Differential regulation of human *YY1* and *caspase 7* promoters by prohibitin through E2F1 and p53 binding sites

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Prohibitin is a 30 kDa growth suppressive protein that has pleiotropic functions in the cell. Although prohibitin has been demonstrated to have potent transcriptional regulatory functions, it has also been proposed to facilitate protein folding in the mitochondria and promote cell migration in association with Raf-1. Our previous studies have shown that prohibitin physically interacts with the marked-box domain of E2F family members and represses their transcriptional activity; in contrast, prohibitin could bind to and enhance the transcriptional activity of p53. Here, we show that promoters of human *YY1* (Yin and Yang 1) as well as *caspase 7* genes are modulated by prohibitin. *YY1* promoter activity was reduced upon overexpression of prohibitin, while it was enhanced when prohibitin was depleted by small interfering RNA techniques. The repressive effects of prohibitin on the *YY1* promoter were mediated through E2F binding sites, as

seen by mutational analysis and chromatin immunoprecipitation assays. Further, depletion of E2F1 prevented prohibitin from repressing the *YY1* promoter. In contrast with *YY1*, prohibitin overexpression led to enhanced levels of *caspase 7*, whereas depletion of prohibitin reduced it. Interestingly, the *caspase 7* promoter was found to have p53-binding sites and prohibitin activated this promoter through p53. These studies show that prohibitin can have diverse effects on the expression of different genes and the activity of various cellular promoters is affected by prohibitin. Further, it appears very likely that prohibitin carries out many of its cellular functions by affecting the transcription of different genes.

Key words: E2F1, prohibitin, p53, retinoblastoma (Rb), transcription, Yin and Yang 1 (YY1).

INTRODUCTION

The maintenance of the normal phenotype of mammalian cells involves a fine balance between events such as proliferation, differentiation, senescence and apoptosis [1–3]. These vital biological events are controlled by a wide variety of cellular proteins that can respond to appropriate extracellular stimuli and elicit the proper response. Regulated and balanced functioning of growth promoting, as well as growth suppressing molecules, ensures that the above events occur at a desired magnitude and frequency [4,5]. The E2F transcription factors belong to a family of molecules that can affect different aspects of the above functions, mainly by modulating the expression of multiple genes whose functions are necessary for these biological processes [6,7]. Indeed, recent studies have suggested that approx. 7% of mammalian promoters may be regulated by E2Fs [8], showing their importance in mammalian cell biology. The best-studied mechanism of regulation of E2F is repression by the Rb (retinoblastoma) family of tumour suppressor proteins [7,9,10]; this illustrated a canonical example of a growth suppressing protein binding to and inactivating a class of molecules involved in cell proliferation and apoptosis [11]. Rb physically interacts with and represses transcriptionally active E2F1, 2 and also 3, by recruiting a variety of co-repressors such as HDAC1 (histone deacetylase 1), CtBP, Brg1 [12,13] and histone methyl transferases as well as DNA methyl transferases [14]. The phosphorylation of Rb in the G₁-phase by various cyclin-dependent kinases releases free, transcriptionally active E2F, thus facilitating the expression of genes necessary for S-phase entry [15,16].

Our search for additional proteins that might regulate E2F function led to the identification of prohibitin as a repressor of E2F activity [17]. Prohibitin is a 30 kDa protein whose gene was originally cloned based on its ability to suppress cell proliferation in mammalian cells [18,19]. Prohibitin was found to physically interact with Rb as well as E2F family members in mammalian cells [20]. The finding that prohibitin could repress the transcriptional activity of E2F provided a molecular explanation for its growth suppressive properties [17,20,21]. Prohibitin recruited HDAC1 as well as N-CoR for this repression [21]; studies from other laboratories have shown that chromatin remodelling proteins such as Brg1 and Brm are also required for prohibitin-mediated repression of E2F1 [22]. It was found that while Rb targets the transcriptional activation region of E2F1–3, prohibitin targets the conserved marked-box region of E2F1–5 [20]. Prohibitin-mediated repression of E2F1 function as well as suppression of cell proliferation required two distinct domains: the first spanning residues 74–116 involved in Rb binding; and the second spanning residues 184–214 that bound E2F1 and HDAC1, which can also potentially fold into coiled-coil structure [23]. In addition, Rb- and prohibitin-mediated repression of E2F1 is reversed by different extracellular signals: for example, Rb-mediated repression of E2F1 can be reversed by adenovirus E1A protein, but does not respond to IgM stimulation; the reverse is true for prohibitin-mediated repression of E2F1 [20]. Additional studies from our laboratory [24] have demonstrated the recruitment of different co-repressors on E2F-regulated promoters in proliferating, quiescent and senescent cells. Whereas, induction of quiescence leads to the recruitment of Rb, prohibitin, HDAC1

Abbreviations used: CAT, chloramphenicol acetyltransferase; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility-shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDAC1, histone deacetylase 1; Rb, retinoblastoma; RNAi, RNA interference; RT, reverse transcriptase; siRNA, small interfering RNA; YY1, Yin and Yang 1.

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and Brg1 to proliferative promoters, induction of senescence led to the recruitment of Rb and prohibitin along with HP1 γ and Suv39H to the same promoters [24]. These findings suggest that while Rb and prohibitin can both regulate E2F function, their underlying molecular mechanisms are different [20,24].

Recent data showed that chemotherapeutic drugs like camptothecin inactivate Rb family members while prohibitin remains functional, further emphasizing the difference between these two tumour suppressors [25]. Attempts to elucidate how prohibitin modulates camptothecin-induced apoptosis led to the finding that prohibitin, which is nuclear in MCF-7 and T47D cells, was exported to the cytoplasmic compartment upon camptothecin treatment [26]. This required the functioning of the exportin, CRM1 [27]. Further, these studies showed that prohibitin could physically interact with and enhance the transcriptional activity of p53 [26]. These results suggested that prohibitin could be affecting cell proliferation and apoptosis by affecting the transcriptional activity of E2F1 and p53.

The studies described here show that the human *YY1* (Yin and Yang 1) promoter is E2F1-responsive and responds to the endogenous levels of prohibitin. It was found that prohibitin could repress the *YY1* promoter through the E2F-binding sites and the depletion of prohibitin enhanced YY1 levels. Our results also show that the *caspase 7* promoter is p53-responsive and that prohibitin can enhance the transcription of this promoter through the p53-binding sites. Thus it appears that prohibitin can affect multiple cellular functions by modulating the activity of E2F family proteins as well as p53.

EXPERIMENTAL

Cell lines

MCF-7 cells were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) foetal bovine serum. An MCF-7 cell line stably overexpressing prohibitin was grown in the presence of blastocidin and zeomycin (both from Invitrogen) as recommended by the manufacturer's protocol. For tetracycline stimulation experiments, MCF-7 cells were stimulated with 1 μ g/ml of tetracycline for the required periods of time.

RNA isolation and Northern blotting

Tetracycline-inducible prohibitin-overexpressing MCF-7 cells were subjected to stimulation with 1 μ g/ml tetracycline for the appropriate times. Unstimulated cells were used as control. Total RNA was isolated by the RNeasy mini-prep kit from Qiagen following the manufacturer's protocol and was used for Northern blotting. YY1, prohibitin and caspase 7 cDNA probes were radiolabelled with [α -³²P]dATP and [α -³²P]dCTP using the Prime-Gene labelling kit (Promega). Total RNA (10 μ g) was denatured, separated on a formaldehyde agarose gel, capillary blotted on to Zeta-probe charged nylon membrane (Bio-Rad), and UV cross-linked and subjected to prehybridization and hybridization following the manufacturer's protocol. Signals were visualized by autoradiography.

Preparing siRNA (small interfering RNA) for prohibitin and E2F1

Prohibitin cDNA was TA-cloned into pCR3.1 (Invitrogen) and clones carrying prohibitin cDNA in *cis* and *trans* orientation were used in prohibitin siRNA preparation. E2F1 deletion constructs for *cis* and *trans* orientation cloned into pCR3.1 that carries amino acids 89–284 + 304-end were used for E2F1 siRNA preparation. siRNAs were prepared using Dicer and BLOCK-iT RNAi (RNA interference) purification kits purchased from Invitrogen.

Briefly, complementary RNA strands were synthesized for both using T7 RNA polymerase (Promega), annealed in equal molar concentration and subjected to dicer reaction for 17 h at 37°C. Diced oligonucleotides were purified using BLOCK-iT RNAi purification kit (Invitrogen) according to the supplied protocol and the purity of the siRNAs were checked on a 4% agarose gel. siRNA oligonucleotides were divided into aliquots and stored at –80°C for use as needed. Control and p53 siRNAs were obtained from Santa Cruz Biotechnology.

Western blotting

Tetracycline-inducible prohibitin-overexpressing MCF-7 cells were grown as described above and lysates from uninduced and tetracycline-induced cells were prepared. To test the gene silencing by dicer siRNAs for prohibitin and E2F1, MCF-7 cells were transiently transfected with 250 ng of the appropriate siRNA using Lipofectamine™ 2000 lipid-based carrier vehicle following the manufacturer's protocol. Lysates were prepared 24, 48 and 72 h after transfection. Western blotting for E2Fs, prohibitin, p53 and actin was performed as described previously [26].

Plasmids and generation of mutants

The *YY1* promoter vector and expression vectors pDCE2F1 and pCDNA3-Phb (where Phb stands for prohibitin) have been described before [20,23,28]. In order to prepare YY1–CAT (chloramphenicol acetyltransferase), a 1000 bp *YY1* promoter fragment was cloned by PCR using a forward primer that introduced an MluI restriction enzyme site (5'-ACGCGT-3') and a reverse primer that introduced an XhoI site (5'-CTCGAG-3') into the promoter. The TA-cloned PCR product was subcloned into pCAT-Basic vector (Promega) to generate the YY1–CAT reporter for use in transient transfection experiments. To prepare deletion mutants, forward primers (that introduce an MluI restriction site) annealing at –600 bp [YY1(518)–CAT], –480 bp [YY1(200)–CAT] and –120 bp [YY1(120)–CAT] were designed. PCR products were TA-cloned and subsequently subcloned into pCAT-Basic vector for transient transfection experiments. In order to generate a tetracycline-inducible construct for prohibitin overexpression, prohibitin cDNA was excised from pCDNA3-Phb by BamHI–XbaI and subcloned into pCDNA4/TO/myc–His A. The resulting plasmid is referred to as pcDNA4/TO/myc–His–Phb A.

The caspase 7 promoter fragment was cloned by PCR using a forward primer that introduced an MluI site (5'-ACGCGT-3') 5'-ACGCGTATCTCACACCAGTTAGAATGGCG-3' and a reverse primer that introduced an XhoI site (5'-CTCGAG-3') 5'-CTCGAGTGGTGGAGATGCTGCTCCT-3' into the promoter. The TA-cloned PCR product was subcloned into the pCAT-Basic vector (Promega) to generate the caspase 7–CAT reporter for use in transient transfection experiments. The details of expression vector pCDNA3-p53 have been described previously [26].

Transient and stable transfections

Transient transfections of 1×10^7 MCF-7 cells were performed by electroporation using a Bio-Rad Gene Pulser at 250 mV constant current; 4 μ g of the promoter reporter plasmids, 2 μ g of E2F and 8 μ g of prohibitin and its deletion mutants were used. A β -galactosidase-expressing plasmid (2 μ g) was used as an internal control for transfection efficiency. Assays for β -galactosidase and CAT activity were performed 72 h after transfection using standard protocols as described previously [23]. Experiments were repeated multiple times and results from two representative experiments are shown.

In order to establish a tetracycline-inducible prohibitin-overexpressing MCF-7 cell line, cells were transfected with 2 µg of pcDNA6/TR by electroporation. Medium was changed 24 h after transfection, and cells were maintained in 1.5 µg/ml blastocidin for selection until resistant colonies emerged; approx. 10 positive colonies were picked and expanded. These clones were used for further transfection in order to establish the tetracycline-inducible prohibitin-overexpressing MCF-7 cell line. Then, 2 µg of pcDNA4/TO/myc-His-Phb A (inducible expression vector) was transfected into TR-positive clones by electroporation. Medium was changed 24 h after transfection, and cells were allowed to grow for an additional 24 h. After that, they were subjected to selection with 1.5 µg/ml blastocidin and 250 µg/ml zeocin; fresh medium was supplied every 3 days until resistant colonies emerged. Colonies were picked and expanded for the analysis.

Semiquantitative RT (reverse transcriptase)-PCR

One microgram of RNA was DNase-treated using RQ1 DNase (Promega), followed by first-strand cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad). Of the final cDNA reaction, 1/20th volume was used in each PCR reaction. Primers sequences are as follows: *caspase 7* (forward primer) 5'-CTACCGCCGTG-GGAACGATGGCAGA-3'; *caspase 7* (reverse primer) 5'-CGA-AGGCCATACCTGTCACTTTATC-3'; *YY1* (forward primer) 5'-GCGGAGCCCTCAGCCATGGCCTCG-3'; *YY1* (reverse primer) 5'-CAGCGGTGCAGAGCGATCATGG-3'; *β-actin* (forward primer) 5'-ATCCTCACCTGAAGTACCC-3'; and, *β-actin* (reverse primer) 5'-TAGAAGGTGTGGTGCCAGAT-3'.

All PCR reactions were run at an annealing temperature of 53°C in the presence of 10% (v/v) DMSO. The PCR products were run on 3% agarose gel and the quantification of expression levels was performed using AlphaEase FC version 3.1.2 (Alpha Innotech).

ChIP (chromatin immunoprecipitation) assays

ChIP assays were performed as described previously [26,29] using chromatin from MCF-7 cells and prohibitin-overexpressing clone 4 MCF7 cells to test the interaction between E2F1, prohibitin or p53 and the *YY1* promoter, and prohibitin and the *caspase 7* promoter. Antibodies to E2F1-5 were purchased from Santa Cruz Biotechnology, prohibitin monoclonal antibody was purchased from NeoMarker and secondary anti-mouse antibody was purchased from Pierce. The sequences of the primers used in the PCR reactions were as follows: hYY1-promoter (forward primer) 5'-ACGCGTTCTAGGCTCTGTTGCAGG-3'; hYY1 promoter (reverse primer) 5'-CTCGAGCTCGATTCTCTCTCG-3'; hFOS promoter (forward primer) 5'-TGTTGGCTGCAGCCCGCAG-CAGTTC-3'; hFOS promoter (reverse primer) 5'-GGCGCGTG-TCCTAATCTCGTGAGCAT-3'; hYY1-EF-D-F (forward primer) 5'-ACGCGTGTCCGTTCCGTCCTCCACACTC-3'; hYY1-CDEF-D-F (forward primer) 5'-ACGCGTGGAAGCACAGGC-GATTCTCG-3'; hYY1-All-F (forward primer) 5'-ACGCGTGC-TGACGTCACGCGC-3'; YY1-A-ChIP-F (forward primer) 5'-AGGCGCGGCTGTAGCGGCAGC-3'; YY1-A-ChIP-R (reverse primer) 5'-CCGCGGCGCGTGACGTCAGC-3'; YY1-B-ChIP-F (forward primer) 5'-GTCCGTTCCGTCCTCCACAC-TCC-3'; YY1-B-ChIP-R (reverse primer) 5'-CGCGAATCGCCTG-TGCTTCC-3'; YY1-C-ChIP-F (forward primer) 5'-CCAACG-GCTGCGTCTCAG-3'; YY1-C-ChIP-R (reverse primer) 5'-CG-GCGCGAGCTCGGCGGTTG-3'; Caspase 7-ChIP-F (forward primer) 5'-ACTGGAATCCGTGTGTGTGTG-3'; Caspase 7-

ChIP-R (reverse primer) 5'-ATGCGGCCGGGAGTCAGTCA-AAC-3'.

EMSA (electrophoretic mobility-shift assay)

An EcoRI-HindIII fragment (120 bp) of adenovirus E2 promoter containing E2F-binding sites was end labelled with [α -³²P]dATP by Klenow enzyme (Promega) and used as E2F probe [25]. Whole cell extract (6 µg) from MCF-7 cells was incubated with 0.2 ng of labelled E2F probe in a buffer containing 20 mM Hepes (pH 7.9), 40 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 1 mM NaF, 1 mM Na₃VO₄, 0.5% Nonidet P40, 1.5 µg/ml of salmon sperm DNA and 10 µg/ml of BSA. For the non-radioactive competition, a 282 bp fragment of the *YY1* promoter containing E2F sites was added to the reaction (50 and 100 ng) prior to the incubation. After incubating at room temperature for 15 min, the reactions were separated on a 4% polyacrylamide gel in 0.25% TBE buffer (1 × TBE is 45 mM Tris/borate/1 mM EDTA) at 300 V for 2.5 h at 4°C [17,25,26]. The gels were then dried and the signals were visualized by autoradiography.

RESULTS

Tet-inducible expression of prohibitin in MCF-7 cells

Since prohibitin is known to repress the transcriptional activity of E2F family members and enhance the activity of p53 protein, we decided to examine the effect of prohibitin on gene expression. As a first step, we established an MCF-7 cell line that overexpresses prohibitin in a tetracycline-inducible fashion (Figure 1A). Several clones were expanded and induced by tetracycline. Prohibitin levels in these clones were examined by Northern and Western blotting. Northern-blot analysis and corresponding Western blot of clones 4 and 7 showed induction of prohibitin expression, whereas clone 1 had little or no induction (Figures 1B and 1C). We selected clone 4 for further analysis. Time course of prohibitin expression in clone 4 after tetracycline induction for 6, 12 and 24 h was determined by Northern-blot analysis, which showed up-regulation of prohibitin message level after 6 h of tetracycline induction (Figure 1D). However, Western blotting showed a predominantly high level of prohibitin protein at the 24 h time point (Figure 1E). Based on these observations, we chose 24 h post-tetracycline stimulation as the time point for further experiments.

A preliminary examination of sequence of human *YY1* promoter revealed potential E2F1-binding sites; similarly, the *caspase 7* promoter was found to have p53-binding sites. Since these promoters were not known to be regulated by E2F1 or p53, we chose them for studying response to E2F1 and p53, as well as how prohibitin affects their function in the stably transfected cells.

Prohibitin affects *YY1* and *caspase 7* expression

The regulation of *YY1* gene by prohibitin was first examined by Northern blotting. A 720 bp PstI fragment isolated from *YY1* cDNA, the complete prohibitin coding sequence and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) cDNAs were radiolabelled and used to probe total RNA from uninduced and tetracycline-induced clone 4. As shown in Figure 2(A), *YY1* was found to be down-regulated in tetracycline-induced samples. This was also verified by Western blotting, and as shown in Figure 2(B) the increase in prohibitin levels led to a corresponding reduction in the levels of *YY1* protein, which confirmed the Northern-blot result.

A similar analysis was carried out to investigate caspase 7 expression. As a first step, *caspase 7* cDNA was radiolabelled and used for Northern blotting. Figure 2(C) shows a moderate

Figure 1 Establishment of MCF-7 cell lines stably transfected with tetracycline-inducible prohibitin

(A) Schematic diagram showing the preparation of constructs and cell lines. **(B)** Northern blot showing the induction of the *prohibitin* gene upon tetracycline (Tet) stimulation in three selected clones. GAPDH mRNA was used as a loading control. **(C)** Western blot showing the tetracycline response of the three clones. Actin was used as the loading control. **(D)** Time course analysis of tetracycline induction of prohibitin. Northern-blotting showed that *prohibitin* mRNA levels increase within 6 h of tetracycline induction. GAPDH mRNA was used as the loading control. **(E)** Western blot shows the highest prohibitin protein level is at 12 and 24 h post-tetracycline induction.

up-regulation of *caspase 7* expression in clone 4 at 6 and 12 h post-tetracycline stimulation. This was verified by Western blotting (Figure 2D) where a gradual and continuous increase in caspase 7 protein level corresponded with the increased expression of prohibitin.

As shown earlier, *prohibitin* mRNA was up-regulated upon tetracycline stimulation, while *GAPDH* mRNA was unchanged; similarly in the Western blots, β -actin was used as the loading control and was unaffected by tetracycline stimulation. We further confirmed these results by performing semiquantitative RT-PCR. As shown in Figure 2(E) the expression levels of *YY1* are greatly reduced in tetracycline-induced cells, while *caspase 7* was more highly expressed in these cells compared with uninduced cells. The quantification of expression levels performed using AlphaEase FC version 3.1.2 (Alpha Innotech) is depicted in Figure 2(F). These results suggest that prohibitin overexpression can differentially affect genes such as *YY1* and *caspase 7*.

The *YY1* promoter is E2F1-responsive

A close examination of the *YY1* promoter sequence revealed presence of at least six potential binding sites for E2F1 (Figure 3A) in both orientations, as present in many promoters that are regulated by E2F. We hypothesized that the down-regulation of *YY1* by prohibitin overexpression could be E2F-mediated, since

prohibitin is known to regulate E2F transcriptional activity. The E2F transcription factor family is involved in the expression of many genes expressed in response to growth factor stimulation [9]. In order to test the functionality of the observed E2F-binding sites, a 1000 bp fragment corresponding to the *YY1* promoter region was cloned by PCR from the original fragment and placed upstream of a CAT reporter gene. It was found that while the cloned *YY1* promoter fragment had only minimal activity, co-transfection of E2F1 could induce the activity of *YY1* promoter significantly (Figure 3B), suggesting that the *YY1* promoter is E2F-responsive. Interestingly, co-transfection of prohibitin significantly suppressed E2F1-mediated transcriptional induction of the *YY1* promoter, suggesting that prohibitin is capable of modulating the *YY1* promoter through the E2F1-binding sites.

Attempts were made to identify the region of the *YY1* promoter that mediated the E2F1 response. Towards this purpose, we generated deletion mutants of the promoter to identify the shortest E2F-responsive fragment. Three deletion mutants that retained 600, 480 and 120 bp of the promoter were generated by PCR and cloned into pCAT-Basic reporter vector and named YY1 (518)-CAT, YY1 (200)-CAT and YY1 (120)-CAT respectively (Figure 3C, left panel). Transient transfection experiments were performed in MCF-7 cells with and without pDCE2F1. Cells were harvested after 72 h of growth and subjected to the CAT assay. As shown in Figure 3(C) (right-hand panel), the - 518 and

Figure 2 Prohibitin overexpression affects YY1 and caspase 7 levels

(A) Northern-blot analysis showing down-regulation of YY1 upon prohibitin induction. As shown in the top panel, *prohibitin* mRNA levels increase after 6 h of induction. Reprobing for YY1 showed a gradual decrease in mRNA expression as prohibitin level increased. GAPDH mRNA levels remained unchanged. (B) Western blot showing the induction of prohibitin, while YY1 protein level decreased. (C) Northern blot showing the up-regulation of *caspase 7* upon prohibitin induction. GAPDH mRNA levels remain unchanged (bottom panel). However, *caspase 7* expression appeared to be going up until 6 h and then decreased. (D) Corresponding Western blots for prohibitin and caspase 7 showed a gradual increase in protein levels. (E) Semi-quantitative RT-PCR for YY1 and *caspase 7* shows cDNA from prohibitin-overexpressing MCF-7 cells in the presence or absence of tetracycline. Prohibitin overexpression down-regulates YY1 expression, while it up-regulates *caspase 7*. PCR for actin was used as the control. (F) Quantification of the PCR data shown in (E). Tet, tetracycline.

–200 bp fragments of the YY1 promoter were fully active and responded to E2F1, whereas the –120 bp fragment did not respond to E2F1, suggesting that the binding sites between –120 and –518 are involved in mediating the E2F1 response of YY1.

Prohibitin regulates YY1 through E2F

We next examined whether the cloned YY1 promoter could respond to prohibitin overexpression by tetracycline induction in clone 4. To test this, MCF-7 clone 4 cells were transiently transfected with YY1-CAT with or without E2F1; after 24 h of growth, cells were treated with tetracycline for 24 h. A CAT assay showed that E2F1 could stimulate YY1 promoter activity in the unstimulated control cells (Figure 4A), whereas the reporter alone had only minimal activity. In contrast, the tetracycline treatment led to the repression of YY1 promoter activity, even when E2F1

was co-transfected. This shows that the levels of prohibitin in the cells can affect the transcriptional activity of the YY1 promoter and supports the earlier results obtained by transient transfection of prohibitin (Figure 3B).

Further experiments were conducted to assess whether the E2F-binding domain of prohibitin was necessary to repress the YY1 promoter. Towards this purpose, MCF-7 cells were transiently co-transfected with YY1-CAT, with or without E2F1 and series of deletion mutants of prohibitin, shown in Figure 4(B). After 72 h, cells were harvested and subjected to the CAT assay. It was found that all the mutants that carry E2F-binding domain (184–215) efficiently repress YY1 promoter activity mediated by E2F1. The deletion mutant that lacks this domain (Δ 184–214) could not repress E2F1 activity on YY1 promoter (Figure 4B, right panel), which implies a direct contact between prohibitin and E2F1 for YY1 promoter regulation.

Figure 3 YY1 promoter is E2F-responsive

(A) Schematic diagram of human YY1-promoter showing — 1000 bp region and putative E2F-binding sites; their orientation is indicated by arrowheads. Actual positions of sites are shown below the arrowheads. (B) Induction of YY1-CAT by E2F1. YY1-CAT reporter alone showed little CAT activity, but co-transfection of 2 μ g of E2F1 induced the promoter significantly. Co-transfection of prohibitin (Phb) suppressed E2F1-mediated promoter activity. YY1(518)-CAT (4 μ g) having approx. 600 bp and YY1(200)-CAT (4 μ g) having approx. 400 bp of the promoter show E2F1 response comparable with the full-length promoter, while YY1(120)-CAT (4 μ g) having 120 bp of the promoter is not E2F1-responsive. Schematic diagrams of the reporters are shown in the left panel and CAT activities are shown in the right panel.

Putative E2F-binding sites on YY1 promoter can bind E2F *in vitro*

Since it appeared that the E2F-binding sites could respond to E2F1 in transient transfection experiments, attempts were made to assess whether E2F does indeed bind to this promoter. This was first examined by EMSA. For this, a 282 bp fragment (corresponding to the underlined region on the YY1 promoter; Figure 4C) covering at least four potential E2F-binding sites were used in an unlabelled competition experiment. An adenovirus E2 promoter fragment, which has been used extensively to study the DNA binding activity of E2Fs [17,25,26] was radiolabelled and incubated with MCF-7 extracts for this experiment. As shown in Figure 4(D), we observed a binding pattern with a 120 bp probe derived from adenovirus E2 promoter that is consistent with published E2F binding patterns. Further, addition of 282 bp YY1 promoter fragment in reaction mixture along with labelled E2 probe competed for the binding of E2F protein complexes. This competition was dose-dependent, showing more than 90 % competition at 100 ng of unlabelled YY1 promoter.

siRNA against prohibitin and E2F1 have opposite effects on YY1 expression

The above results indicated that the YY1 promoter was E2F-responsive and that prohibitin overexpression down-regulated the YY1 promoter by targeting E2F. We tested this further by utilizing siRNAs that target prohibitin and E2F1. Briefly, 250 ng aliquots of the appropriate siRNA molecule were transfected, using Lipofectamine™ 2000, into MCF-7 cells in duplicate sets; cells were harvested after 24, 48 and 72 h and protein levels

analysed by Western blotting. As shown in Figure 5(A), prohibitin expression gradually decreased with time and was significantly reduced 72 h after transfection with the siRNA. The same blot was stripped and reprobed for YY1 as well as caspase 7 levels. It was found that YY1 levels were up-regulated after 72 h, correlating with the reduction in prohibitin levels. In contrast, caspase 7 levels were decreased significantly when prohibitin levels were reduced. There was no change in the expression level of actin, which was examined as a loading control. Such changes in the levels of prohibitin, YY1 or caspase 7 were not observed in cells that were mock transfected with Lipofectamine™ 2000 alone or with a control siRNA molecule. Taken together, these results suggest that depletion of prohibitin *in vivo* leads to an increase in YY1 expression levels, while caspase 7 expression is reduced. Thus YY1 expression is reduced by prohibitin *in vivo*, whereas caspase 7 expression is enhanced by prohibitin.

The effect of depleting E2F1 on YY1 was opposite to that of depleting prohibitin. As shown in Figure 5(B), E2F1 levels were reduced 24–48 h after transfection of the siRNA (Figure 5B). Interestingly, reprobing of the blot showed a significant down-regulation of YY1 at 24 h, when E2F1 levels were the lowest, while there was no change in actin expression, which was used as the loading control. The specificity of E2F1 siRNA was verified by reprobing the blot for two other E2F family members, E2F2 and E2F3, which showed no change; further, transfection of a control siRNA did not alter the levels of E2F1 or prohibitin, indicating the specificity of the siRNAs used. Taken together, it may be concluded that E2F1 acts as a regulator of YY1 and this regulation can be affected by prohibitin.

Figure 4 Prohibitin regulates YY1 promoter by suppressing E2F

(A) YY1-CAT alone showed little or no CAT activity but co-transfection of E2F1 induced the promoter. Stimulation with tetracycline (tet) suppressed E2F1-mediated induction of the YY1 promoter. (B) E2F-binding domain of prohibitin is necessary to suppress YY1 promoter. MCF-7 cells were transiently transfected with YY1-CAT alone or with pDCE2F1; wild-type prohibitin or mutants lacking Rb-binding or E2F1-binding domains were co-transfected. As seen in the right panel, YY1 promoter is induced by pDCE2F1; this was repressed by full-length prohibitin (Phb) as well as prohibitin-(1–243). Mutants lacking Rb-binding domain or E2F-binding domain could not repress E2F1-mediated YY1 promoter activity. (C) YY1 promoter fragment showing the regions used in EMSA as well as ChIP assays. (D) An EMSA using MCF-7 whole cell extracts on a probe derived from adenovirus E2 promoter. This probe has been used extensively in our laboratory and other laboratories for assaying E2F. ³²P-labelled E2 probe was mixed with whole cell extract in the absence or presence of YY1 cold probe in two different concentrations, and EMSA was carried out as described in the Experimental section. As can be seen, YY1 cold probe could significantly compete out binding of E2 probe with E2F complexes in a dose-dependent fashion.

siRNA against p53 affects caspase 7 expression

The results presented in this paper, show that caspase 7 is up-regulated in response to the overexpression of prohibitin. To see the effect of p53 on the expression levels of caspase 7, we transfected the cells with either control siRNA or siRNA against p53. The cells were incubated for 24 h in complete medium. One set of transfected cells was induced with tetracycline and lysates were prepared after 24 h. A Western blot for caspase 7 revealed a decrease in expression levels of caspase 7 on silencing of p53 compared with control siRNA-transfected cells, suggesting that prohibitin and p53 act synergistically to affect caspase 7 expression (Figure 5C).

We performed semiquantitative RT-PCR for *caspase 7* to evaluate the effect of silencing p53 on *caspase 7* expression. MCF-7 tetracycline-inducible clone 4 was transfected with either control siRNA or siRNA against p53. The transfected cells, one plate each, were treated with camptothecin in the presence or absence of tetracycline. After 24 h of treatment with camptothecin, cDNA was prepared from the treated cells to perform semiquantitative RT-PCR for *caspase 7*. As shown in Figures 5(D) and 5(E), we found that the expression of caspase 7 was elevated upon tetracycline stimulation. This increase was also observed with camptothecin treatment, indicating an apoptotic response. Interestingly, the expression of caspase 7 was further induced in control siRNA-

transfected cells upon camptothecin treatment and tetracycline stimulation, in contrast with cells transfected with siRNA against p53. We did not observe this increase in p53 siRNA-transfected cells, suggesting that prohibitin can further activate p53 activity and this leads to the added induction of caspase 7.

The YY1 promoter interacts with E2Fs and prohibitin *in vivo*

ChIP assays were carried out to examine whether E2F1 binds to the YY1 promoter *in vivo*. ChIP assay lysates were prepared from prohibitin overexpressing MCF-7 clone 4 cells that were stimulated with tetracycline for 0, 6, 12 and 24 h. The lysates were immunoprecipitated with a control antibody or antibodies against E2F1 and prohibitin. The presence of a 282 bp YY1 promoter fragment (Figure 6A) in the immunoprecipitate was examined by PCR. As shown in Figure 6(B), there was no change in E2F1 association with the human YY1 promoter upon tetracycline stimulation at various time points, as seen by the recovery of comparable amounts of the promoter fragment in the E2F1 immunoprecipitation. However, the association of prohibitin with the promoter gradually increased with a maximum binding occurring after 24 h of tetracycline stimulation. This supports the earlier observation that YY1 expression levels decrease gradually upon induction of prohibitin. The YY1 promoter fragment could not

Figure 5 siPhb and siE2F1 show opposite effect on YY1 expression

(A) MCF-7 cells were transiently transfected with 250 ng of siPhb or siE2F1 using Lipofectamine™ 2000 following the manufacturer's protocol. Cells were harvested after 24, 48 and 72 h and lysates Western blotted for various proteins. There was a gradual decrease in prohibitin levels compared with mock transfected cells; in contrast, there was a corresponding increase in YY1 protein levels. On the other hand, caspase 7 levels decreased along with prohibitin. (B) Transfection of siE2F1 led to the reduction of E2F1 protein by 24 h, which reappeared by 72 h. siE2F1 did not affect the levels of E2F2 and E2F3. There was a significant decrease in YY1 protein level at 24 h when the E2F1 is lowest. Loading control actin remained unchanged. (C) Transfection with sip53 led to decreased expression of caspase 7; however, induction with tetracycline (tet) to overexpress prohibitin could still up-regulate the caspase 7 expression while the levels of E2F1 and actin remained unchanged. (D) Semiquantitative RT-PCR for caspase 7 to evaluate the effect of silencing p53 on caspase 7 expression. MCF-7 tetracycline (tet) inducible clone 4 was transfected with either si control or sip53. Camptothecin (Campto) treatment induces caspase 7 expression and addition of tetracycline further increases the levels of caspase 7 mRNA while cells transfected with sip53 do not show this induction. (E) Quantification of the data presented in (D).

be detected by immunoprecipitation with an irrelevant antibody, confirming the specificity of the antibodies used in the ChIP assay.

Since E2F1 was found to be associated with the promoter, we asked whether other E2F family members could also bind to the *YY1* promoter. This was also tested by ChIP assays and lysates were prepared from MCF-7 clone 4 cells treated with tetracycline for 0 or 24 h. Immunoprecipitation was performed using antibodies against various E2F members, prohibitin and an irrelevant antibody as control. As shown in Figure 6(C), all E2F family members appeared to be binding to the promoter region and they were not altered upon tetracycline stimulation. As expected, a significant amount of prohibitin bound to the promoter upon tetracycline stimulation. The irrelevant antibody control did not pull down the promoter fragment; further, the unrelated pro-

motor *c-fos* was also absent from immunoprecipitations with antibodies to prohibitin and E2Fs, indicating the specificity of the assay. We had found that depletion of E2F1 by siRNA reduced the expression of YY1 (Figure 5B); given that observation, it appears that, although multiple E2F family members bind to the *YY1* promoter, it is probably E2F1 that plays a major role in its expression.

As mentioned earlier, there were at least six potential E2F-binding sites in the *YY1* promoter; we therefore tested which sites were associating with E2Fs *in vivo*. To test this, sets of primers flanking two E2F sites each were designed; it was extremely difficult to design independent primer sets for each binding site, since they were very close to each other and overlapping in some cases. The regions spanned by the primer sets were named A, B

Figure 6 ChIP assays showing *in vivo* association of E2F1 and prohibitin with the YY1 promoter

(A) Schematic diagram showing E2F-binding sites and areas selected for ChIP assays. (B) MCF-7 clone 4 overexpressing prohibitin was grown with or without tetracycline; induction was for 6, 12 and 24 h and ChIP assays performed with prohibitin, E2F1 or irrelevant antibodies. PCR was performed using a primer set that flanked 282 bp region of promoter. There was a consistent increase in the association of prohibitin with the YY1 promoter, with maximum at 24 h. However, there was no or little increase in E2F1 association. Irrelevant antibody or negative control (no input DNA) did not show any bands suggesting the specificity of the assay. (C) Other E2F family members can also bind to YY1 promoter. All the tested E2F family members appeared to be associated with YY1 promoter. Also, prohibitin level was significantly higher in the tetracycline-induced sample. (D) Proximal four sites of YY1 promoter bind to E2F. ChIP assays were performed using E2F1 and prohibitin antibodies and primers covering three different areas of promoter (A, B and C) spanning E2F-binding sites as shown in (A). E2F and prohibitin bound to the proximal four sites falling in areas A and B in induced or un-induced cells. There was no association of E2F or prohibitin with area C comprising the distal E2F sites in the promoter. Tet, tetracycline; Phb, prohibitin; IAb, irrelevant antibody; Fos, c-fos.

or C (Figure 6A). As can be seen in Figure 6(D), ChIP assays showed that promoter regions A and B covering four potential E2F-binding sites were immunoprecipitated by E2F1 as well as prohibitin antibodies in both tetracycline stimulated or unstimulated cells; however, promoter region C covering two distal sites was not precipitated with E2F1 or prohibitin antibody. These results imply that the proximal four potential E2F-binding sites are perhaps the functional sites and respond to E2F transcription factor driving the *YY1* expression; also these are the sites where prohibitin binds the promoter through E2Fs. The irrelevant antibody did not immunoprecipitate *YY1* promoter fragments and

similarly, the unrelated control *c-fos* promoter was absent from the ChIP assay PCRs indicating the specificity of the assay.

Prohibitin enhances p53-induced caspase 7 activity

The analysis of the *caspase 7* promoter revealed several potential p53-binding sites (Figure 7A). Since studies from our laboratory have shown that prohibitin could physically interact with and enhance the transcriptional activity of the p53 protein, we made an attempt to study the role of p53, and also the influence of prohibitin on transcriptional activity of p53, on the *caspase 7* promoter. Towards this purpose, the *caspase 7* promoter was amplified by PCR and cloned upstream of the CAT reporter gene in pCAT-Basic. This reporter was transfected into MCF-7 cells where it showed basal activity. Co-transfection of p53 increased the reporter activity significantly (Figure 7B). Interestingly, co-transfection of the prohibitin further enhanced p53-mediated activation of the *caspase 7* promoter (Figure 7B). We compared the co-operative effect of p53 and prohibitin on *caspase 7* promoter with that on *MDM2* promoter and found that prohibitin had a similar effect on both the promoters (Figure 7C). This confirms that prohibitin could up-regulate the *caspase 7* promoter through p53. Further, transfection of a variety of prohibitin mutants showed that mutating any region of prohibitin other than the extreme C-terminal region abolished the ability of prohibitin to 'superactivate' p53-mediated transcription (Figure 7D). These results suggest that prohibitin can superactivate p53 leading to increased transcription of caspase 7.

We further confirmed our results by using the stably transfected MCF-7 clone 4 that overexpresses prohibitin under tetracycline stimulation. Transfection of this cell line with the *caspase 7*-CAT promoter reporter construct showed basal CAT activity (Figure 8A). Transfection of p53 increased the reporter activity, and transfection of the p53 construct in cells treated with tetracycline led to a further increase in the reporter activity (Figure 8A), whereas tetracycline alone did not alter the reporter activity. We ascertained the binding of prohibitin and p53 to the *caspase 7* promoter by ChIP assays on lysates from MCF-7 cells using an antibody against prohibitin. Anti-mouse secondary antibody served as negative control of immunoprecipitation and the *c-fos* promoter was used as a negative control for prohibitin binding (Figure 8A). The PCR revealed recruitment of prohibitin to the *caspase 7* promoter. Further we performed ChIP assays in MCF-7 cells prior to and after tetracycline induction. The results in Figure 8(C) suggest a quantitative increase in binding of prohibitin and p53 to the promoter (120 bp upstream) (175 base-pairs) after induction with tetracycline. These results confirm that p53 in association with prohibitin significantly up-regulates the *caspase 7* promoter.

DISCUSSION

Prohibitin, a potential tumour suppressor gene, has been proposed to have functions in the nucleus, mitochondria and the cell membrane. Its proposed functions range from transcriptional regulation, protein folding, cell motility and inhibition of apoptosis. Given these varied functions of prohibitin, it is not surprising that it is capable of modulating the expression of genes involved in many of these processes. Studies from our and other laboratories have shown that prohibitin is present in the nucleus of several breast cancer cell lines where it can bind to p53 as well as stimulate p53 transcription [26,31,32]. p53 plays a pivotal role in cellular response to genotoxic damage and other forms of stress by inducing cell-cycle arrest and apoptosis [14,15]. Tang et al. [32a] have demonstrated that the down-regulation of prohibitin

Figure 7 Caspase 7 promoter is p53-responsive

(A) Schematic diagram of human caspase 7 promoter showing the putative p53-binding sites. Actual positions are shown above the arrowheads; at least nine potential p53-binding sites can be discerned in the promoter region. (B) Induction of caspase 7 CAT by p53. MCF-7 cells were transiently transfected with the indicated vectors by electroporation. Caspase 7-CAT vector (4 μ g) showed little CAT activity, whereas co-transfection of 2 μ g of p53 induced the promoter; co-transfection of prohibitin vector increased p53-mediated induction of caspase 7-CAT. (C) Comparison of caspase 7 promoter with MDM2 promoter reveals that both promoters are induced by p53; co-transfection of prohibitin caused further activation of both promoters. (D) Deletion mutants of prohibitin showing that only full-length and 1–243 construct can superactivate p53-mediated induction of caspase 7 promoter. Phb, prohibitin.

and p53 was concomitantly reduced on silencing of the stress-responsive gene *BRE* (brain and reproductive organ-expressed), suggesting an integrated role of prohibitin and p53 in cell-cycle regulation. Although earlier studies from our laboratory have shown that prohibitin could affect the transcription of reporter genes regulated by E2F1 and p53, the cellular targets of prohibitin remained elusive. The studies described in the present paper, have shown that promoters of the YY1 transcription factor and caspase 7 are prohibitin-responsive. Interestingly, the down-regulation of *YY1* by prohibitin is mediated through E2F-binding sites in the genes promoter, whereas the up-regulation of caspase 7 is through p53-binding sites. These results are in complete agreement with our earlier findings that prohibitin can repress E2F1-mediated transcription while up-regulating p53 function [26].

It is interesting that a protein with multiple functions and the ability to regulate both transcriptional activation and repression is modulating the expression of the YY1 transcription factor. The bifunctional YY1 transcription factor is a 65 kDa protein that is ubiquitously expressed, highly conserved among human, mouse and *Xenopus*, and is classified as GLI-Krüppel family member with zinc-finger domains [33,34] that represses and activates a variety of promoters such as m-Myc, c-Fos, p53, surf-2, grp78, IgH enhancer, β -casein, Ig κ 3' enhancer, IFN- γ (interferon γ), rpl30, rpl32, P5 of AAV, E6 and E7 of HPV, Mx2 and many more [35,36]. It is hypothesized that the potential binding of HDAC1 or HATs (histone acetyl transferases) such as p300/CBP [CREB (cAMP-response-element-binding protein)-binding protein] to YY1 could be the key to answer how it variably regulates different genes [37,38]. Association of YY1 with high-molecular-mass Notch complexes has been reported to suppress the transactivation activity of Notch, thereby modulating the

Notch signalling pathway [39]. Other evidence has suggested a role for YY1 in amplifying p53 promoter activity. Conversely, in response to genotoxic stress, YY1 can also inhibit the activation of p53 [37,38,40]. Evidence shows that in HeLa cells, YY1 could play a part in tumour suppression, whereas it could also help in discerning the specificity of E2F function by binding with E2Fs, specifically with E2F2 and E2F3 through RYBP (RING1 and YY1 binding protein) [41]. Thus YY1 is a versatile multifunctional transcription factor. Although the *YY1* promoter had been cloned earlier, its regulation by E2F family members had not been observed. Here, our finding that human *YY1* promoter is E2F-responsive sheds light on a new scenario where the levels of prohibitin modulate the expression of the bifunctional transcription factor YY1, which in turn can up- or down-regulate various genes facilitating proliferation, differentiation or apoptosis. These findings also place YY1 downstream of E2F family members, some of whose activity is affected by YY1 levels. Thus there appears to be a complex network of transcription factors and associated proteins that variably modulates the proliferative state of the cell, allowing it to divide, senesce, differentiate or apoptose efficiently.

Apoptosis is an important mechanism involved in many biological processes where caspases play a crucial role [42]. An interesting observation made in this regard is the transcriptional regulation of *caspase 8* by p53. Liedtke et al. [43] have shown by computer-assisted analysis with the p53-responsive element 5'-TCCTGCCTGCCTG-3' a 100% identity to a sequence located within the murine *caspase 8* promoter; also their study reveals the transcriptional regulation of the *caspase 8* gene by basal (Sp1- and Ets-dependent) and inducible (p53-dependent) mechanisms. In this context our finding that the *caspase 7* promoter is responsive

Figure 8 p53 and prohibitin associate with the caspase 7 promoter

(A) MCF-7 clone 4 cells were transiently transfected with caspase 7 CAT and p53 in the presence or absence of tetracycline (tet). Caspase 7–CAT was induced by p53; induction of prohibitin by tetracycline stimulation further induced the caspase 7 promoter. Stimulation with tetracycline in absence of p53 did not alter basal CAT activity. (B) ChIP assays showing *in vivo* association of prohibitin (Phb) with the caspase 7 promoter. MCF-7 clone 4 cells were stimulated with tetracycline for the indicated periods; lysates were immunoprecipitated with prohibitin or irrelevant antibodies (IRAb). PCR was performed on the indicated region of the caspase 7 promoter (Figure 7A) or the irrelevant *c-fos* (Fos) promoter. As can be seen, prohibitin could associate with the caspase 7 promoter. (C) ChIP assays showing *in vivo* association of p53 and prohibitin (Phb) with the putative p53 binding site on a 120 bp proximal caspase 7 promoter fragment. Induction with tetracycline increased the binding of both prohibitin and p53 to this particular binding site, suggesting their co-operative activity. Blank, lane with no input DNA; IrAb, irrelevant antibody.

to p53 and prohibitin is relevant and novel. It also sheds light on how prohibitin might be affecting the apoptotic process. Studies from other laboratories have shown that prohibitin could modulate the amount of apoptosis induced by agents like camptothecin and androgens [22,44]. Caspase 7 is a member of CED-3 (cell-death determining-3) subfamily; it is a 303-amino-acid protein with high similarity to caspase 3 [45–47]. Caspase 7 is activated by apoptosis induced by DNA damaging agents that are dependent on p53 [48–50]. We have shown previously that prohibitin could superactivate p53 on the *MDM2* promoter [26]. The present paper shows that prohibitin can affect a pro-apoptotic protein, by up-regulating p53 function.

The paper essentially throws light on the potential mechanisms by which prohibitin can modulate cell proliferation and apoptosis by affecting the expression of cellular genes. Thus it appears that the functions of prohibitin in transcription regulation may mediate many of the reported effects on the biology of the mammalian cell. It can be expected that further studies will expand these observations in the context of deregulated cell proliferation and enhanced survival of cancer cells.

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