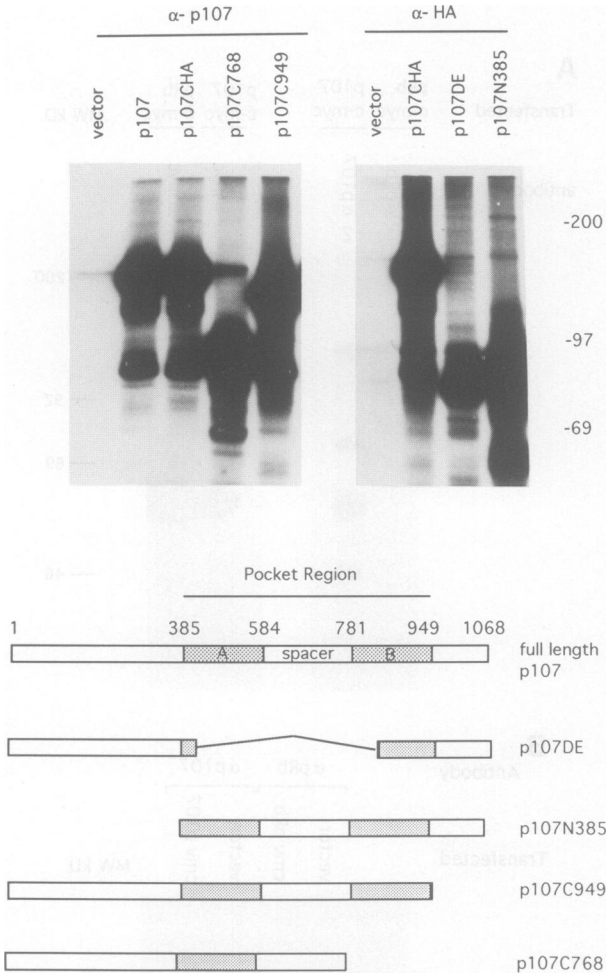

Fig. 4. Expression of p107 and p107 mutants. U-2 OS cells were transfected with wild type p107, HA-tagged wild type p107 (p107HA), or with different p107 mutants: p107N385, p107C768, p107DE and p107C949. The transfected cells were labeled and immunoprecipitated with p107 antibodies (p107, p107C949 and p107C768) or with 12CA5 (p107HA, p107DE and p107N385). Immunoprecipitates were separated on a 7.5% SDS–polyacrylamide gel and subjected to autoradiography. The lower panel gives a schematic representation of the different p107 mutants used. The 'pocket' region of p107 consists of two subdomains, A and B, separated by a spacer.

with the Myc-specific antibody (Figure 2). From these studies we estimate that 5–10% of the c-Myc protein is associated with p107. We have not been able to detect the p107–c-Myc interaction by using antibodies directed against c-Myc. The lack of detection of the p107–Myc complex by c-Myc antibodies could be caused by the fact that the c-Myc antibodies used are directed against the N-terminus of c-Myc that is involved in the interaction with p107.

It has been shown that adenovirus E1A associates with both pRb and p107 *in vivo* (Whyte *et al.*, 1989). As a further control, we therefore co-transfected pRb and p107 expression vectors with an E1A expression vector. As expected, E1A proteins co-immunoprecipitated with both p107 and pRb (Figure 2, lanes 10 and 12). The association of transfected E1A and endogenous pRb was observed after long exposure of the same gel (data not shown). Taken together, these data show that c-Myc can form a specific complex with p107 *in vivo*, and not with pRb.

p107 binds the transactivation domain of c-Myc

We have shown previously that pRb requires amino acids 41–178 of c-Myc for interaction *in vitro*, a domain of c-Myc involved in transactivation. Since p107 shares homology with pRb, we tested whether the N-terminal transactivation domain of c-Myc is also required for the interaction with p107. We constructed a mutant c-Myc expression vector, c-Myc del9, that lacks the region of c-Myc required for binding to pRb. This mutant was then assayed for its ability to interact with p107 *in vivo*. To measure the expression of the c-Myc del9 mutant, we first transfected wild type c-Myc and c-Myc del9 expression vectors transiently in U-2 OS cells. Forty-eight hours after transfection, cells were ^{32}P-labeled and immunoprecipitated with c-Myc antibody. Figure 3 shows that wild type c-Myc and c-Myc del9 were expressed at the same level. However, in a sequential immunoprecipitation, only wild type c-Myc, and not c-Myc del9, could be co-immunoprecipitated with p107 (Figure 3). We conclude that the p107 binding site is located within the N-terminal transactivation domain of c-Myc.

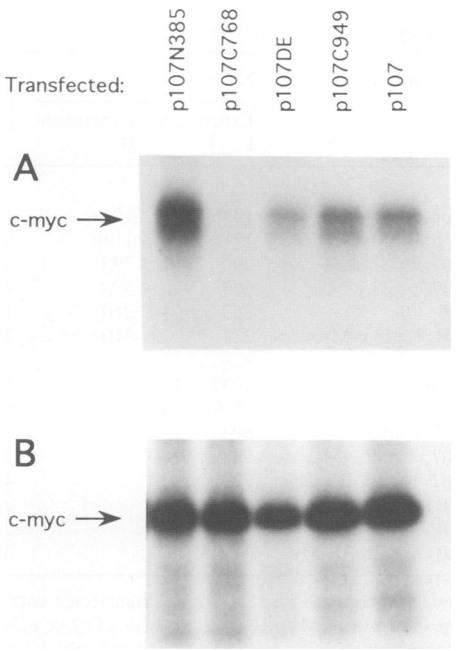

Fig. 5. The pocket region is required for binding to c-Myc. U-2 OS cells were transfected with wild type p107 or different p107 mutants: p107N385, p107C768, p107DE and p107C949, together with pJ3Ω c-Myc. (**A**) The transfected cells were labeled and 90% of the lysate was immunoprecipitated with p107 antibodies (p107, p107C768 and p107C949) or with 12CA5 (p107DE and p107N385). Proteins were released from the first immunoprecipitations and re-immunoprecipitated with pan Myc antibody. (**B**) 10% of the lysates were immunoprecipitated with pan Myc antibody. Immunoprecipiates were separated on a 7.5% SDS−polyacrylamide gel and subjected to autoradiography.

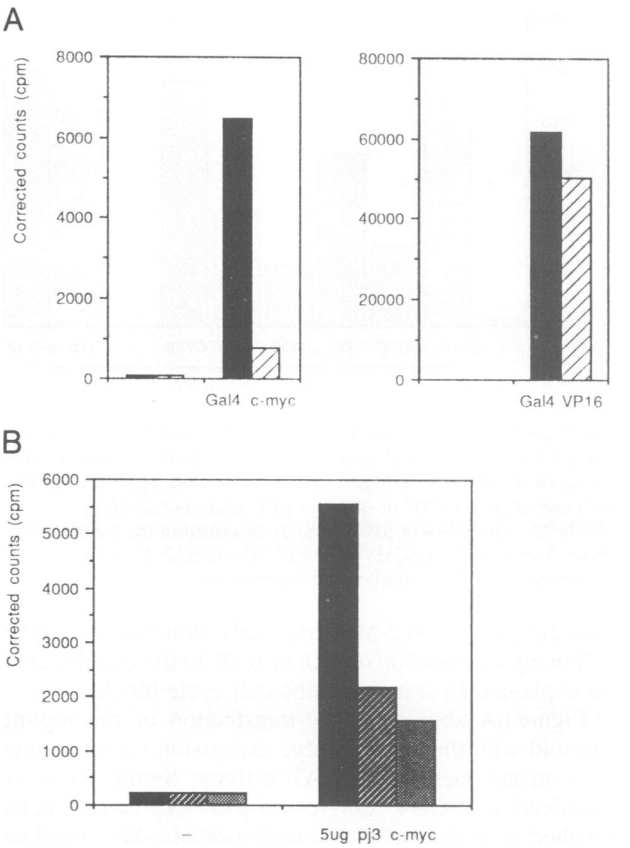

Fig. 6. Repression of c-Myc-mediated transcription by p107. (**A**) U-2 OS cells were transfected with the Gal4 reporter plasmid alone or co-transfected with reporter plasmid and 0.5 μg pSVGal4−c-Myc 1–262 (left panel) or 0.5 μg pSVGal4−VP16 (right panel) together with 2 μg of pCMVp107 antisense (black bars) or 2 μg pCMVp107 (hatched bars). CAT activity was determined as described. pRSV-luciferase was included as an internal control. CAT activities were corrected with the internal control and are the average of duplicate samples. Data are representative for at least three independent experiments. (**B**) CAT assays were performed as described for Gal4 except that a reporter plasmid containing c-Myc/Max binding sites was used. 5 μg of this reporter plasmid was co-transfected with 5 μg pJ3Ω c-Myc. In addition to these plasmids, increasing amounts of pCMVp107 were transfected: either 10 μg pCMVp107 antisense (black bars), 10 μg pCMVp107 (hatched bars) or 20 μg pCMVp107 (stippled bars).

c-Myc binds the pocket region of p107

The structural homology between pRb and p107 is mainly located in the 'pocket' region (Ewen *et al.*, 1991) the region required for binding to viral oncoproteins and for growth suppressive activity (Kaelin *et al.*, 1990; Qin *et al.*, 1992; Zhu *et al.*, 1993). To investigate which part of p107 is required for the interaction with c-Myc, we used a set of four p107 deletion mutants. These mutants lack most of the pocket region (DE) or the N-terminal or C-terminal region (N385 and C949, respectively). The mutant p107C768 lacks the C-terminus and B part of the pocket. Two of these p107 mutants were not efficiently recognized by the p107 antibodies. To circumvent this problem, these mutants were tagged with the hemagglutinin (HA) epitope, which is recognized by the 12CA5 monoclonal antibody. The expression of wild type p107 and the mutants was examined in transient transfection and immunoprecipitation with either p107 antibodies or the 12CA5 monoclonal antibody. All mutants of p107 were expressed at a similar level (Figure 4). Figure 5A shows that c-Myc co-immunoprecipitates with wild type p107. Deletion of the N-terminal 385 amino acids of p107 in p107N385 had no deleterious effect on binding to c-Myc. Deletion of the C-terminus of p107 outside the pocket in p107C949 also did not greatly affect binding to c-Myc. However, C-terminal deletion of parts of the 'pocket' region in p107C768 completely abolished c-Myc binding. The mutant p107DE retained some c-Myc binding capacity. The absence of association was not caused by the decreased expression of c-Myc (Figure 5B). From these data we conclude that the 'pocket' region of p107 is essential for the interaction with c-Myc, and that the B part of the pocket is more important for this interaction.

Inhibition of c-Myc transactivation by p107

Because p107 binds to the transactivation domain of c-Myc, we asked whether c-Myc transactivation was affected by p107 binding. We used two different approaches to study the effect of p107 on c-Myc transactivation. In the first experiment we used a chimeric protein which contains the transactivation domain of c-Myc (amino acids 1–262) linked to the Gal4 DNA domain. A reporter plasmid harboring a core promoter and five upstream Gal4 binding sites linked to the CAT reporter gene was co-transfected with the vector encoding the Gal4−c-Myc chimeric protein in the human U-2 OS osteosarcoma cell line. This cell line was chosen because U-2 OS cells are insensitive to growth arrest induced by overexpression of both pRb and p107 (Zhu *et al.*, 1993).

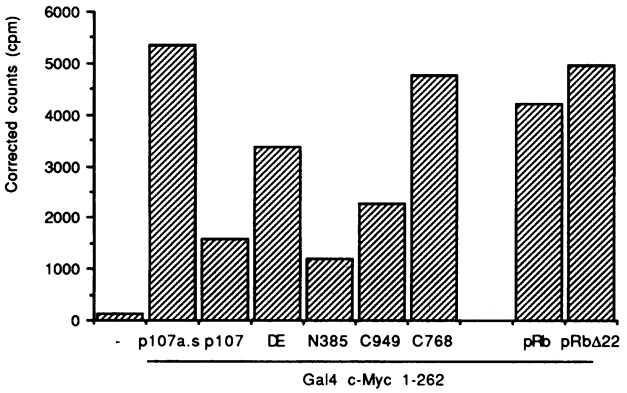

Fig. 7. Binding of p107 correlates with inhibition of transactivation. The Gal4 reporter plasmid used in Figure 5A was co-transfected with 0.5 μg of pCMVGal4−c-Myc 1–262 together with 2 μg pCMVp107 antisense or pCMVp107 or different p107 mutants (p107DE, p107N385, p107C949 or p107C768) as described in the legend to Figure 4 or with 2 μg pCMVpRb or pCMVpRbΔ22. Data are representative of three independent experiments.

Thus, any effect on c-Myc transactivation that is observed following transfection of pRb or p107 in these cells cannot be explained by a non-specific cell cycle block.

Figure 6A shows that co-transfection of the reporter plasmid with the Gal4−c-Myc expression vector resulted in a strong increase in CAT activity. Significantly, co-transfection of Gal4−c-Myc and p107 expression vectors resulted in an almost 10-fold inhibition of c-Myc-mediated transactivation, but not of Gal4−VP16 which was used as a control (Figure 6A). Since Gal4−c-Myc can be supershifted with p107 in gel retardation assays (data not shown), we conclude that p107 inhibits c-Myc transactivation by interfering with the function of the transactivation domain and not by interfering with DNA binding of the fusion protein.

In a second, independent experiment we investigated whether p107 could inhibit the transactivation of the wild type c-Myc protein when it is in complex with its dimerization partner, Max. We co-transfected a wild type c-Myc expression vector with a reporter plasmid in which the CAT gene is driven by a core promoter with four upstream CACGTG c-Myc/Max binding sites. Figure 6B shows that introduction of wild type c-Myc expression vector with the reporter plasmid resulted in a 20-fold activation of the CAT reporter gene. Again, co-transfection of full-length c-Myc and p107 expression vectors resulted in a significant decrease in the activity of a reporter plasmid harboring c-Myc binding sites (Figure 6B).

To test whether the ability of p107 to bind c-Myc correlated with the ability to inhibit c-Myc transactivation, we also tested the panel of p107 mutants, pRb and the mutant pRbΔ22 for their ability to inhibit c-Myc transactivation. Figure 7 shows that mutants of p107 that were unable to bind c-Myc, were also unable to inhibit c-Myc transactivation. Conversely, mutants of p107 that could still interact with c-Myc, were also able to inhibit c-Myc transactivation (compare Figures 5 and 7). Also pRb and pRbΔ22, which are both unable to bind to c-Myc, did not significantly affect c-Myc transactivation. We conclude that p107 inhibits c-Myc transactivation through direct binding of the p107 pocket to the N-terminus of c-Myc.

Table I. Colony formation assay with SaoS-2 cells

SaoS-2 cells transfected with	No. of colonies		Colony formation (%)
	Experiment I	Experiment II	
(A)			
pCMV alone	1416	1284	100
pCMV + pJ3 c-Myc	1216	1140	87
pCMVp107	290	390	25
pCMVp107 + pJ3 c-Myc	663	590	46
pCMVpRB	253	210	17
pCMVpRB + pJ3 c-Myc	227	310	20
(B)			
pCMV alone	1782 ± 82		100
pCMV	1510 ± 71		84.6 ± 4
pCMVp107 + pSP	693 ± 12		38.9 ± 0.7
pCMVp107 + pSP c-Myc	908 ± 28		50.9 ± 1.6
pCMVp107 + pSPΔBR Myc	720 ± 14		40.4 ± 0.8
pCMVpRB + pSP	440 ± 26		24.6 ± 1.5
pCMVpRB + pSP c-Myc	403 ± 21		22.6 ± 1.1
pCMVpRB + pSPΔBR Myc	379 ± 21		21.2 ± 1.1

(A) The osteosarcoma cell line SaoS-2 was transfected with pCMVneo or pCMVp107neo or pCMVpRB together with pJ3Ω or pJ3Ω c-Myc. 24 h after transfection, cells were trypsinized and split 1:5 and cultured in medium containing 500 mg/ml G418. After 3 weeks, cells were fixed and stained and the number of G418-resistant colonies was counted. Each experiment was done in duplicate and the average number of colonies of two plates is given for each experiment.
(B) SaoS-2 cells were transfected with pCMVneo or pCMVp107neo or pCMVpRB together with pSP, pSP c-Myc or pSP c-MycΔBR. The cells were treated as described above. The average number of four plates is given for each experiment.

c-Myc releases p107-induced growth arrest

Both c-Myc and p107 appear to play a role in the transition from G_1 to the S phase of the cell cycle: c-Myc stimulates entry into S phase (Eilers *et al.*, 1991), whereas p107 can arrest sensitive cells at the G_1 to S phase transition (Zhu *et al.*, 1993). To test whether the direct interactions between c-Myc and p107 are antagonistic, we investigated whether a p107-induced growth arrest could be overcome by high expression of c-Myc. We transfected SaoS-2 osteosarcoma cells either with a neomycin resistance marker alone, or with a p107/neo or pRb/neo expression vector and selected for outgrowth of G418-resistant colonies. Table I shows that transfection of p107 and pRb led to a reduction in colony formation, presumably through the growth suppressive effects of p107 and pRb overexpression on SaoS-2 cells. Co-transfection of c-Myc and p107 expression vectors, however, resulted in a partial rescue from the p107-induced growth arrest (Table IA). Significantly, overexpression of c-Myc did not release SaoS-2 cells from a pRb-induced growth arrest (Table IA).

The release of the p107-induced growth arrest by c-Myc could be brought about in two ways. First, expression of c-Myc may increase the amount of free (i.e. not bound to p107) c-Myc protein in the cell, allowing the free c-Myc to promote cell cycle progression. Alternatively, c-Myc might act to bind the p107 pocket, thereby releasing other cellular proteins from p107. To discriminate between these possibilities, we used a mutant of c-Myc, ΔBR. This mutant lacks the basic region (deleted amino acids 353–367) and is therefore unable to bind DNA (Blackwood and Eisenman, 1991), but has retained the N-terminal p107 binding domain. In the second set of experiments

(Table IB) p107 and pRb were cotransfected with wild type c-Myc or mutant c-MycΔBR. Again, c-Myc did rescue the p107 block but was ineffective in releasing pRb-arrested cells. The rescue in the second set of experiments is less pronounced, which may be due to the different c-*myc* expression vectors used in these two experiments. Importantly, however, the mutant c-MycΔBR did not rescue the p107-induced growth arrest. This indicates that rescue of p107 growth suppression cannot be reversed by filling the p107 pocket with an inactive form of c-Myc that can still bind p107. These results suggest that the growth suppressive effect of p107 may, at least in part, reflect inactivation of c-Myc through direct binding.

Discussion

In this paper, we demonstrate that binding of p107 to the c-Myc transactivation domain leads to a dramatic decrease in the ability of c-Myc to activate transcription. c-Myc resembles another cellular transcription factor, E2F, in that both c-Myc and E2F are transcription factors that are implicated in cell cycle control. Our present data extend these similarities by demonstrating that transactivation by c-Myc, like E2F (Schwarz *et al.*, 1993), can be down-modulated by p107.

Unfortunately, the detection of c-Myc−p107 complexes in non-transfected cells is hampered by two complicating factors. First, p107, when compared with pRb, is expressed only at low levels in all cell types tested to date (for example see Figure 1B). Furthermore, the poor solubility of c-Myc protein under mild lysis conditions greatly reduces the sensitivity of detection of c-Myc−protein complexes. As a result of this, we have only detected c-Myc−p107 complexes in transiently transfected cells. Nevertheless, we have several reasons to believe that the interaction between c-Myc and p107 may be relevant to control of normal cell proliferation. First, it is well established that the ability of c-Myc to transform is intimately linked to its ability to activate transcription (Stone *et al.*, 1987; Barrett *et al.*, 1992). It is likely, therefore, that inhibition of c-Myc transactivation by p107 suppresses c-Myc's ability to stimulate cell proliferation. Secondly, the domain of p107 that is required for interaction with c-Myc is also essential for p107's growth suppressive activity. This suggests that the growth suppressive activity of p107 is mediated, at least in part, through interaction with c-Myc. In support of this notion is our finding that in SaoS-2 osteosarcoma cells a p107-induced growth arrest could be partially rescued by overexpression of c-Myc. We interpret these data as indicating that the decision whether to progress through a cell cycle depends in part on the amount of free c-Myc protein. If most of c-Myc is in complex with p107 (in the p107-transfected SaoS-2 cells) the cells will be arrested in the G_1 phase of cell cycle. If c-Myc is co-transfected, sufficient free c-Myc remains to allow progression into S phase. Taken together, these data suggest that inhibition of c-Myc transactivation through interaction with p107 mediates, at least in part, p107-induced growth suppression.

Expression of c-Myc could not rescue a pRb-induced growth arrest (Table I). The absence of an effect on a pRb-induced growth arrest is in contrast with the results described by Goodrich and Lee (1993). They found that micro-injection of bacterially synthesized c-Myc protein into SaoS-2 cells could release cells from a pRb-induced growth arrest. This discrepancy is most likely caused by the different levels of c-Myc used in these two experiments. In the stably transfected SaoS-2 osteosarcoma cells, we obtain only low levels of c-Myc expression, which is apparently sufficient to release cells from a p107 block, but not from a pRb-induced growth arrest. Goodrich and Lee used micro-injection, which may have introduced much higher levels of c-Myc protein in the cells that allowed for the release of the pRb block.

Recently, it was reported that a pRb-induced cell cycle block can be rescued by overexpression of E2F-1, cyclin A or cyclin E (Zhu *et al.*, 1993). In contrast, p107-mediated growth arrest could not be overcome by co-expression of E2F-1 or cyclins. These results indicate that the targets of pRb differ from those of p107. The experiments described in this paper indicate that c-Myc is one of the p107-specific targets in growth suppression.

Recent data show that c-Myc is able to interact with the TATA binding protein (TBP) *in vivo* (Hateboer *et al.*, 1993). Interestingly, it appears that p107 and TBP share overlapping interaction sites on c-Myc. This raises the possibility that p107 can prevent binding of TBP to the transactivation domain of c-Myc, providing a potential mechanism for p107-induced inhibition of c-Myc transactivation.

The activity of pRb is regulated by cell cycle-dependent phosphorylation by protein kinases. Binding of cellular proteins, such as E2F, is abolished by phosphorylation of pRb, resulting in the cyclical association of pRb with E2F. Similarly, p107 is found in complexes with cyclin-dependent kinases, and cell cycle-regulated association of p107 with E2F has been reported (Lees *et al.*, 1992; Shirodkar *et al.*, 1992; Cobrinik *et al.*, 1993). These data raise the possibility that complex formation between p107 and c-Myc could also be regulated by phosphorylation of either p107 and/or c-Myc. Whether p107 binding to c-Myc is regulated during the cell cycle is currently under investigation. An interaction between c-Myc and p107 has also been observed by Gu *et al.* (1994).

Materials and methods

Cell culture and transfections

Osteosarcoma cell lines U-2 OS and SaoS-2 were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum. Transfections were performed using the standard calcium phosphate precipitation technique (Graham and Van der Eb, 1973).

Plasmids

The sequence coding for c-Myc was excised from psp64 c-myc (a gift from J.Woodgett) and cloned in pJ3Ω (Morgenstern and Land, 1990) creating pJ3Ω c-Myc. pJ3Ω c-Myc del9 was made by polymerase chain reaction using pM21 human c-Myc D41–178 as a template (Stone *et al.*, 1987) and cloned in pJ3Ω. The vectors pSP, pSP c-MYC and pSP c-MycΔBR have been described previously (Blackwood *et al.* 1991). The expression vectors for pCMVpRB, pCMVp107AS, pCMVp107 and pCMVp107 deletion mutants are described previously (Zhu *et al.*, 1993). For CAT assays, reporter plasmids containing five Gal4 sites upstream of a minimal promoter linked to the chloramphenicol acetyl transferase (CAT) reporter gene (Kato *et al.*, 1990) or four CACGTG sites fused to a minimal promoter and linked to the CAT reporter gene (Kretzner *et al.*, 1992) were used. The activator plasmids pSVGal4−c-Myc 1–262 and pSVGal4−VP16 have been described previously (Kato *et al.*, 1990).

For pCMVGal4–c-Myc the Gal4–c-Myc fragment was subcloned in pCMVNEO (Baker *et al.*, 1990).

Antibodies

The following antibodies were used: (i) against pRb: XZ 91, XZ77 and C36 (Hu *et al.*, 1991); (ii) against p107: SDmix containing SD2, SD4, SD6, SD9 and SD15 (Zhu *et al.*, 1993); (iii) against c-Myc: pan Myc (Cambridge Research Biochemicals) and Ab 2&3 (Oncogene Science); (iv) against E1A: M73 (Harlow *et al.*, 1985) and 12CA5 (directed against the HA epitope).

Immunoprecipitations

Forty-eight hours after transfections the cells were starved in phosphate-free medium supplemented with 10% dialyzed fetal calf serum or methionine-free medium supplemented with 10% fetal calf serum for 1 h and labeled for 4 h at 37°C with, per plate, 5 mCi [^{32}P]orthophosphate in 5 ml phosphate-free medium or 0.5 mCi *trans*-^{35}S label in 5 ml of methionine-free medium. The cells were collected in 1 ml ELB^{+} buffer (Whyte *et al.*, 1988), supplemented with 10 mM NaF, 10 mM sodium orthovanadate, 0.2 mM sodium pyrophosphate, 1 μg/ml chymostatin, aprotinin, antipain and leupeptin and 1 mM phenylmethylsulfonyl fluoride, and lysed by sonication. The lysate was precleared with 5 μl of normal mouse serum coupled to protein A–Sepharose beads and the supernatant was used in immunoprecipitations with 4 μl antiserum or 100 μl of tissue culture supernatant from hybridoma cells. After 45 min at 4°C immune complexes were collected with protein A–Sepharose and washed four times in 1 ml of ELB buffer. For sequential immunoprecipitations, immunoprecipitates were resuspended in 100 μl of ELB containing 2% SDS and 15 mM DTT and boiled for 10 min. After this, protein A–Sepharose beads were removed by centrifugation and the supernatant diluted to 1 ml in ELB buffer and precleared with protein A–Sepharose beads. The released proteins were then immunoprecipitated with the second antibody. The immunoprecipitated proteins were collected by binding to protein A–Sepharose beads, boiled in SDS-containing buffer, separated on a 7.5% SDS–polyacrylamide gel, dried and subjected to autoradiography (^{32}P) or fluorography (^{35}S).

CAT assays

U-2 OS cells were transiently transfected with 5 μg of reporter construct, 0.2 μg of pRSV-luciferase (van Zonneveld *et al.*, 1988) and the indicated amounts of activator plasmids, and pCMV vector DNA was added to a total of 20 μg DNA in each precipitate. Forty hours after transfection cells were collected and CAT activity was determined using the phase extraction assay (Seed and Sheen, 1988). Luciferase activity was determined by scintillation counting (Promega, luciferase assay system) to correct for transfection efficiency.

Colony formation assay

SaoS-2 cells in 100 mm dishes were transfected using the calcium phosphate method. In experiment I, cells were transfected with 10 μg pCMVneo, pCMVp107neo or pCMVpRbneo together with either 10 μg pJ3Ω or pJ3Ω c-Myc. In experiment II, cells were transfected with 10 μg pCMVneo, pCMVp107neo or pCMVpRbneo together with either 10 μg pSP, pSP c-Myc or pSP c-MycΔBR. Twenty-four hours after transfection, cells were trypsinized, diluted 1:5 and seeded on 100 mm plates. Twenty-four hours later medium was replaced by medium containing 500 μg/ml G418 and selected for 3 weeks. The cells were then fixed and stained and colonies consisting of >30 cells were counted.

Acknowledgements

We thank J.Woodgett, C.V.Dang and R.N.Eisenman and L.Kretzner for providing plasmids, and K.A.Simmen and L.J.van't Veer for discussion and critical reading of the manuscript. This work was supported by a grant of the Netherlands Organization for Scientific Research (NWO) and by a grant from the National Institutes of Health. L.Z. is the recipient of a fellowship from the Leukemia Society of America.

References

Ayer,D.E., Kretzner,L. and Eisenman,R.N. (1993) *Cell*, **72**, 211–222.
Baker,S.J., Markowitz,S., Fearon,E.R., Willson,J.K.V. and Vogelstein,B. (1990) *Nature*, **249**, 912–915.
Barrett,J., Birrer,M.J., Kato,G.J., Dosaka,A.H. and Dang,C.V. (1992) *Mol. Cell. Biol.*, **12**, 3130–3137.
Blackwood,E.M. and Eisenman,R.N. (1991) *Science*, **251**, 1211–1217.
Cobrinik,D., Whyte,P., Peeper,D.S., Jacks,T. and Weinberg,R.A. (1993) *Genes Dev.*, **7**, 2392–2404.
Eilers,M., Schirm,S. and Bishop,J.M. (1991) *EMBO J.*, **10**, 133–141.
Einat,M., Resnitzky,D. and Kimchi,A. (1985) *Nature*, **313**, 597–600.
Ewen,M.E., Xing,Y., Lawrence,J.B. and Livingstone,D.M. (1991) *Cell*, **66**, 1155–1164.
Goodrich,D.W and Lee,W. (1992) *Nature*, **360**, 177–179.
Graham,F.L. and Van der Eb,A.J. (1973) *Virology*, **52**, 456–467.
Gu,W., Bhatia,K., Magrath,I.T., Dang,C.V. and Dalla-Favera,R. (1994) *Science*, **264**, 251–254.
Gupta,S., Seth,A. and Davis,R.J. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 3216–3220.
Hann,S.R., Thompson,C.B. and Eisenman,R.N. (1985) *Nature*, **314**, 366–369.
Harlow,E., Franza,R. and Schley,C. (1985) *J. Virol.*, **55**, 533–546.
Hateboer,G., Timmers,H.T.M., Rustgi,A.K., Billaud,M., van't Veer,L.J. and Bernards,R. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 8489–8493.
Heikela,R., Schwab,G., Wickstrom,E., Loong Loke,S., Pluznik,D.H., Watt,R. and Neckers,L.M. (1987) *Nature*, **328**, 445–449.
Hu,Q., Bautista,C., Edwards,G., Defeo-Jones,D., Jones,R. and Harlow,E. (1991) *Mol. Cell. Biol.*, **11**, 5792–5799.
Kaelin,W.G., Ewen,M.E. and Livingston,D.M. (1990) *Mol. Cell. Biol.*, **10**, 3761–3769.
Kato,G.J., Barrett,J., Villa,G.M. and Dang,C.V. (1990) *Mol. Cell. Biol.*, **10**, 5914–5920.
Kelly,K., Cochran,B.H., Stiles,C.D. and Leder,P. (1983) *Cell*, **35**, 600–610.
Kretzner,L., Blackwood,E.M. and Eisenman,R.N. (1992) *Nature*, **359**, 426–429.
Lees,E., Faha,B., Dulic,V., Reed,S.I. and Harlow,E. (1992) *Genes Dev.*, **6**, 1874–1885.
Li,Y., Graham,C., Lacy,S., Duncan,A.M.V. and Whyte,P. (1993) *Genes Dev.*, **7**, 2366–2377.
Morgenstern,J.P. and Land,H. (1990) *Nucleic Acids Res.*, **18**, 1068.
Qin,X., Chittenden,T., Livingston,D. and Kaelin,W.,Jr (1992) *Genes Dev.*, **6**, 953–964.
Rustgi,A.K., Dyson,N. and Bernards,R. (1991) *Nature*, **352**, 541–544.
Schwarz,J.K., Devoto,S.H., Smith,E.J., Chellappan,S.P., Jakoi,L. and Nevins,J.R. (1993) *EMBO J.*, **12**, 1013–1020.
Seed,B. and Sheen,J. (1988) *Gene*, **67**, 271–277.
Seth,A., Alvarez,E., Gupta,S. and Davis,R.J. (1991) *J. Biol. Chem.*, **266**, 23521–23524.
Shirodkar,S., Ewen,M., DeCaprio,J.A., Morgan,J., Livingston,D.M. and Chittenden,T. (1992) *Cell*, **68**, 157–166.
Stone,J., de Lange,T., Jakobovits,E., Bishop,J., Varmus,H. and Lee,W. (1987) *Mol. Cell. Biol.*, **7**, 1697–1709.
Thompson,C.R., Challoner,P.B., Neiman,P.E. and Groudine,M. (1986) *Nature*, **314**, 363–366.
van Zonneveld,A., Curriden,S. and Loskutoff,D. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 5525–5529.
Whyte,P., Buchkovich,K.J., Horowitz,J.M., Friend,S.F., Raybuck,M., Weinberg,R.A. and Harlow,E. (1988) *Nature*, **334**, 124–129.
Whyte,P., Williamson,N.M. and Harlow,E. (1989) *Cell*, **56**, 67–75.
Zhu,L., van den Heuvel,S., Helin,K., Fattaey,A., Ewen,M., Livingston,D., Dyson,N. and Harlow,E. (1993) *Genes Dev.*, **7**, 1111–1125.

Received on February 7, 1994; revised on June 20, 1994