The Y-box-binding Protein, YB1, Is a Potential Negative Regulator of the p53 Tumor Suppressor*

Annette Lasham‡, Stephanie Moloney‡, Tracy Hale§§, Craig Homer§§, You Fang Zhang§§, J. Greg Murison‡, Antony W. Braithwaite§§, and James Watson‡

From the ‡Genesis Research and Development Corporation Limited, P. O. Box 50, Auckland 1001, New Zealand and the §Cell Transformation Group, Pathology Department, Box 913, Dunedin School of Medicine, University of Otago, Dunedin 9001, New Zealand

The p53 tumor suppressor plays a major role in preventing tumor development by transactivating genes to remove or repair potentially tumorigenic cells. Here we show that the Y-box-binding protein, YB1, acts as a negative regulator of p53. Using reporter assays we show that YB1 represses transcription of the p53 promoter in a sequence-specific manner. We also show that YB1 reduces endogenous levels of p53, which in turn reduces p53 activity. Conversely, inhibiting YB1 in a variety of tumor cell lines induces p53 activity, resulting in significant apoptosis via a p53-dependent pathway. These data suggest that YB1 may, in some situations, protect cells from p53-mediated apoptosis, indicating that YB1 may be a good target for the development of new therapeutics.

Apoptosis, or programmed cell death, is the mechanism by which damaged, modified or superfluous cells are removed from a complex organism (1). Another important role for apoptosis is preventing the development of cancer by removing cells with mutated or damaged DNA in order to preserve the integrity of the genome (2). The tumor suppressor protein p53 plays a pivotal part in this and consequently has been termed the “guardian of the genome” (2). In response to DNA damage, p53 is “activated” and initiates either growth arrest or apoptosis pathways (3). This allows DNA damage to be repaired or potential tumor precursor cells to be removed from tissues. Both pathways are initiated probably via the transcriptional activation of specific genes by p53. These genes include the WAF1/CIP1 (4), GADD45 (5), and 14-3-3σ (6), which induce cell cycle arrest, and Bax (7), killerIR5 (8), and PIG3 (9), which induce apoptosis.

Consistent with its important tumor suppressor role, more than 50% of human cancers contain mutations in the p53 gene, encoding a protein that is inactive for some or all of the functions of p53 (10). In the other 50% of cancers there is selection against other components of the p53 signaling pathway, suggesting that p53 may be functionally inactive in the vast majority of cancers. Thus, there appears to be a powerful selection against functional p53 during tumor development.

The Y-box-binding protein, YB1, belongs to the family of cold shock proteins, which is highly conserved from bacteria to man. YB1 is multifunctional and appears to regulate gene expression at both the transcriptional and translational levels (11, 12). With regard to transcription, we noticed that YB1 regulates some of the same genes as wild type (wt) p53 (13–19) but in an opposing manner. For example, the fas gene promoter is repressed by YB1 (13) and stimulated by wt p53 (19), whereas the multidrug resistance (mdr1) gene promoter is activated by YB1 (14) and repressed by wt p53 (16). These data suggest that YB1 might negatively regulate p53.

In this study we present evidence that YB1 represses the p53 promoter and down-regulates endogenous p53 expression. We then show that a reduction of YB1 in several tumor cell lines results in an induction of apoptosis via the activation of a p53 pathway. If this occurs in vivo, then these observations suggest that YB1 may play a role in the development of some tumors by protecting cells from apoptosis induced by p53.

EXPERIMENTAL PROCEDURES

Oligonucleotides—For electrophoretic mobility shift assays, oligonucleotides were synthesized as follows: 5′- to 117 of the mouse p53 promoter (20), strand 1, 5′-AGAGGCCCCCTGCAGGCTGTCATGGCTGGTGAGG-3′, and strand 2, 5′-AGAGTATAGAGCTGTGAGGGCAAAATGGGTGGAAACCGATTCGGAGG-3′. For transfection, phosphorothioated oligonucleotides were synthesized as follows. YB1 cis-element decay was derived from the fas silencer region (−1035 to −1008 bp of the 5′-flanking sequence of the human fas gene (13)), 5′-GAACCTGATTTGGGATATCGACAGAC-3′. For transfection, phosphorothioated oligonucleotides were synthesized as follows. YB1 cis-element decay was derived from the fas silencer region (as described above): 5′-GGAGCTGAATTTGGGATATCGACAGAC-3′; and antisense YB1 oligonucleotides were used as a combination of five: 5′-GGGCCCCGGCGTTCCTGTCGGCCTGG-3′, 5′-GCGAAGAGGAGTTGGAATGAC-3′, 5′-GGAACTGATTTGGGATATCGACAGAC-3′, 5′-GGGGTCTTCTGACAGAC-3′, 5′-GGGGGATTCCTGACAGAC-3′; and negative control oligonucleotide-5′-GGGATACAAATCCATTCGAGC-3′. The antisense YB1 oligonucleotides were designed to cover the region encoding the C-terminal portion of YB1, termed the multitargetization domain (21). Phosphorothioated oligonucleotides corresponding to the p53 binding site of the human GADD45 promoter (22) were synthesized (5′-TACAGAAGATCATTGGAATTTGAGG-3′ and 5′-CCCCACATGTTGAGATTTCCGAGC-3′) and annealed by heating to 100 °C in 0.1 M NaCl followed by slow cooling to room temperature. Negative control double-stranded phosphorothioated oligonucleotides were synthesized (5′-GGGATACAAATCCATTCGAGCAGG-3′ and 5′-GGGGGATTCCTGACAGAC-3′) and annealed as described above.

Plasmids—Rat YB1 was cloned into the mammalian expression vector pcDNA3 as described previously (13). The promoterless reporter construct (pCAT3M) contains the CAT gene but no upstream eukaryotic promoter sequences (20). The 446-bp mouse p53 promoter construct (pCAT3M) contains the CAT gene but no upstream eukaryotic promoter sequences (20). The 446-bp mouse p53 reporter construct

* This work was supported by grants from the New Zealand Lottery Board (to T. H.) and the Cancer Society of New Zealand (to C. H. and Y. F. Z.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Present address: Breast Center, Baylor College of Medicine, Houston, TX 77030.

‡ To whom correspondence should be addressed. Tel.: 64-3-479-7165; Fax: 63-3-479-7136; E-mail: antony.braithwaite@stonebow.otago.ac.nz.

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contains 320 to 116 of the promoter cloned into pCAG3M as described previously (20). The adenovirus-5 E3 promoter-CAT reporter construct (pKCAT23) was as described previously (23). The Y-box deletion construct was prepared by deleting the region 143 to 121 from the mouse p53 promoter using inverse PCR as described previously (24). The −93 to −51 deletion mouse p53 promoter reporter construct has been described (24). The p21 reporter constructs contain the promoter of the p51E-box gene linked to the luciferase reporter and a variant promoter (p21A53) without the p53 binding site.

Cell Culture—The following cell lines were maintained in complete RPMI 1640 medium (Invitrogen) supplemented with 5% fetal bovine serum: melanomas SK Mel 5 and NZM8, lymphoblastic leukemia Jurkat, histiocytic lymphoma U937, and promyelocytic leukemia, HL60. The remaining cell lines were maintained in complete Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% sodium deoxycholate, 0.1% SDS, 158 mM NaCl, 50 mM Tris-HCl pH 7.5, protease inhibitors 1 mM phenylmethylsulfonyl fluoride, 10 g/ml leupeptin, 10 g/ml aprotinin) at 37°C (10% CO2). Cells were cryopreserved using FuGENE 6 reagent (Roche Molecular Biochemicals). Control oligonucleotide and luciferase activity was measured according to the manufacturer’s instructions (Promega) and then standardized to cell number.

Electrophoretic Mobility Shift Assay—Nuclear extract preparation and electrophoretic mobility shift assays were performed as described previously (13).

Oligonucleotide Transfections—Cells were transfected with 1 g of YB1 plasmid and actin mRNA levels (data not shown). Finally, the effect of transfected YB1 (data not shown). This observation was extended to a further three cell lines of mouse and human origin, where between 75 and 95% of the p53 promoter activity was abolished following co-transfection with YB1 (Fig 1D). Similar results were obtained when a human p53 reporter construct (32) was used in these same cells (data not shown). These data show that YB1 is capable of represing transcription of both mouse and human p53 promoters in what appears to be a sequence-specific manner.

To further explore the sequence specificity of this repression, YB1 was transfected into SK Mel 5 cells along with two deletion constructs of the mouse p53 promoter, one of which is deleted for the putative Y-box-binding site (−143 to −121 bp relative to the transcriptional start site; see Fig 1A) and another deleted between −93 and −51 bp relative to the start site. Results (Fig 1E) show that YB1 was able to repress neither the Y-box deleted promoter nor a smaller deletion (−138 to −121 bp) within this region but was still able to repress transcription from the −93 to −51 bp construct. This latter result excludes structural effects as being responsible for loss of YB1 repression. We conclude that YB1 is capable of repressing transcription of the p53 promoter in vitro.

YB1 Reduces p53 Expression and Activity in Transfected Cells—To determine whether YB1 also reduces expression of endogenous p53, A549 and SK Mel 5 cells, both expressing wild type p53 protein, were transfected with a YB1 expression construct, and p53 levels were measured. In the first part of this investigation, Western blotting was carried out. Results for A549 cells (Fig 2A) show that actin protein levels were essentially invariant after transfection of YB1, whereas 18 h p53 levels were reduced to 75% (1 g of YB1 plasmid) and 65% (2 g of YB1 plasmid) of control and were further reduced to about 50% of control by 48 h after transfection. This was also evident at the same time after transfection of SK Mel 5 cells (Fig 2A) in which, for example, p53 levels were reduced to 50% of control by 18 h after transfection with 2 g of YB1 plasmid and actin levels were unchanged. A Northern blot was also done, and consistent with the protein experiments, YB1 reduced p53 mRNA levels (data not shown). Finally, the effect of transfect-
tion of YB1 on p53 function was measured. A549 cells were transfected with YB1 along with a luciferase reporter construct linked to the \( p21WAF1 \) promoter, known to be transactivated by wt p53. In parallel, a similar experiment was carried out using a mutant \( p21^{\text{NAP1CIP1}} \) reporter construct without the p53 response element (p21Δ53). Results (Fig 2B) show that YB1 markedly reduces activity of the \( p21^{\text{NAP1CIP1}} \) promoter but has no effect on the activity of the p21Δ53 reporter. In other
experiments, YB1 does not inhibit p21WAF1/CIP1 promoter activity in Skov3 and Saos2 cells, both of which are p53-deficient (data not shown). These data show that the inhibition of p21WAF1/CIP1 promoter activity by exogenous YB1 parallels the decline in p53 expression and is not a direct effect of YB1. The experiments shown in both Figs. 1 and 2 are consistent with the interpretation that YB1 down-regulates p53 expression, which in turn leads to reduced p53 activity.

Reduction of YB1 Induces Cells to Undergo Apoptosis—Because over-expression of YB1 leads to reduced p53 activity, the converse experiment, in which YB1 levels are reduced, may lead to up-regulation of p53. To test this possibility we used a YB1 cis-element from the human fas promoter (13), which should effectively function as a YB1 decoy to sequester YB1 from endogenous promoters. This approach has been used successfully for several transcription factors (33, 34). An equimolar concentration of a random sequence oligonucleotide was also transfected as a negative control. First, the endogenous levels of p53 and the pro-apoptotic p53-regulated gene Bax (7) were determined by Western blotting. The results for transfected SK Mel 5 cells (Fig 3A) show that the levels of p53 increased 3-4-fold in cells receiving the decoy oligonucleotide, peaking at 4 h post-transfection. The levels of Bax also increased about 3-fold, peaking at 4-6 h post-transfection, whereas the levels of ERK1 remained unchanged. In contrast, there was essentially no change in the levels of p53, Bax, or ERK1 after transfection with control oligonucleotides (Fig. 3A).

We next asked whether the YB1 decoy would increase a p53 response as measured by the p21WAF1/CIP1 reporter constructs used in Fig 2A. A549 cells were transfected with the p21WAF1/CIP1 reporter constructs along with decoy or control oligonucleotides or with a human p53 expression construct. The results (Fig 3B) show that both human p53 and YB1 decoy increased the activity of the p21WAF1/CIP1 reporter, but this did not happen with control oligonucleotides. Furthermore, only the reporter with the p53 response element was up-regulated, showing that the effect of the oligonucleotides was p53-specific.

To confirm that the decoy oligonucleotide was specifically able to target YB1, SK Mel 5 cells were transfected with biotinylated decoy or control oligonucleotides. At 18 h post-transfection, oligonucleotides were removed by incubating lysates from transfected cells with streptavidin beads. The level of YB1 remaining in the supernatant of cells that had received the decoy oligonucleotide was 60% less than that found in control lysates (Fig 3C), but ERK1 levels were the same. This experiment therefore confirms the specificity of the decoy oligonucleotide.

Reduction of YB1 Induces Cells to Undergo Apoptosis—The results described above show that reducing YB1 causes an up-regulation of p53. We therefore asked whether reducing YB1 can cause cells to undergo apoptosis in a p53-dependent manner. At 18 h post-transfection of SK Mel 5 cells with either antisense YB1 or YB1 decoy oligonucleotides, about 30% of cells were in the late stages of apoptosis as assessed by in situ TUNEL assay (Fig 4A) and DNA laddering (Fig 4B). However, much less apoptosis was observed with the control oligonucleotide. To confirm that the antisense YB1 oligonucleotides did in fact reduce the level of YB1 protein, SK Mel 5 cell lysates were prepared from antisense and negative control oligonucleotide transfectants. Western blotting with a YB1 antibody showed that in cells transfected with the YB1 antisense oligonucleotide, 75% less YB1 protein was detected than from cells transfected with control oligonucleotide (Fig 4C), whereas ERK1 levels were unchanged. This confirmed that the antisense YB1 oligonucleotides were able to target YB1 specifically.

To determine whether wt p53 is required for the apoptosis observed when YB1 is reduced by the antisense or decoy oligonucleotides, RKO and RKO p53.13 cells were used. RKO are colorectal cells expressing wt p53, and RKO p53.13 are a variant cell line containing a dominant-negative mouse p53 mutant (25). Thus RKO p53.13 are functionally p53 null. Both cell lines were transfected with the YB1 decoy or negative control oligonucleotides. Results (Fig 4D) show that at 18 h post-transfection there was a 53% decline in the viability of RKO cells when transfected with the YB1 decoy compared with the control oligonucleotide, however, there was no reduction in viability of RKO p53.13 cells. These results provide evidence that the induction of apoptosis caused by reduction of YB1 is via a p53-mediated pathway. This experiment was repeated in a panel of human and mouse tumor cell lines that differed in their p53 status. Apoptosis/cell death was measured by TUNEL assay and/or by trypan blue exclusion. Some of the TUNEL staining results are shown in Fig 4E, and a summary of these and other results is shown in Table I. Collectively these experiments show that at 18 h post-transfection, >50% apoptosis
occurred after the addition of decoy oligonucleotide to cells expressing wt p53, but no apoptosis was observed in cells with a mutant p53 (e.g. HaCAT, Fig 4E, Table I) or in cells expressing no p53 (e.g. HL60, Table I). Additionally, we were also able to prevent YB1 decoy-induced apoptosis by co-transfecting a p53 decoy (a double-stranded p53 oligonucleotide) from the human GADD45 promoter (see “Experimental Procedures” for the sequence) but not with the control oligonucleotide (data not shown).

The effect of transfection of antisense and decoy oligonucleotides was also examined at later times. We generally found that more extensive cell death occurred by 42 h post-transfection (about 50%), but was no greater beyond this time (data not shown). This may be because of oligonucleotide instability, but the reasons for this are not clear. This qualification notwithstanding, the above experiments show that apoptosis/cell death is induced by inhibiting YB1 and that this appears to require wt p53.

DISCUSSION

There is growing evidence that the Y-box-binding protein, YB1, may be important in tumor biology. YB1 is activated in response to genotoxic stress (35, 36) and is associated with drug resistance (35), and both YB1 protein level (37) and nuclear localization (38) appear to be prognostic for some human cancers. In addition, YB1 regulates expression of several tumor-associated genes. These include epidermal growth factor receptor (EGFR or c-ErbB1), matrix metalloproteinase-2 (MMP-2), fas, mdr1, DNA topoisomerase IIa and MHC Class II (13–15, 39, 40). As an activator of MMP-2, elevated levels of YB1 would facilitate tumor cell invasion and metastasis, and as a promoter of EGFR and mdr1 gene expression, YB1 would enhance cell growth and resistance to chemotherapeutic agents. The p53 tumor suppressor regulates a similar cluster of genes (16–19, 41, 42), but unlike YB1, it represses the expression of MMP-2, EGFR, and mdr1 and promotes the expression of fas. However, as p53 regulates these genes to (presumably) protect against tumor development, we postulated that YB1 may function as a dominant-negative regulator of p53. This is the subject of the present study, for which we provide some evidence.

In this paper we have shown that YB1 represses transcription of the promoter of the p53 gene and that this occurs in a sequence-specific manner (Fig. 1). We also have shown that YB1 bound preferentially to the upper pyrimidine-rich strand, consistent with other data showing it to be a single-stranded DNA-binding protein (13, 30, 31). Despite binding single-stranded DNA, our data suggesting that YB1 transcriptionally regulates the p53 promoter are not unique, as YB1 has been reported to transcriptionally regulate a number of genes (13, 21, 30, 31, 43) as do other single strand binding proteins such as Purα, Purβ (44, 45), and the polypyrimidine tract-binding

FIG. 3. Up-regulation of p53 by reduction of YB1. A, analysis of p53, Bax, and ERK1 levels in transfected cells. SKMel 5 cells were transfected with negative control or YB1 decoy oligonucleotides. Cells were harvested at 2, 4, 6, 14, and 20 h post-transfection. Lysates were analyzed by Western blotting with anti-p53, anti-Bax, or anti-ERK1 on the same blot. Similar results were observed in two independent experiments. B, up-regulation of the p21<sup>WAF1/CIP1</sup> promoter by YB1 decoy oligonucleotides. A549 cells were transfected with control or decoy YB1 oligonucleotides or human p53, along with the p21<sup>WAF1/CIP1</sup>-luciferase reporter constructs with or without (p21Δ53) the p53 response element. Cells were harvested 48 h later and luciferase activities measured. C, YB1 decoy oligonucleotide sequesters YB1 protein. SK Mel 5 cells transfected with 3'-biotinylated negative control (ctrl) or YB1 decoy oligonucleotides were harvested at 18 h post-transfection. Lysates were incubated with streptavidin beads for 3 h, and supernatant was removed. Supernatants were analyzed by Western blotting with anti-YB1 or anti-ERK1 on the same blot. Similar results were observed in two independent experiments.
FIG. 4. Reducing YB1 induces apoptosis/cell death in a p53-dependent manner. A, identification of apoptotic cells by in situ TUNEL assays using a fluorescein label. SK Mel 5 cells were transfected with antisense YB1 oligonucleotides (A), YB1 decoy oligonucleotide (B), negative control oligonucleotide (C), or no DNA (D). A, cells were fixed and analyzed by fluorescence microscopy with Nomarsky optics at 18 h post-transfection. Cells undergoing apoptosis are indicated by arrowheads. Magnification, ×40. Similar results were obtained in 10 independent experiments. B, evidence of apoptosis by DNA laddering. SK Mel 5 cells transfected with negative control oligonucleotide (ctrl), antisense YB1 oligonucleotides (a/s), or YB1 decoy oligonucleotide (decoy) were harvested after 18 h. About 10⁶ cells from each transfection were analyzed for DNA fragmentation on a 2% agarose gel. Similar results were obtained in three independent experiments. C, YB1 antisense oligonucleotides reduce YB1 protein levels. SK Mel 5 cells transfected with negative control or antisense YB1 oligonucleotides were harvested at 18 h post-transfection. Lysates were analyzed by Western blotting with anti-YB1 or anti-ERK1 on the same blot. Similar results were observed in four independent experiments. D, transfection of RKO cell lines with negative control and YB1 decoy oligonucleotides. Wild type p53 cell line RKO (filled bars) and dominant-negative p53 derivative RKO p53.13 (hatched bars) were transfected with negative control or YB1 decoy oligonucleotides as indicated. Cells were harvested after 18 h, and cell viability was determined by trypan blue staining. The percentage of live cells was determined relative to cells treated with the negative control oligonucleotide. Values are the mean and standard errors obtained for quadruplicate counts of two individual experiments. E, induction of apoptosis in other tumor cell lines. Cells were transfected with antisense YB1 oligonucleotides (a/s YB1) or negative control oligonucleotide and fixed at 18 h post-transfection. Apoptosis was assessed by in situ TUNEL assay followed by fluorescence microscopy. Cells undergoing apoptosis show bright fluorescein labeling. Two classes are shown: wild type p53 tumor cells (A549 and B10.5) and mutant p53 tumor cells (HaCaT). Magnification ×40.
protein (46). In addition to the reporter experiment, we also showed that transfection of YB1 caused a down-regulation of endogenous p53 and a concomitant reduction in the ability of p53 to transactivate the p21\(^{WAF1/CIP1}\) promoter (Fig. 2). These data suggest that YB1, among other genes it regulates, might also be a negative regulator of p53, consistent with our hypothesis. In other experiments, the reduction of YB1 levels using antisense oligonucleotides, or an inhibition of YB1 activity, also be a negative regulator of p53, consistent with our hypothesis. Data suggest that YB1, among other genes it regulates, might pregnancy caused by YB1 in the nucleus (53). It was also shown that transfection of an antisense YB1 expression construct led to a reduction in p53-dependent promoter activity (47), completely the opposite of what we report here. However, our experiments have been repeated several times in different cells and with other p53-dependent promoters (48). We have never seen an up-regulation of p53 activity with YB1 in any of our experiments, although our cells were not treated with cisplatin as was used in the above report (47), which may well be an important difference. Nonetheless, a priori it is possible that the down-regulation of the p53 promoter and endogenous p53 levels (Figs. 1 and 2) is due to a p53-YB1 complex. However, this seems unlikely, as the p53 promoter repression by YB1 also occurs in the p53-defective cells (HeLa (Fig. 1), RKO p53.13, Saos2, and Skov3 (data not shown)). Nevertheless, there are reports that YB1 and p53 regulate expression from the same site in the \(mdr1\) promoter (14, 47, 49) and also interact with a third transcription factor, AP2α, to transactivate the \(MMP-2\) promoter (49). Thus, although YB1 and p53 may not interact to regulate expression of 53 itself, YB1 may bind p53 to regulate expression of other genes.

Although the down-regulation of p53 as we have reported here appears to be best explained by a transcriptional mechanism, consistent with other reports implicating a role for YB1 in transcriptional regulation (14, 47, 49), such an interpreta-

| Cell line | Source       | p53 status | Induction of apoptosis |
|-----------|--------------|------------|-----------------------|
| SK Mel5   | Melanoma     | wt         | +                     |
| HepG2     | Hepatoma     | wt         | +                     |
| A549      | Adenocarcinoma | wt       | +                     |
| NZM9      | Melanoma     | wt         | +                     |
| RKO       | Colon carcinoma | wt        | +                     |
| B10.2     | Fibrosarcoma | wt         | +                     |
| B10.5     | Fibrosarcoma | wt         | +                     |
| Jurkat U937 | T-cell leukemia | Mutant     | –                     |
| HLE90    | Promyelocytic leukemia | Null | –                     |
| RKO p53.13 | Colon carcinoma | Dominant negative | –                     |
| HaCaT     | Transformed keratinocyte | Mutant | –                     |
| SaOS2     | Osteosarcoma | Null       | –                     |
| IICF/Cc   | LiFraumeni skin fibroblasts | Null | –                     |

**Acknowledgments**—We thank Dr. Prudence Grandison for glutathione S-transferase-YB1 purification, Alan Wolfe for the antibody to FRG2, Dr. Michael Kastan for RKO and RKO p53.13 cells, Dr. David Lynch for cell lines B10.2 and B10.5, Dr. Bruce Baguley for cell line NZM9, and Lorna Strachan and Dr. Hilary Sheppard for reading the manuscript.

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