Interaction between YY1 and the Retinoblastoma Protein

REGULATION OF CELL CYCLE PROGRESSION IN DIFFERENTIATED CELLS*

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Overexpression of the transcription factor YY1 activates DNA synthesis in differentiated primary human coronary artery smooth muscle cells. Overexpression of the retinoblastoma protein together with YY1 blocked this effect. In growth-arrested cells, YY1 resides in a complex with the retinoblastoma protein, but the complex is not detected in serum-stimulated S phase cultures, indicating that the interaction of the retinoblastoma protein and YY1 is cell cycle-regulated. Recombinant retinoblastoma protein directly interacts with YY1, destabilizing the interaction of YY1 with DNA and inhibiting its transcription initiation function in vitro. We conclude that in differentiated cells elevation of the nuclear level of YY1 protein favors progression into the S phase, and we propose that this activity is regulated by its interaction with the retinoblastoma protein.

Differentiation is a coordinated process in which cells cease proliferation and express genes encoding specialized functions. A failure to differentiate or maintain a differentiated phenotype can be deleterious. For example, restenotic lesions result from an abnormal proliferation of several cell types, including vascular smooth muscle cells (1). The earliest event in the medial vascular smooth muscle cell, leading to neointimal formation, is likely to be altered transcription of genes that control the differentiated phenotype (2). Indeed, mitogenic stimuli not only stimulate proliferation but also repress transcription of muscle-specific genes, including the smooth muscle α-actin gene. CarG boxes are critical DNA promoter elements for the regulated expression of many muscle-specific genes (3–5), and YY1 is one of the transcription factors involved in this regulation (6, 7). Transcription repression of the smooth muscle α-actin gene requires both an intact CarG box and YY1 (8).

YY1 is a DNA-binding transcription factor that influences expression of a wide variety of genes (9, 10). It can function as an activator or repressor, depending on the context of its binding site within a promoter. It acts as a transcriptional initiator when bound at the initiator element of the adenov-associated virus P5 promoter (11, 12). YY1 interacts with transcription regulators such as Sp1 (13, 14), p300 (15), and the mammalian homologue of the yeast global regulator RPD3 (16). Deletion of the murine YY1 gene results in embryonic lethality, suggesting an essential function for YY1 in development (17).

Here we report a new function of YY1, a physical and functional association of YY1 with the retinoblastoma protein (Rb)1 in primary human coronary artery smooth muscle (CASM) cells. Overexpression of YY1 in growth-arrested CASM cells activates DNA synthesis, stimulating them to enter the S phase of the cell cycle. The overexpression of Rb together with YY1, however, abolished this effect. In extracts of growth-arrested CASM cells a significant portion of YY1 resides in a complex with Rb. Little of the complex is detected in S phase cells. Highly purified, recombinant YY1 and Rb form a complex in solution via a direct physical interaction. Rb inhibits the interaction of YY1 with DNA and blocks YY1-dependent transcription in vitro. We propose that YY1 may participate in checkpoint functions that regulate cell cycle transitions in differentiated cells.

MATERIALS AND METHODS

Cells and Plasmids—Primary CASM cells (CLONTECH), Saos 2 cells (ATCC), and Sf9 cells (Invitrogen) were grown as recommended by the suppliers. A cDNA-encoding YY1 was inserted into the pCEP4 expression vector (Invitrogen), which then produced YY1 with an HA tag at its N terminus under control of the cytomegalovirus major immediate early promoter. All other expression vectors were described previously (18, 28).

Protein Interactions and Immunofluorescence—Rb was purified from Sf9 cells (32). Recombinant YY1 and other proteins were purified from Escherichia coli (12, 28). To test for a YY1-Rb interaction in solution, Rb (50 μg) and YY1 (30 μg) were incubated alone or in a mixture for 30 min at 22 °C and then separated by gel filtration on Superose 6 (Amersham Pharmacia Biotech) as described (12). Chymotrypsinogen A (18 kDa), bovine serum albumin (68 kDa), and myosin (206 kDa) were used as markers. To test for a YY1-Rb interaction in cells, extracts were prepared (40) from cells that were growth-arrested by serum starvation for 48 h (G0/G1, phase cells) or from cells that were first growth-arrested and then treated for 24 h with 5% fetal calf serum (S phase cells). YY1, Rb, and control pre-immune IgG immunoprecipitation reactions were performed and used as described (12). For Western blot assays, YY1 was localized using a rabbit polyclonal antibody, Rb was identified with monoclonal antibody to Rb (a gift from E. Harlow, Harvard Medical School), and E2F1 was monitored with monoclonal antibody to Rb (a gift from E. Harlow, Harvard Medical School).

Cells were prepared for immunofluorescence by fixation with 4% paraformaldehyde in phosphate-buffered saline. Expression of HA-YY1 fusion protein was monitored with a monoclonal antibody to the HA tag

The abbreviations used are: Rb, retinoblastoma protein; CASM, coronary artery smooth muscle; HA, hemagglutinin; BrdUrd, bromodeoxyuridine; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorter; GST, glutathione S-transferase; TBP, TATA box-binding protein.
(Roche Molecular Biochemicals), and Rb was monitored using the antibody described above. Both were visualized with Texas Red-conjugated goat anti-mouse antibody (Jackson Immunoresearch). BrdUrd-specific monoclonal antibody and fluorescein isothiocyanate-conjugated anti-mouse antibodies (PharMingen) were employed to identify cells that synthesized DNA in the presence of the nucleotide analogue.

FACS Analysis—Flow cytometric analysis (FACS) was performed on a Becton Dickinson FACScan instrument using CellQuest software as described previously (20). FuGENE 6 (Roche Molecular Biochemicals) was used to deliver DNA to CASM cells using the protocol recommended by the manufacturer, and the efficiency of transfection was 20–25%.

Gel Shift Assays and Transcription Reactions—The gel shift assays and in vitro transcription reactions were performed as described previously (12).

RESULTS

Overexpression of YY1 Induces DNA Synthesis in Growth-arrested CASM Cells, and Overexpression of Rb Blocks the YY1-mediated Induction—The adenovirus E1A protein, which stimulates cell cycle progression (18), antagonizes transcription repression by YY1 (19). Its effect on YY1 correlates with the ability of E1A to bind to the transcription factor (18). YY1 has also been reported to regulate the expression of several muscle-specific genes involved in the switch from proliferation to quiescence (8). The regulation of YY1 by E1A and its role in expression of differentiation-specific genes raised the possibility that YY1 might function in cell cycle regulation. Accordingly, we tested the effect of YY1 overexpression on cell cycle progression in primary CASM cells. These cells contain a low endogenous level of YY1.2

After maintenance in 0.1% serum for 48 h, growth-arrested cells were transfected with a plasmid expressing HA-tagged YY1 plus a second plasmid encoding a membrane-localized derivative of the green fluorescent protein (GFP) (20), which served as a marker for transfected cells. After transfection cells were refed with the fresh medium with low serum to prevent serum-induced cell cycle progression. Twenty-four h later, cells were harvested, and the DNA content of GFP-positive cells was determined by FACS. In response to transfection with the HA-YY1 expression plasmid, 38% of the serum-starved cells entered the S phase, and 51% remained in G0/G1 (Fig. 1A, panel a, 2). In a control experiment with the vector lacking a YY1 insert plus the GFP-expressing plasmid, only 5% of the cells entered the S phase, and 86% of the population remained in G0/G1 (Fig. 1A, panel a, 1).

Rb is a key inhibitor of progression from the G1 to S compartment. Therefore, we asked whether overexpression of Rb could block the YY1-mediated induction of DNA synthesis. Following transfection with a mixture of Rb and YY1 expression plasmids, only 11% of the cells reached the S phase, and 81% remained in G0/G1 (Fig. 1A, panel a, 2). Transfection with the expression plasmid lacking an Rb insert in the absence of the YY1 expression vector did not significantly change the cell cycle profile, and the majority of the cells (82%) remained in G0/G1 (Fig. 1A, panel a, 4). No significant change in the G0/M population was observed in response to YY1 or Rb, either alone or together. Western blot analysis verified the presence of the ectopically expressed proteins (Fig. 1B).

We next verified the correlation of protein overexpression and changes in DNA synthesis in individual cells (Fig. 1C). The ectopically expressed proteins were visualized by immunofluorescence using antibody to the HA tag or Rb plus a Texas Red-conjugated secondary antibody. DNA synthesis was monitored by BrdUrd incorporation, which was assayed with a BrdUrd-specific antibody plus a fluorescein isothiocyanate-conjugated second antibody. Following transfection with the HA-

\footnotesize{2 V. Petkova and A. Usheva, unpublished result.}

\begin{figure}[h]
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\caption{Ectopic expression of YY1 induces growth-arrested CASM cells to enter the S phase of the cell cycle. A, DNA content of growth-arrested cells after transfection with YY1 and Rb expression plasmids. All cells received 1 μg of GFP expression plasmid, and the total amount of DNA in each transfection reaction was maintained at 5 μg by adding expression vector without an insert when necessary. A, panels a and b, 1, cells received 4.0 μg of expression vector with no insert; A, panels a and b, 2, cells received 2.0 μg of HA-tagged YY1 expression plasmid DNA; A, panels a and b, 3, cells received 2.0 μg each of YY1 and Rb expression vectors; A, panels a and b, 4, cells received 2.0 μg of Rb plasmid. Each experiment is illustrated by the FACS profile measuring cellular DNA levels (A, panel a) and by diagrams in which the bars present the quantity of GFP-positive cells in G0/G1, S, and G2 compartments (A, panel b). Four independent experiments produced consistent results (S.D. = ±5%). B, verification of protein overexpression in transfected CASM cultures by Western blot. Lanes 1 through 4 correspond to cellular lysates prepared from the cellular populations shown in A. Rb and HA-YY1 were visualized with antibodies identified to the right of the panels. C, immunofluorescence was used to monitor cells for simultaneous expression of ectopic YY1 and/or Rb plus DNA synthesis. Growth-arrested cells were transfected with expression plasmids, and after 24 h cultures were incubated with BrdUrd for 2 h prior to fixation. YY1 and Rb were visualized by reacting with monoclonal antibodies followed by a Texas Red-conjugated secondary antibody. DNA synthesis was visualized with a BrdUrd-specific monoclonal antibody followed by fluorescein isothiocyanate-conjugated secondary antibody. Left, ectopic YY1 expression (C, 1) and BrdUrd incorporation in the same field of cells (C, 4); center, ectopic YY1 plus Rb expression identified with a YY1-specific antibody (C, 2) and BrdUrd incorporation in the same field of cells (C, 3); right, ectopic Rb expression (C, 3) and BrdUrd incorporation in the same field of cells (C, 6).}
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Functional and Physical Interaction of YY1 and Rb

**Fig. 2. Interaction between YY1 and Rb.** A and B, YY1 binds to Rb in solution. Purified recombinant YY1 and Rb alone or after mixing were subjected to size exclusion chromatography on a Superose 6 matrix. A, 1, YY1 alone; A, 2, Rb alone; A, 3, Rb plus YY1; B, 1, Rb alone; B, 2, ΔYY1 alone; B, 3, ΔYY1 plus Rb separation. Fractions (numbers at the top of the gels) were analyzed by Western blot with antibody to YY1 or Rb as indicated on the right-hand side of the gels. C, capture of Rb by GST-YY1. Affinity beads and recombinant Rb were mixed in buffer containing 0.2 M NaCl and incubated at 22 °C for 40 min. The beads were separated from the supernatant by centrifugation, and Rb protein was analyzed by Western blot. Samples are as follows: lane 4, input (IN) Rb; lane 5, Rb that was not bound (NB); lanes 6 and 7, beads sequentially washed with buffer containing 0.6 and 1.0 M NaCl, respectively; lanes 8 and 9, 20 and 40% ethylene glycol (EG), respectively; lane 10, boiled in sample buffer containing 1% SDS. In a control experiment Rb was incubated with control GST beads: lane 1, input (IN) Rb; lane 2, Rb that was not bound (NB); lane 3, Rb eluted with SDS. D, capture of Rb by GST-E1A: lane 1, input (IN) Rb; lane 2, Rb that was not bound (NB); lane 3, Rb eluted by boiling in buffer with 1% SDS. E, the purity of the recombinant proteins was evaluated by electrophoresis and silver staining: lane 1, ΔYY1; lane 2, GST-E1A; lane 3, YY1; lane 4, Rb. The migration of size markers is indicated to the left of the gel.

whether purified recombinant YY1 and Rb form a complex in solution. Purified YY1 and Rb were incubated alone or together for 30 min at 22 °C and then subjected to gel filtration chromatography. The fractions were analyzed by Western blot for the presence of YY1 and Rb. When YY1 and Rb were mixed before chromatography, a substantial portion of YY1 and Rb coeluted as a large complex (Fig. 2A, 3), well before either Rb or YY1 alone eluted (Fig. 2A, 1 and 2). The size of the YY1-Rb complex, relative to that of marker proteins, was consistent with the formation of a heterodimer. The C-terminal domain of YY1 containing its zinc finger motifs (ΔYY1, amino acids 297–414) was sufficient to form a complex with Rb in solution (Fig. 2B), and, again, the size of the complex indicated that it was likely a heterodimer. Fractionation of the recombinant protein preparations by electrophoresis followed by silver staining of the separated protein bands verified the purity of the proteins (Fig. 2E) making it very unlikely that contaminating prokaryotic proteins were present that fortuitously bridged between YY1 and Rb.

Our second assay for the YY1-Rb interaction employed YY1 affinity chromatography (Fig. 2C). A bacterially expressed and purified glutathione S-transferase (GST)-YY1 fusion protein was bound to glutathione-Sepharose and used as a YY1 affinity matrix. GST without a fusion partner was bound to glutathione-Sepharose and used as a control matrix. The YY1 affinity beads were first incubated with recombinant Rb. Unbound protein was removed, and the beads were washed under various conditions and finally boiled in sample buffer containing 1% SDS to remove bound protein. Eluted Rb was assayed by Western blot. When Rb was applied to control beads with the GST protein, >95% of the Rb remained in the unbound fraction (Fig. 2C, lanes 1 and 2). In contrast, the GST-YY1 affinity matrix captured >90% of the Rb (Fig. 2C, lanes 4 and 5). The captured protein was not released by washing with buffer containing as much as 1 M NaCl (Fig. 2C, lanes 4 and 7). It was released by buffer containing 20% ethylene glycol (Fig. 2C, lane 8), and no further Rb was released upon boiling in buffer containing SDS (lane 10), suggesting that the Rb-YY1 interaction is most likely hydrophobic in nature.

It is known that Rb binds to E1A, and we used an affinity matrix with GST-E1A fusion protein as a positive control for binding of the recombinant protein (21); >85% of the input Rb, as expected, was captured on the E1A affinity matrix (Fig. 2D, lanes 1–3). The E1A and YY1 matrices bound Rb to a similar extent.

Our experiments argue that highly purified recombinant YY1 and Rb interact directly to form a heterodimer in solution, mainly through hydrophobic contacts. The C-terminal domain of YY1 is sufficient to mediate Rb binding.

**YY1 and Rb Exist in a Complex in Growth-arrested CASM Cells—**The physical interaction of purified recombinant YY1 and Rb and their opposing effects on cell cycle progression suggested that they might form a complex in CASM cells. To probe for YY1-Rb complexes, extracts were prepared from serum-starved G0/G1 CASM cultures (Fig. 3A, panel 1) and from serum-stimulated S phase cultures (Fig. 3B, panel 1). We tested the ability of antibody to either YY1 or Rb to capture the presumed partner protein. In the cellular extract from growth-arrested cells, YY1-specific antibody captured ~40% of the Rb together with YY1 (Fig. 3A, panel 2, lane 4). However, in extracts from S phase CASM cultures, the YY1 antibody captured <5% of the Rb (Fig. 3B, panel 2, lane 4). Consistent with this result, Rb-specific antibody captured ~50% of the YY1 from extracts of growth-arrested CASM cells (Fig. 3A, panel 3, lane 4), whereas it captured <3% of the YY1 from an S phase extract (Fig. 3B, panel 3, lane 4). As a positive control for the Rb antibodies, we demonstrated that E2F1 (22, 23) was captured (Fig. 3A, panel 3, and B, panel 3, lane 4); pre-immune mouse antibody served as a negative control (Fig. 3A, panels 2 and 3, and B, panels 2 and 3, lane 1). We conclude that a significant portion of the Rb protein in extracts from G0/G1- or S-phase CASM cells, but not S phase cells, exists in a complex with YY1.

We also tested extracts from G0/G1- or S-phase Saos-2 cells (Fig. 3C). These cells contain an abnormal endogenous Rb protein of 95 kDa that has a mutation in its C-terminal domain (24). This mutant Rb fails to interact with SV40 large T-antigen, adenovirus E1A, and cellular E2F (25). The mutant Rb was not captured by YY1-specific antibody; it was mainly present in the unbound fraction (Fig. 3C, lane 3). Although we cannot rule out a more complex explanation, it is likely that the C-terminal domain of Rb is important for the interaction with YY1.

**Rb Prevents YY1 Binding to DNA and Inhibits YY1-dependent Transcription in Vitro—**Given that YY1 and Rb interact in vitro and exist in a complex in CASM cell extracts, it seemed possible that Rb might alter the transcription activity of YY1. The functional consequence of the YY1-Rb interaction was explored in two assays. We first employed gel shift reactions. Purified recombinant YY1 was mixed with a 32P-labeled, dou-
ble-stranded oligonucleotide containing the initiator element from the adenov-associated virus P5 promoter to which YY1 binds (12), and it generated a shifted complex (Fig. 4A, lane 2). When Rb was included in the reactions, we observed a dose-dependent inhibition of YY1-P5 initiator DNA complex formation (Fig. 4A, lanes 3–5). Rb alone did not form a complex with the YY1-specific binding site under the conditions of the reaction (Fig. 4A, lane 6), and heat treatment of Rb at 85 °C for 15 min inactivated its ability to inhibit the gel shift reaction (Fig. 4A, lanes 9 and 10). We also probed the effect of Rb on the YY1 interaction with a double-stranded oligonucleotide corresponding to the CarG DNA box of the smooth muscle α-actin promoter, which contains a binding site for YY1 (26). YY1 bound to this DNA, and Rb inhibited formation of the YY1-CarG DNA complex in a dose-dependent manner (Fig. 4B). In contrast to its effect on YY1 binding, Rb did not visibly affect TBP-DNA complex formation (Fig. 4C, lanes 2–5). As a control for a proper function of the recombinant Rb, we assembled reactions with recombinant E2F-1 protein and 32P-labeled E2F-specific oligonucleotide (27), where we observed the formation of the Rb-E2F1-DNA triple complex (Fig. 4D).

As a second assay we tested the effect of Rb on YY1-dependent transcription in a reconstituted system with purified transcription factors, where YY1 functions as an inhibitor protein (28). Transcription reactions were assembled with the AAV P5 promoter serving as template DNA (28), and Rb was included at different concentrations. Rb inhibited the YY1-dependent activity of the AAV P5 promoter in a dose-dependent manner (Fig. 4E, 2, lanes 2–5). Transcription from the AdMLP template in the reconstituted system was not altered in the presence of
Rb (Fig. 4E, 1, lanes 2–5), which argues that the Rb inhibition of the YY1-specific reaction is specific.

These results demonstrate that Rb can alter YY1 function, preventing it from interacting with its DNA binding site and inhibiting YY1-dependent transcription in vitro.

DISCUSSION

Ectopic expression of YY1 induces quiescent CASM cells to progress to the S phase of the cell cycle, synthesizing DNA (Fig. 1). Consequently, the main conclusion to be drawn from our experiments is that YY1 can induce the transition from a quiescent to a proliferative phenotype in CASM cells. We observed a similar but less pronounced effect of ectopic YY1 expression in growth-arrested U2OS and Molt4 lymphocytes, which suggests that YY1 has the capacity to induce DNA synthesis, and therefore cell cycle progression, in a variety of cell types.

At present we do not know how YY1 promotes the activation of DNA synthesis. However, given its transcription regulatory functions, it is likely that YY1 regulates the expression of genes the products of which function directly or indirectly in cellular DNA replication. Indeed, it has been reported that YY1 activates several genes encoding proteins involved directly in DNA synthesis (29). Consistent with a role in the induction of proliferation, YY1 regulates the expression of some ribosomal protein (9) and histone genes (30).

The best characterized Rb protein partners are the E2F transcription factors (31). Rb converts E2F from a transcription activator into a repressor, and this contributes to cell cycle arrest. However, E2F is not the only target for Rb; Rb also requires for its specific DNA recognition for interaction with YY1. YY1 no longer binds to DNA after Rb expression vector, E. Harlow for monoclonal antibody to Rb, R. Mudryj, H. Pines, M. D. (1995) J. Virol. 69, 1628–1636.

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