The c-myc Oncoprotein Forms a Specific Complex with the Product of the Retinoblastoma Gene

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myc proteins are involved in the regulation of cell proliferation and differentiation. Deregulated expression of *myc* family genes has been implicated in the genesis of a variety of cancers (Lüscher and Eisenman 1990). *myc* proteins share significant sequence homology in the carboxyl terminus with a number of cellular transcription factors. These basic domain-helix-loop-helix and leucine zipper motifs have been shown to be involved in protein dimerization and DNA binding (Murre et al. 1989; Blackwell et al. 1990; Blackwood and Eisenman 1991). Indeed, evidence now exists to indicate that the c-*myc*, N-*myc*, and L-*myc* proteins can form a heterodimer with a cellular protein, named MAX, and that the *myc*-MAX heterodimer can bind to DNA in a sequence-specific fashion (Blackwood and Eisenman 1991). In addition, *myc* proteins have several amino-terminal regions that are highly conserved between *myc* family members. These regions of homology are not found in other cellular proteins but are essential for transformation (Stone et al. 1987).

On the basis of these observations, it would appear likely that *myc* proteins function in cell growth by regulating the activity of a number of cellular genes whose products control cell proliferation. Several groups have identified cellular genes that are subject to regulation by *myc* proteins (for review, see Lüscher and Eisenman 1990). However, none of these genes is likely to be directly involved in the control of cell cycle progression.

We show here that amino-terminal sequences in the c-*myc* protein mediate specific binding to the product of the retinoblastoma gene, pRB. We propose a model in which activity of the c-*myc* protein is modulated through binding to the retinoblastoma gene product.

EXPERIMENTAL PROCEDURES

Preparation of glutathione S-transferase (GST) fusion protein-loaded beads and binding assays. GST fusion proteins were prepared basically according to the method of Kaelin et al. (1991) with the modification that GST fusion proteins bound to glutathione-agarose beads were eluted with 5 mM glutathione. After elution, fusion proteins were dialyzed overnight in 0.1 M $NaPO_4$ buffer (pH 7.8), and protein concentration was measured by modified Bradford assay. Dialyzed proteins were checked for integrity by SDS-PAGE followed by Coomassie staining. This procedure of protein elution followed by rebinding to glutathione agarose beads makes it possible to check protein integrity and quantify the amount of GST fusion protein loaded on the beads. To generate GST fusion protein-loaded beads, 20 μl of glutathione agarose beads (50% slurry, obtained from Sigma) was incubated with 1 μg of GST fusion protein for 30 minutes at 4°C. After this, beads were washed three times with 1 ml of ELB buffer (Whyte et al. 1988). Beads were then incubated with in-vitro-translated ^{35}S-labeled protein for 2 hours at 4°C and washed five times in 1 ml of ELB buffer. Bound proteins were released by boiling in SDS buffer and were separated on a polyacrylamide-SDS gel. pRB and c-*myc* protein were generated by sequential in vitro transcription and translation (reagents obtained from Stratagene, La Jolla, California).

Coimmunoprecipitation of pRB with GST fusion proteins. In-vitro-translated pRB protein was diluted with 250 μl of ELB buffer, and 1 μg of GST fusion protein was added. After incubation for 2 hours on ice, mixes were spun for 15 minutes in a microfuge, and the supernatants were immunoprecipitated with 5 μl of mouse anti-GST polyclonal antibody.

Competition of pRB/GSTΔmyc binding with adenovirus E1A peptide. In-vitro-translated pRB (15 μl) was diluted with 125 μl of ELB and incubated for 30 minutes on ice with competitor polypeptides (either 200 μg of control peptide or adenovirus E1A peptide). After this, 0.5 μg of GST-E1A or GSTΔ*myc* was added to each mix. Protein complex formation and precipitation of complexes was as described above. The polypeptides used were: HFEPPTLHELYDLDQEVIDL TCHEAGFPPS and HFEPPTVHEVYDVQPEVIEV TSHDAGFPPS (control; a mutated version of pRB binding sequence of Ad5 E1A that fails to bind to pRB (N. Dyson et al., in prep.).

RESULTS

Binding of the Retinoblastoma Protein to c-*myc*

myc proteins functionally resemble the E1A proteins of adenovirus. Both encode nuclear phosphoproteins that can immortalize primary rodent fibroblasts (Land et al. 1983; Ruley 1983). Furthermore, both *myc* and

E1A can cooperate with an activated *ras* oncogene in the oncogenic transformation of primary rat embryo fibroblasts. The E1A proteins appear to function, at least in part, by binding a number of cellular proteins, one of which was recently identified as the product of the retinoblastoma gene (Whyte et al. 1988). We therefore tested whether c-*myc*, like adenovirus E1A, can form a specific complex with pRB. The study of *myc*-associated proteins has been greatly hampered by the fact that *myc* proteins are highly insoluble nuclear proteins that cannot be readily extracted from the cell homogenates (Moore et al. 1987). Thus, conditions that solubilize *myc* will be too harsh to preserve protein interactions, whereas conditions that preserve protein-protein interactions will not solubilize *myc* from cell lysates. To circumvent this problem, we constructed a bacterial expression vector that directs the synthesis of a GST-c-*myc* fusion protein. In this GSTΔ*myc* protein, the first 204 amino acids of human c-*myc* are linked to GST. Initial experiments with this fusion protein indicated that GSTΔ*myc* protein, but not GST-full-length *myc* fusion protein, was highly soluble and could be readily purified from bacterial cell lysates by binding to glutathione agarose beads (data not shown).

To investigate whether the amino-terminal conserved motifs of the c-*myc* protein mediate binding to the product of the retinoblastoma gene, pRB, we incubated in-vitro-translated ^{35}S-labeled RB protein with GSTΔ*myc* protein. Proteins bound to GSTΔ*myc* were coimmunoprecipitated with a polyclonal mouse anti-GST serum. Bound proteins were released by boiling in an SDS-containing buffer, separated on a 12% polyacrylamide gel, and detected by fluorography. As a positive control we used GST-E1A fusion protein. As shown in Figure 1, in vitro translation of human RB protein yields a series of amino-terminal truncated products generated by initiation of translation at internal AUG start codons (lane labeled IVT-RB). High-affinity binding of E1A to pRB requires two regions of the RB protein, spanning amino acids 393 to 572 and 646 to 772, respectively (Hu et al. 1990). In vitro translation products of pRB of approximately 56 kD and larger will therefore harbor both E1A-binding regions; those between 28 kD and 56 kD will harbor only the carboxy-terminal E1A-binding region; and those smaller than 28 kD will lack both E1A-binding regions.

Figure 1 shows that GST-E1A and GSTΔ*myc* both bind pRB in vitro translation products of 28 kD and larger. GST-E1A binding to pRB differs from *myc* binding to pRB in that E1A binds with higher affinity to pRB in vitro translation products of 56 kD and larger, whereas *myc* does not appear to have such a preference (Fig. 1). No binding of pRB in vitro translation products was seen with GST protein alone, nor with five other randomly chosen GST fusion proteins (data not shown). We conclude that the amino-terminal 204 amino acids of the c-*myc* protein mediate specific binding to the product of the retinoblastoma gene.

The finding that *myc* and E1A bind the same pRB in vitro translation products suggested that *myc* and E1A

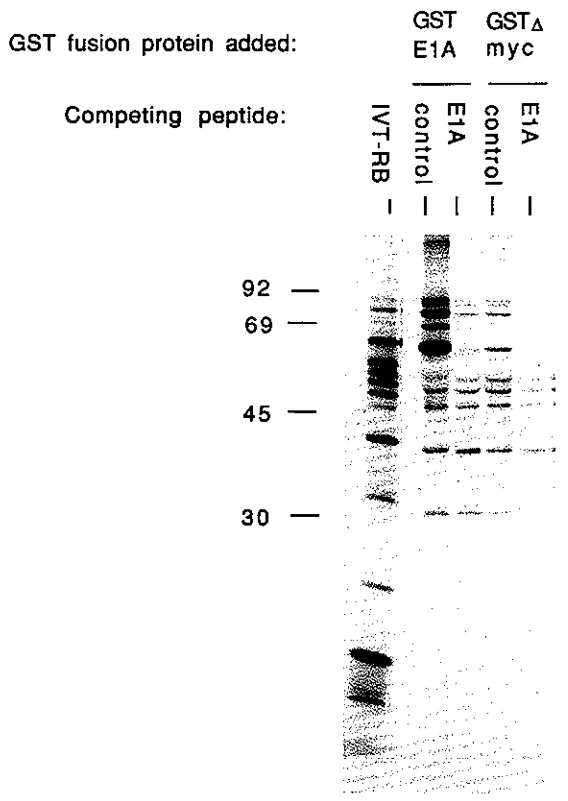

Figure 1. Binding of in-vitro-translated pRB to GST fusion proteins. Control peptide (200 μg) (lanes labeled control) or 200 μg of E1A peptide (lanes labeled E1A) was incubated on ice for 30 min with in-vitro-translated pRB protein. After this, 1 μg of GST fusion protein was added as indicated and incubated for 2 hr at 4°C. Complexes were immunoprecipitated with polyclonal mouse anti-GST antibody. For comparison, 1 μl of total in-vitro-translated RB protein was loaded on the gel (lane labeled IVT-RB). Bound proteins were separated on a 12% polyacrylamide gel and detected by fluorography.

bind approximately to the same site on pRB. To investigate this further, we performed a competition experiment with a peptide of E1A that contains the pRB-binding site of E1A. Figure 1 shows that E1A peptide, but not control peptide, interferes with the binding of both E1A and c-*myc* to the larger than 56 kD pRB in vitro translation products. These data indicate that E1A and *myc* interact with similar sites on pRB.

High- and Low-affinity Binding Sites

In our assay, GST-E1A binds to pRB in vitro translation products of 28 kD and larger (Fig. 1). These results differ from those reported by Hu et al. (1990), who showed that E1A binds only to pRB in vitro translation products of 56 kD and larger. One difference between our assay and the one used by Hu et al. is that we used significantly more E1A protein in our pRB-binding assay. The binding of E1A to smaller than 56 kD pRB in vitro translation products could thus be explained by low-affinity binding of E1A to the smaller pRB in vitro translation products. To test this hypoth-

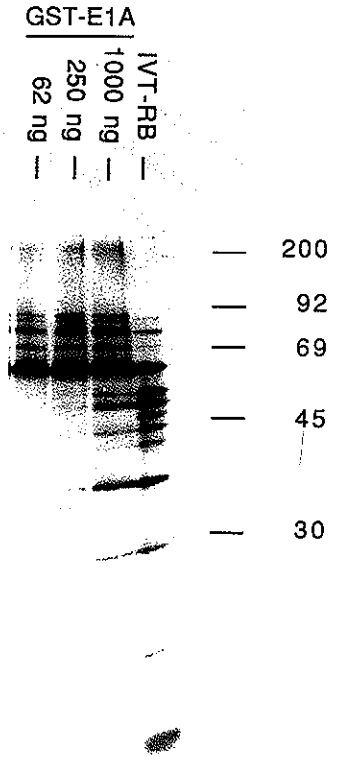

Figure 2. Binding of pRB to GST-E1A. ^{35}S-labeled in-vitro-translated human pRB protein was incubated with the indicated amounts of GST-E1A fusion protein for 2 hr at 4°C. Bound proteins were immunoprecipitated with polyclonal anti-GST antibody and analyzed on a 12% polyacrylamide gel. For comparison, 1 μl of total in-vitro-translated pRB was loaded (lane labeled IVT-RB).

esis, we incubated serial dilutions of GST-E1A fusion protein with in-vitro-translated pRB and precipitated complexes with anti-GST antibodies. Bound pRB protein species were separated on a 12% polyacrylamide gel and detected by fluorography. Figure 2 shows that when 1000 ng of GST-E1A fusion protein is incubated with in-vitro-translated pRB, pRB species of 28 kD and larger bind to the GST-E1A protein. When only 62 ng of GST-E1A was used, the pRB species of 56 kD and larger were almost exclusively bound. These data support the notion that E1A binds with high affinity to pRB protein species of 56 kD and larger, with lower affinity to pRB protein species that are between 28 kD and 56 kD, and has no detectable affinity for pRB species smaller than 28 kD.

Myc Binding to GST-RB

To further evaluate the specificity of the c-*myc*-pRB interaction, we tested whether in-vitro-translated c-*myc* protein could bind to a GST-pRB fusion protein that harbors only the pRB domain required for binding to E1A (amino acids 379–792, Kaelin et al. 1991). Glutathione agarose beads were loaded with 1 μg of GST-RB fusion protein as described in Experimental

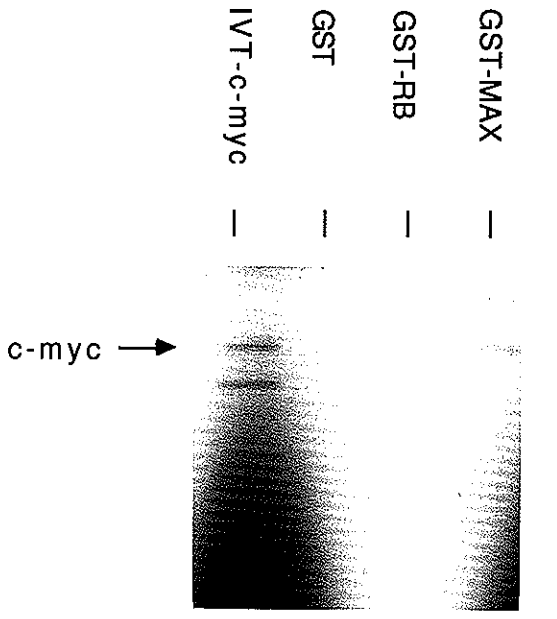

Figure 3. Binding of in-vitro-translated c-*myc* to GST fusion protein beads. ^{35}S-labeled in-vitro-translated human c-*myc* protein was incubated with agarose beads loaded with either glutathione S-transferase protein (lane labeled GST), GST-RB fusion protein (lane labeled GST-RB), or GST-MAX fusion protein (lane labeled GST-MAX). Bound proteins were released by boiling in SDS and separated on a 10% polyacrylamide gel. Bound proteins were detected by fluorography. The 45-kD protein species seen in the c-*myc* in vitro translation is a nonspecific protein also found in unprogrammed rabbit reticulocyte lysates.

Procedures. Fusion protein-loaded beads were subsequently incubated with ^{35}S-labeled in-vitro-translated c-*myc* protein. Bound proteins were released by boiling in SDS and analyzed by electrophoresis through a 10% polyacrylamide gel. As a negative control, we used beads loaded with the glutathione S-transferase protein only (GST beads), and as a positive control we used GST-MAX beads, since the MAX protein binds c-*myc* with high affinity (Blackwood and Eisenman 1991).

Figure 3 shows that in-vitro-translated c-*myc* is bound by both GST-RB beads and GST-MAX beads with roughly equal efficiency, but not by GST control beads. Taken together, our data indicate that c-*myc* can form a specific complex with the product of the retinoblastoma gene.

DISCUSSION

We show that the c-*myc* protein can bind to the product of the retinoblastoma gene. Because of the insolubility of full-length c-*myc* protein, we used a truncated soluble form of c-*myc* to study the interaction between c-*myc* and pRB. At present, our observations are based on in vitro protein affinity assays. Despite this obvious limitation, our findings are intriguing because the domain of pRB that is involved in binding to c-*myc* is frequently deleted in human tumors (Horowitz et al. 1989; Yandell et al. 1989). This suggests that

c-*myc* interacts with a physiologically important domain of pRB. Consistent with this notion is our recent finding that the RB protein expressed by the J82 bladder carcinoma cell line, which carries a 35-amino-acid deletion in the carboxyl terminus of pRB, failed to bind to c-*myc* (Rustgi et al. 1991).

The members of the *myc* gene family share several regions of high homology. The carboxyl termini, which carry the DNA binding and helix-loop-helix/leucine zipper motifs, are highly conserved, whereas the amino termini of *myc* family members have three conserved motifs (Sugiyama et al. 1989). It has been shown that the region between the conserved amino and carboxyl termini is largely dispensable for transforming activity of *myc* (Stone et al. 1987). *myc* proteins thus appear to be modular transcription factors with two distinct effector domains: carboxy-terminal DNA binding and amino-terminal *trans*-activation and pRB binding. It is interesting that a *myc*-like protein named B-*myc* has been described that has the conserved amino-terminal domains found in c-*myc* but lacks the carboxy-terminal DNA-binding domain (Ingvarsson et al. 1988). Similarly, a carboxy-terminal truncated form of L-*myc* has been found in some tumor cell lines (Ikegaki et al. 1989). These truncated *myc* proteins may prove to be important modulators of the activity of *myc* family members.

Our preliminary results indicate that c-*myc* can only bind the unphosphorylated form of pRB (Rustgi et al. 1991). Since pRB undergoes a cell-cycle-dependent phosphorylation, this suggests that binding of c-*myc* to pRB is also cell-cycle-regulated. Recently, Eilers et al. (1991) have shown that activation of c-*myc* alone is sufficient to drive quiescent cells into the S phase of the cell cycle. Conversely, suppression of c-*myc* with antisense oligonucleotides has been shown to prevent entry into S phase (Heikkila et al. 1987). It is thus possible that pRB controls cell cycle progression by cell-cycle-regulated binding to c-*myc*. It is noteworthy that the region of c-*myc* that mediates binding to pRB was recently shown to have transcription activating ability (Nato et al. 1990).

These observations suggest a model for the c-*myc* and pRB interaction that is schematically represented in Figure 4: Early in the G_1 phase of the cell cycle, pRB is unphosphorylated and is complexed to the amino terminus of c-*myc*. Phosphorylation of pRB releases pRB from c-*myc*, which is now free to interact with another, as yet unknown, cellular protein. This interaction results in activation of transcription of a number of cellular genes whose expression drives cells into S phase (Fig. 4, bottom). Overexpression of pRB by DNA transfection results in a block in cell cycle progression at the G_1 to S boundary (S. Friend, pers. comm.). Our model predicts that high-level expression of pRB prevents entry into S phase, because overexpressed pRB competes with other cellular factors for binding to c-*myc*, thereby blocking c-*myc* function, which is required for entry into S phase (Heikkila et al. 1987). Taken together, our data suggest that dominant and recessive nuclear oncogenes can cooperate through direct binding to control progression through the cell cycle.

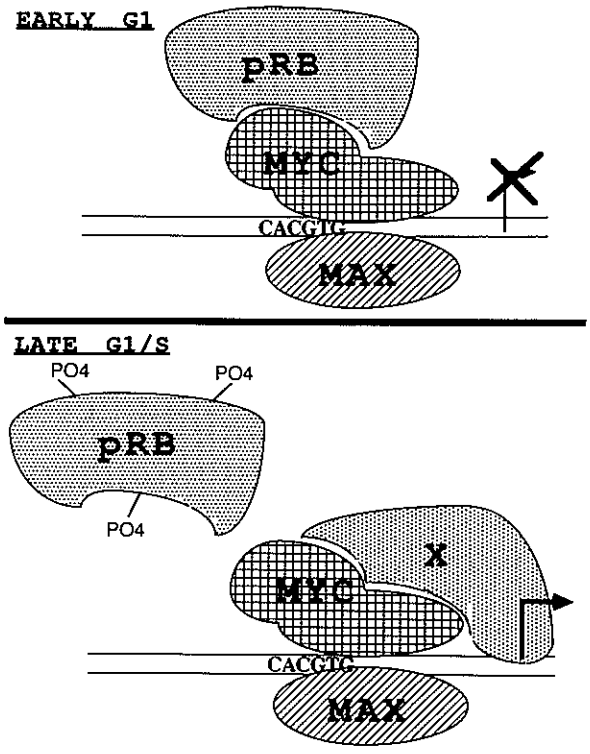

Figure 4. Schematic representation of the c-*myc*-pRB interaction. (*Top*) In early G_1 phase of the cell cycle, c-*myc* is complexed to MAX through the carboxy-terminal dimerization domain. The *myc*/MAX heterodimer is bound to DNA. The amino terminus of c-*myc* is complexed to pRB, thereby suppressing the *trans*-activating ability of c-*myc*. (*Bottom*) Phosphorylation of pRB causes release of pRB from c-*myc*, which is now free to interact with another cellular protein (designated X), which mediates the *trans*-activating effect of c-*myc*.

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