

Human Homologue of Yeast Rad23 Protein A Interacts with p300/Cyclic AMP-responsive Element Binding (CREB)-binding Protein to Down-Regulate Transcriptional Activity of p53¹

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Abstract

The tumor suppressor protein p53 regulates various cellular responses to DNA damage and plays a significant role in DNA repair. The nuclear p300/cyclic AMP-responsive element binding (CREB)-binding protein (CBP) proteins act as coactivators in supporting the transcription function of p53. We examined the role of the human homologue of yeast Rad23 protein A (hHR23A), one of the two human homologues of the *Saccharomyces cerevisiae* nucleotide excision repair gene product Rad23, in the p300/CBP-associated regulation of p53 activity. Overexpression of wild-type hHR23A inhibits the p53 transcriptional activity and results in a decreased steady-state protein level of cellular p53. The inhibitory effect of hHR23A can be overcome by the concomitant expression of p300, CBP, and p300 segments harboring C/H1 domain and neutralized by the coexpression of HIV accessory protein Vpr, which binds COOH terminus of hHR23A/B. Additionally, hHR23A was shown to interact *in vitro* and *in vivo* with p300 segments harboring C/H1 domain. These studies provide evidence for the involvement of hHR23A in the regulation of p53 activity through p300/CBP. Although the precise direct role of hHR23 proteins in regulation of p53 and DNA repair remains to be elucidated, our data suggest that the interaction between hHR23A and p300/CBP has important implications in cross-talk between the p53 pathway and DNA repair.

Introduction

The human tumor suppressor p53 is deemed critical for maintaining genomic stability and homeostasis (1). Diverse mutations in the p53 gene constitute the most common type of genetic alterations in human cancers (2). It is believed that tumor suppressor p53 protein, accumulated in response to DNA damage, transcriptionally activates downstream target genes, thereby signaling a G₁ cell cycle checkpoint for cells to perform DNA repair before the transit from G₁ to S phase (3). Many p53-response genes have been identified as important mediators of p53-dependent cell growth arrest, apoptosis, and p53-regulated DNA repair. For example, p21^{waf1} is mainly responsible for p53 induced G₁ arrest (3), Bax contributes to apoptotic response (4), whereas GADD45 and p48 play a role in p53-regulated DNA repair (5–7).

It is known that shortly after exposure of cells to DNA-damaging agents, p53 protein becomes stabilized and activated. This is achieved mainly through posttranslational modifications of the p53 protein (8). p300/CBP proteins function as the transcriptional coactivators in

supporting p53 transcriptional activity (9–11). p300/CBP³ is known to directly bind to the p53 activation domain to potentiate transcription by p53. Both p300 and CBP are intrinsic histone acetyltransferases (12), and their enzymatic activities have recently been demonstrated to be involved in a novel mechanism of transcriptional regulation through alterations in chromatin structure (13). In addition to these functions, p300/CBP has been shown to be involved in p53 degradation process. The NH₂ terminus of p300 has been shown to interact specifically and independently with p53 and MDM2, and these interactions are required for normal p53 turnover (14). Through such an interaction, MDM2 can also inhibit p53-mediated transactivation (15).

It has become increasingly clear that p53 plays an important role in the regulation of DNA repair (16–21). Nevertheless, the linkage between the p53 pathway and DNA repair largely remains to be elucidated. Among the different DNA repair proteins, hHR23A seems to play a dual role, *e.g.*, in DNA repair and cell cycle progression. Notably, hHR23A has been identified as a substrate for E6AP ubiquitin ligase, and its protein levels are regulated in a cell cycle-dependent manner (22). This report explores for the first time the potential role of hHR23A in the regulation of p53 transcriptional activity. The data demonstrate that hHR23A can interact with p300/CBP to down-regulate p53 transcriptional activity and suggest that hHR23 proteins play a key role in cross-talk between the p53 pathway and DNA repair.

Materials and Methods

Plasmid DNAs. Plasmid PG13-*luc*, a luciferase reporter containing 13 copies of a synthetic p53 binding site derived from the promoter of p21^{waf1} (23), and plasmid WWP-*luc*, containing the human p21^{waf1} native promoter (3), were provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). pcDNA-hHR23A- and hHR23A (C57)-expressing constructs and pcDNA-Vpr expression plasmid were gifts of Dr. George Pavlakis (NCI-Frederick Cancer Research and Development Center, Frederick, MD). pCMV-hHR23A and pCMV-hHR23A dc57 were constructed by insertion of PCR-generated DNA fragments encoding hHR23A or hHR23A dc57 (hHR23A segment lacking the COOH-terminal 57 amino acids) into pCMV-Tag2 vector (Stratagene). pCMV-p300, pCMVβ-HAp300 (1-595), pCMVβ-HAp300 (1-140), pCD-HAp300 (1-340), and pCMVβ-HAp300 (300-528) encode the indicated p300 segments fused to an NH₂-terminal HA tag. These constructs were kindly provided by Dr. David Livingston (Harvard Medical School, Boston, MA). Full-length mouse CBP expression vector was provided by Dr. Richard Goodman (Oregon Health Science University, Portland, OR). pCMVmdm-2 was provided by Dr. Arnold Levine (Princeton University, Princeton, NJ). 12S E1A and mutant E1A constructs were obtained from Dr. Michael Mathews (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

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³ The abbreviations used are: CBP, cyclic AMP-responsive element binding-binding protein; MDM2, murine double minute; hHR23A/B, human homologues of yeast Rad23 proteins A and B; LFS, Li-Fraumeni syndrome; GST, glutathione S-transferase; DBD, DNA-binding domain; NER, nucleotide excision repair.

pGEX-hHR23A and pGEX-hHR23A dc57 constructs were created by cloning PCR-amplified fragments into pGEX-4T-1 vector (Amersham Pharmacia Biotech). DNA was purified from the transformants by a purification kit (Qiagen, Inc) and quantitated by microfluorimetry as described (24, 25).

Cell Culture, Transfections, and Reporter Assay. The LFS fibroblast strain MDAH041 (*p53*-null, harboring a codon 184 frameshift mutation that results in premature termination of translation of p53 protein) was kindly provided by Dr. Michael Tainsky (M. D. Anderson Cancer Center, Houston, TX). These fibroblasts and SaoS2 (from ATCC) cells were grown in DMEM supplemented with 10% FCS and antibiotics at 37°C in a humidified atmosphere of 5% CO₂ (24). For each transfection experiment, exponentially growing cells (2×10^5) were plated in duplicate 35-mm dishes 18–20 h prior to plasmid transfection. Cells were transfected with p53 reporter PG13-*luc* or p21^{waf1} reporter WWP-*luc* and other expression vectors using FuGENE 6 transfection Reagent (Boehringer Mannheim) according to manufacturer's instruction manual. Appropriate amounts of vector DNA were used to maintain a constant total amount of transfected DNA between various experimental samples. After a 24-h posttransfection period, cells were washed twice with PBS and lysed in 100 μ l of luciferase cell culture lysis reagent (Promega). Luciferase activity from 20 μ l of cell lysates was assayed in duplicate using a standard luciferase assay system (Promega). Reference standards and negative controls were run in each experiment, and the luminescence was recorded with a LB 9510 luminometer (Wallac, Inc., Gaithersburg, MD). The luciferase activity from 20 μ l of protein lysate from a 2-ng p53 transfection protocol was routinely $1-5 \times 10^5$ luminescent units. The activation and inhibition levels were normalized in relation to the values of control containing the reporter and p53-expressing constructs. The calculated average values derived from at least three independent experiments, each performed in duplicate, were plotted for various plasmid transfection combinations.

Transient Expression and Western Blot Analysis of p53, p21^{waf1}, and p300 Segments. Fibroblast or SaoS2 cells transfected with individual p53-, hHR23A-, and p300 segment-expressing constructs or combinations of constructs were lysed in SDS sample buffer after 48 h of transfection. The protein concentration was determined using the DC Bio-Rad Assay according to the manufacturer's recommendations; Western blotting was performed as described (26). The anti-HA antibody 12CA5 (Boehringer Mannheim) and anti-FLAG M2 antibody (Stratagene) were used for detection of the HA epitope at the NH₂ terminus of each p300 segment or the FLAG epitope at the NH₂ terminus of each hHR23A segment. In parallel experiments, linear dose responses between transfected gene expression and DNA amount were determined using pCMV-Tag2 control construct and assayed for luciferase activity at a DNA dosage of 0–10 μ g for each transfection.

Detection of *in Vivo* Protein Interactions by Yeast Two-Hybrid System. A yeast two-hybrid assay was performed with the MATCHMAKER GAL4 Two-Hybrid System 3 (CLONTECH). PCR-amplified cDNA for hHR23A or hHR23A dc57 was fused in-frame with the GAL4 DBD in pGBKT7 vector. Various p300 segments, p300 (1-140), p300 (1-340), p300 (1-595), and p300 (300-528), were fused in-frame with the GAL4 activation domain in pGADT7 vector. The resulting plasmids were used for transformation of yeast strain Y187. The transformants were selected and assayed for β -galactosidase activity using a filter lift assay according to the user's manual.

***In Vitro* Protein Interaction Assay.** Various p300 proteins segments were *in vitro* transcribed/translated with the TNT T7-coupled reticulocyte lysate system (Promega) using pGADT7-p300 segment constructs as templates. GST fusion proteins were loaded onto glutathione-Sepharose (Amersham Pharmacia Biotech) by incubating the beads with bacterial lysates containing various GST fusion proteins in $1 \times$ HNT buffer (20 mM HEPES, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 10 μ g/ml leupeptin and pepstatin) for 30 min at 4°C. After loading, the beads were washed three times with HNT buffer and resuspended in GBB100 buffer (20 mM HEPES, 100 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 200 μ g/ml BSA, 2 mM DTT, 10 μ M ZnCl₂, 10 μ M EGTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μ g/ml leupeptin and pepstatin). Glutathione-Sepharose beads (20 μ l; one bed volume) were incubated with 5 μ l of *in vitro* translation mixture containing [³⁵S]-labeled proteins in 200 μ l of GBB100 buffer for 2 h at 4°C. The beads were then washed four times with GBB100 buffer, and the bound proteins were analyzed by SDS-PAGE and autoradiography.

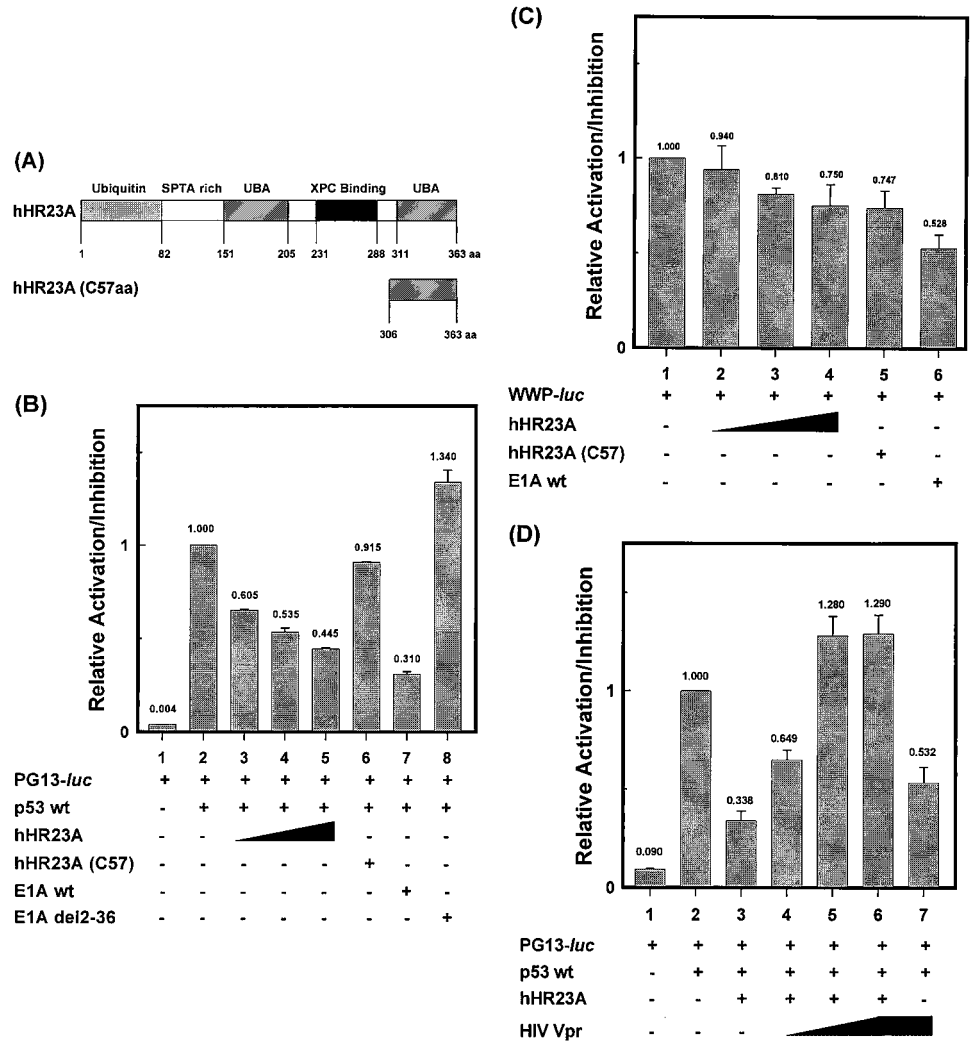
Results

Overexpression of hHR23A Inhibits Transcriptional Activity of p53. To study the role of hHR23A in the regulation of transcriptional activity of p53, we initially performed transient transfections to examine the effects of hHR23A expression on p53 transcriptional activity through quantitative expression of a reporter gene driven by a promoter containing multiple p53 binding sites (PG13-*luc*). Two hHR23A constructs, harboring different protein-binding domains, were used in the transfection experiments (Fig. 1A). p53 transcriptional activity was inhibited by cotransfection of hHR23A expression constructs in a dose-dependent manner (Fig. 1B, columns 2–5). However, coexpression of hHR23A (C57), which contains only the COOH-terminal 57 amino acid residues of hHR23A, had minimal, if any, effect on p53 activity (Fig. 1B, column 6). To determine whether the observed effects of hHR23A on p53 transcriptional activity are specific, we also examined the effect of hHR23A on the p53-independent expression of another p53 responsive reporter driven by p21^{waf1} promoter. As shown in Fig. 1C, when the hHR23A expression construct was cotransfected, only a slight, albeit dose-dependent inhibitory effect of hHR23A on p53-independent expression of p21^{waf1} promoter-driven reporter was observed in p53-null cells (Fig. 1C, columns 1–4). Furthermore, in this experiment, expression of the COOH-terminal 57 amino acid residues of hHR23A seemed to have effect similar to those of the full-length hHR23A (Fig. 1C, column 5 versus column 4). As expected, expression of wild-type E1A reduced the expression of the reporter by ~50% (Fig. 1C, column 1 versus column 6), suggesting involvement of p300/CBP in such a p53-independent reporter expression. Taken together, these results indicate that although the COOH-terminal 57 amino acid residues of hHR23A were enough to inhibit p53-independent and presumably p300/CBP-dependent transcription, this segment of hHR23A was not sufficient to influence the p53-dependent transcription of target genes.

The requirement for the COOH terminus of hHR23A to mediate inhibitory effects on p53 transcriptional activity was further confirmed by separate cotransfection experiments with Vpr, a HIV accessory protein that can bind to the COOH-terminal 57 amino acid residues of hHR23A (27, 28). As shown in Fig. 1D, coexpression of Vpr neutralized the inhibitory effects of hHR23A in a competitive and dose-dependent manner (Fig. 1D, columns 2–6). By contrast, expression of Vpr alone inhibited the transcriptional activity of p53 (Fig. 1D, column 7 versus column 2). These results indicate that the COOH terminus of hHR23A clearly interacts with cellular target(s) to mediate its inhibitory effect but by itself is not sufficient to inhibit p53 transcriptional activity.

Inhibitory Effect of hHR23A Is Overcome by Coexpression of p300, CBP, and p300 Segments Harboring C/H1 Domain. Two cellular factors could be envisaged to be the targets of hHR23A: p53 protein itself or its transcriptional coactivator, p300/CBP. However, hHR23A does not seem to interact with p53, as tested by the yeast two-hybrid system (29). Therefore, to investigate whether p300/CBP mediates inhibition of p53 transcriptional activity by hHR23A, we examined the effect of hHR23A expression on the reporter activity in the presence and absence of p300 or CBP expression. As can be seen in Fig. 2A, expression of p300 allowed a full recovery of the transcriptional activity of p53 inhibited by hHR23A (Fig. 2A, columns 4–6), whereas expression of p300 alone increased the p53-dependent expression of the reporter to a maximum level. These results indicate that exogenous expression of excess p300 quantitatively overrides the inhibitory effects resulting from expression of hHR23A. Accordingly, the expression of hHR23A antagonizes the stimulatory effects of p300 on p53-dependent expression. Similar results were obtained for experiments using coexpression of CBP with hHR23A (Fig. 2B). Thus,

Fig. 1. Inhibition of transcriptional activity of p53 by hHR23A. **A**, domain organization of hHR23A (35). *Ubiquitin*, ubiquitin-like region; *SPTA rich*, four kinds of amino acids (serine, proline, threonine, and alanine, are predominant in this region); *UBA*, ubiquitin-associated domain; *XPC binding*, xeroderma pigmentosum group C protein-binding domain; *aa*, amino acids. *hHR23A (C57aa)* represents the 57-amino acid COOH-terminal fragment of hHR23A. **B**, expression of hHR23A inhibits p53 transcriptional activity. MDAH041 cells were transfected with p53 reporter PG13-*luc* (0.5 μ g), expression vectors for p53 (*p53 wt*; 50 ng), and increasing amounts (0, 0.5, 1.0, and 1.5 μ g) of hHR23A-expressing vector, 1.5 μ g of hHR23A (C57aa)-expressing vector [*hHR23A (C57)*], or 1.5 μ g of E1A (*E1A wt*) or E1A del2-36 expressing vectors as indicated. **C**, effects of expression of reporter driven by p21^{Waf1} promoter. MDAH041 cells were transfected with p53-reporter WWP-*luc* (0.5 μ g) and increasing amounts (0, 0.5, 1.0, and 1.5 μ g) of hHR23A-expressing vector, 1.5 μ g of hHR23A (C57aa)-expressing vector [*hHR23A (C57)*], or 1.5 μ g of E1A-expressing vector (*E1A wt*). **D**, coexpression of Vpr prevents the inhibitory effect of hHR23A. MDAH041 cells were transfected with p53-reporter PG13-*luc* (0.2 μ g), expression vectors for p53 (*p53 wt*; 2 ng) and hHR23A (1.0 μ g), or together with increasing amounts (0, 0.1, 0.4, and 0.8 μ g) of Vpr-expressing vectors (*HIV Vpr*) as indicated. Total DNA amount for each transfection was maintained at 2.0 μ g by vector pcDNA3. Numbers shown are average values. Bars, SE.



these results strongly suggest that p300 and CBP proteins are cellular targets of hHR23A action.

With the aid of several p300 constructs that express different segments of the NH₂ terminus of p300 (Fig. 3A), we further characterized the functional interaction between p300 and hHR23A. As shown in Fig. 3B, when expressed alone, both p300 (1-140) and p300 (1-340) increased the p53-dependent expression of the reporter by ~100 and 20%, respectively, whereas transfection of expression vectors for p300 (1-595) and p300 (300-528) stimulated the transcription from the same reporter construct ~3-fold (Fig. 3B, columns 8 and 10; Ref. 15). Compared with the expression of p300 segments alone, coexpression of hHR23A inhibited the p53-dependent transcription stimulated by p300 (1-140), p300 (1-340), and p300 (1-595) (Fig. 3B, column 5 versus column 4, column 7 versus column 6, and column 9 versus column 8). Coexpression of hHR23A almost completely blocked the stimulatory effects of p300 (1-140) and p300 (1-340), but only decreased enhancement of the expression of the reporter from ~3-fold by p300 (1-595) to 2-fold. hHR23A, however, failed to inhibit p53-dependent transcription stimulated by p300 (300-528). These data indicate that a span of amino acids from 300 to 528 is required for p300 segments to overcome the inhibitory effects of hHR23A in a dominant negative manner (Fig. 3B, column 11 versus column 10).

hHR23A Interacts *in Vivo* and *in Vitro* with NH₂ Terminus of p300 Segments Harboring C/H1 Domain. To demonstrate interaction between hHR23A and the NH₂ terminus of p300, we first

performed a transcriptional assay for detecting protein interactions using the yeast two-hybrid system. As shown in Table 1, hHR23A itself has a transactivation activity when fused with the GAL4 DBD. This activity is greatly compromised by the deletion of 57 amino acids from its COOH terminus, which is not surprising because our subsequent experiments showed that this particular region contributes largely to the interaction between hHR23A and p300 protein. The relatively lower reporter activity of DBB-hHR23A notwithstanding, a strong interaction between hHR23A and p300 (300-528) as well as p300 (1-595) could be easily detected as prompt and more distinct color changes of yeast colonies cotransformed with hHR23A bait and p300 target constructs. Interestingly, deletion of 57 amino acids from the COOH terminus of hHR23A greatly reduced, but did not completely abolish, the interaction between hHR23A and p300 (1-595).

To confirm the interaction between hHR23A and p300, the hHR23A cDNA and its deletion mutant hHR23A dc57 were cloned into pGEX-4T-1 bacterial vector and expressed as GST fusion proteins. A GST pull-down assay was performed with these GST fusion proteins and various ³⁵S-labeled p300 segments synthesized *in vitro* using reticulocyte lysate and different pGADT7 p300 target constructs as templates. As shown in Fig. 4, the GST portion alone failed to bind any p300 segments. However, GST-hHR23A was able to bind p300 (1-595) and p300 (300-528) without any evidence of binding of hHR23A to either p300 (1-140) or p300 (1-340) segments. Removal of the COOH-terminal portion of hHR23A greatly reduced its p300-binding capability, indicating that the COOH-terminal portion of

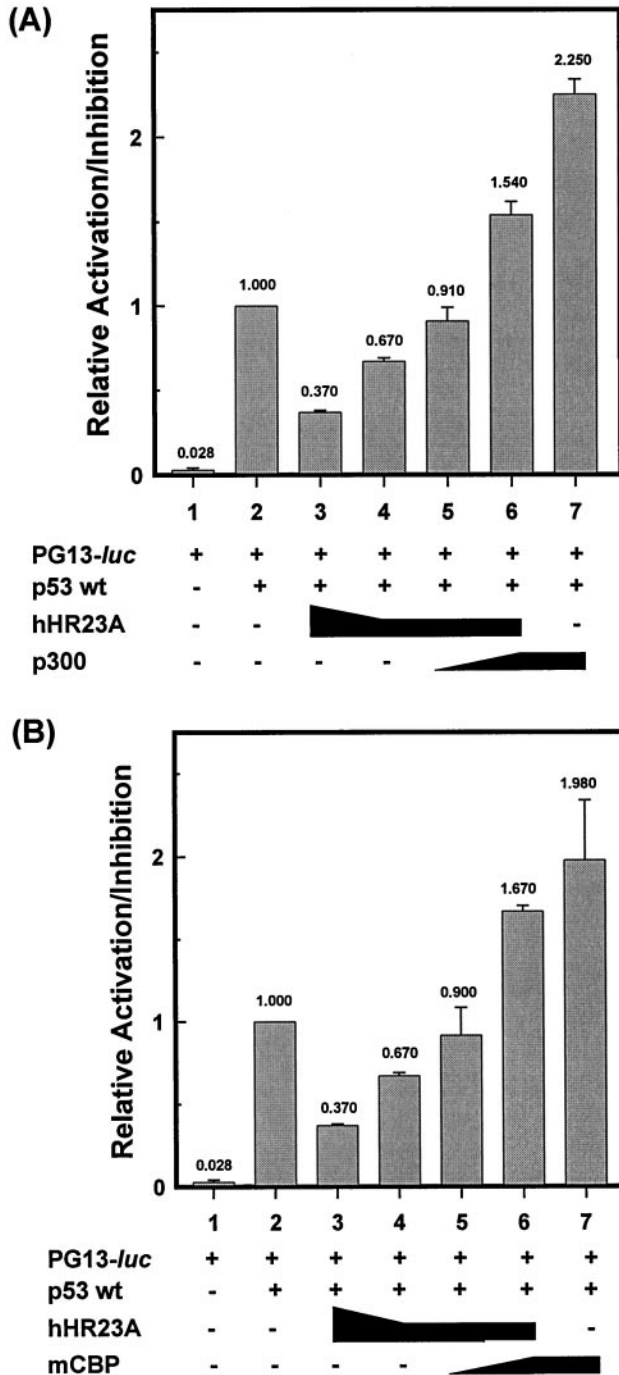


Fig. 2. Exogenous expression of p300 or CBP overcomes the inhibitory effects of hHR23A. MDAH041 cells were transfected with p53-reporter PG13-*luc* (0.2 μ g), expression vectors for p53 (*p53 wt*; 2 ng) and hHR23A (final amount, 1.0 μ g), or together with increasing amounts (0, 1.0, and 1.8 μ g) of p300 (A) or mouse CBP (B). Cotransfection with 1.8 μ g each of p300 (*p300*) or mouse CBP (*mCBP*) expression vector in the absence of hHR23 is also shown. Total DNA amount was kept constant at 3.0 μ g by adding vector pcDNA3. Numbers shown are average values. Bars, SE.

hHR23A is mainly responsible for such an interaction. Binding of GST-hHR23A to p300 (300-528) was similarly stronger than that of GST-hHR23A dc57. Comparing the bound p300 (1-595) and p300 (300-528) to that of 10% input label, it could be seen that p300 (1-595) was able to bind GST-hHR23A more effectively than p300 (300-528). The overall results of protein-protein interaction as determined by *in vitro* experiments further confirmed the parallel results of *in vivo* interaction between hHR23A and p300 as determined by the yeast two-hybrid system.

Overexpression of hHR23A Reduces the Steady-State Levels of p53 and Consequently Decreases Endogenous p21^{waf1} Expression.

It has been shown that interactions among the p300/CBP C/H1 domain, p53, and MDM2 are intimately involved in regulation of p53 transcriptional activity and MDM2-mediated p53 degradation. Hence, the binding of hHR23A to p300 segments harboring the C/H1 domain can suggest a role for hHR23A in controlling p53 transcriptional activity and its abundance within cells. To explore this possibility, p53 expression vector was cotransfected with increasing amounts of hHR23A expression vector into p53-null cells, and the protein levels of p53 and p21^{waf1} were then evaluated by Western blot analysis. A linear dose-response relationship between transfected gene expression and increasing DNA dosage (0–10 μ g per transfection) was established with pCMV-Tag2 control construct containing a luciferase reporter (results not shown). As shown in Fig. 5A, LFS fibroblast strain MDAH041 did not exhibit any detectable p53 protein expression. Introduction of the p53 expression vector into these cells resulted in high levels of expression of both p53 and p21^{waf1} proteins. However, coexpression of increasing amounts of hHR23A caused a dose-dependent reduction in the observed levels of p53 and consequently

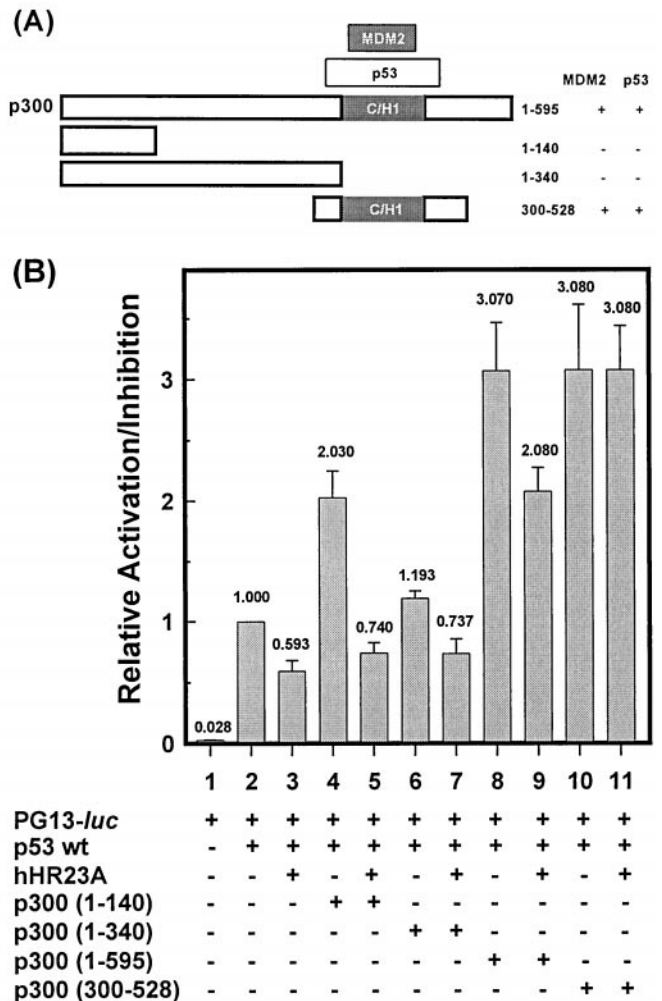


Fig. 3. Effect of overproducing p300 segments on hHR23A induced inhibition of p53 activity. A, schematic of the NH₂-terminal p300 segments and their p53 and/or MDM2 binding abilities. Boxes labeled MDM2 and p53 represent the minimal p300 sequences sufficient to bind each protein. B, MDAH041 cells were transfected with p53-reporter PG13-*luc* (0.2 μ g), expression vectors for p53 (*p53 wt*; 2 ng) and hHR23A (1.0 μ g) without or with 1.8 μ g of different p300 segment-expressing vectors as indicated at bottom of the figure. Total DNA amount was kept constant at 3.0 μ g with vector pcDNA3. Numbers shown are average values. Bars, SE.

Table 1 *In vivo* interactions between hHR23A and p300 segments in the yeast two-hybrid system

Yeast strain Y187 was transformed with pGBKT7 bait construct and pGADT7 target construct. Colonies were streaked on synthetic dropout selection agar and assayed for β -galactosidase activity. Four colonies were assayed for each kind of transformant, and experiments were repeated three times.

Bait construction	Target construction	β -Galactosidase assay ^a
PGBKT7-hHR23A		+
PGBKT7-hHR23A dc57		±
	pGADT7-p300 (1-140)	—
	pGADT7-p300 (1-340)	—
	pGADT7-p300 (1-595)	—
	pGADT7-p300 (300-528)	—
pGBKT7-hHR23A	pGADT7	+
pGBKT7-hHR23A	pGADT7-p300 (1-140)	+
pGBKT7-hHR23A dc57	pGADT7-p300 (1-140)	±
pGBKT7-hHR23A	pGADT7-p300 (1-340)	+
pGBKT7-hHR23A dc57	pGADT7-p300 (1-340)	±
pGBKT7-hHR23A	pGADT7-p300 (300-528)	++
pGBKT7-hHR23A	pGADT7-p300 (1-595)	+++
pGBKT7-hHR23A dc57	pGADT7-p300 (1-595)	++
pGBKT7-Lam	pGADT7-p300 (1-595)	—

^a —, no evidence for color change of the yeast colony; ±, color of colony changed to very light blue at the end of 8-h test; +, ++, and +++, relative blue color intensities of the colonies.

decreased the levels of p21^{waf1} (Fig. 5A). In contrast, cotransfection with increasing amounts of hHR23A dc57 failed to affect the level of p53, but resulted in slightly decreased levels of p21^{waf1} protein (Fig. 5B). Similar results were obtained with another p53-deficient cell line, SaoS2 (results not shown). Because the COOH-terminal portion of hHR23A is mainly responsible for its interaction with p300, it is conceivable that a strong interaction between the 57-amino acid stretch in the COOH-terminal portion of hHR23A and p300 is absolutely required for hHR23A to reduce steady-state p53 levels. However, the weak interaction that was demonstrated between hHR23A dc57 and p300 was still able to inhibit p53 activity directly, albeit less effectively. Additionally, coexpression of hHR23A was also found to cause a prominent decrease in the steady-state level of the p300 segment, p300 (300-528) (data not shown). Thus, it appears that strong binding of hHR23A to p300 might be required for the assembly of p53 degradation complex, with p300 protein used as a platform.

hHR23A and MDM2 Act Cooperatively to Inhibit p53 Transcriptional Activity. To test whether MDM2 and hHR23A could act cooperatively to inhibit p53-dependent transcription, we first evaluated the effects of overproduction of p300 (300-528) on MDM2-mediated inhibition of p53-dependent transcription. As shown in Fig. 6A, transfection of the MDM2 expression construct decreased p53-dependent transcription of the reporter in a dose-dependent manner (Fig. 6A, columns 2–4). However, when the p300 (300-528) expression construct was cotransfected with the MDM2 expression construct, inhibition was alleviated and transcription of the reporter was stimulated again in a dose-dependent manner with increasing amounts of p300 (300-528). The cooperative action of MDM2 and hHR23A was examined by transfecting cells with various amounts of MDM2 expression vector either alone or in combination with hHR23A expression vector. As shown in Fig. 6B, MDM2 participates in a dose-dependent manner with hHR23A to further decrease the expression of p53-regulated reporter (Fig. 6B, columns 2–6). These two sets of results suggest that hHR23A and MDM2 act cooperatively to regulate p53 activity.

Discussion

hHR23 proteins are identified as partners for XPC-p125 protein, which is affected by gene mutations in complement group C of xeroderma pigmentosum. On the basis of sequence homologies with

yeast Rad23, hHR23 proteins are believed to function in NER. Whereas only hHR23B has been found to form a tight complex with XPC protein *in vivo*, both of hHR23 proteins can stimulate XPC repair activity and are functionally interchangeable (30, 31). Recent evidence showing greater preference of binding of XPC-hHR23B complex to damaged DNA than that of XPA argues for the importance of XPC-hHR23B complex as a primary recognition factor for damage within the nontranscribed strand during NER (32, 33).

In addition to participating in NER, hHR23 proteins could also play a role in cell cycle progression. hHR23A has been identified as a substrate for E6AP ubiquitin protein ligase (E3), and its level is regulated in a cell cycle-dependent manner (22). Additionally, studies on HIV-Vpr indicate that expression of Vpr in cells mimics DNA damage and causes an arrest at the G₂-M phase of the cell cycle because of direct interactions between Vpr and hHR23A (27, 28). Our previous studies on the regulation of p53 transcriptional activity in xeroderma pigmentosum cells after UV irradiation indicated that DNA damage recognition could link the p53 pathway to DNA repair (20). In this study, we asked whether hHR23A could affect p53 transcriptional activity. Using the transient transfection/expression reporter assay, we first demonstrated the inhibitory effects of hHR23A on p53 transcriptional activity. We also examined the contribution of the COOH-terminal portion of hHR23A to such inhibitory effects by overexpression of its COOH-terminal segment and by coexpression of Vpr with hHR23A. Results from these experiments showed that the COOH-terminal portion hHR23A interacts with cellular target(s) to mediate such an inhibitory effect but that the COOH-terminal portion itself is not sufficient for efficient inhibition of p53 transcriptional activity. It is well known that LXXLL motif(s), *i.e.*, part of the NH₂ terminus of p300/CBP protein, is also present in HIV-Vpr protein (34). This led us to examine whether p300/CBP could be a cellular

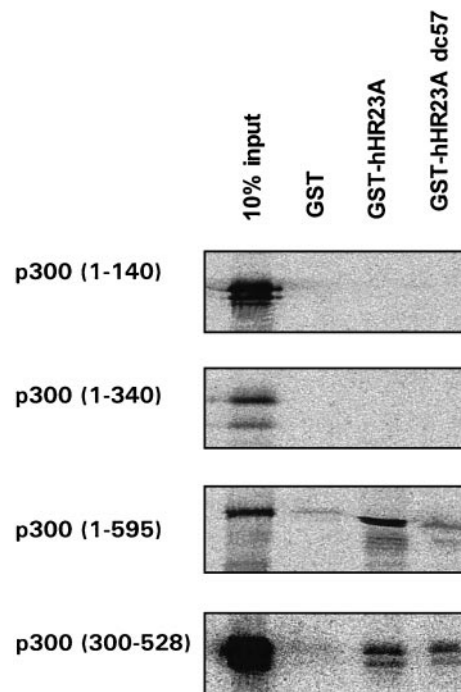


Fig. 4. *In vitro* interaction of hHR23A with p300 segments. Proteins corresponding to various p300 segments were synthesized *in vitro* by reticulocyte lysates in the presence of [³⁵S]methionine. The labeled proteins were incubated at 4°C for 2 h with glutathione-Sepharose beads previously loaded with GST fusion, GST-hHR23A, or GST-hHR23A dc57 proteins to perform a GST pull-down assay as described in "Materials and Methods." The bound proteins were released and resolved by 15% SDS-PAGE. For autoradiography, gels were exposed overnight to a phosphorimaging screen at room temperature and scanned. This figure is representative of two independent experiments.

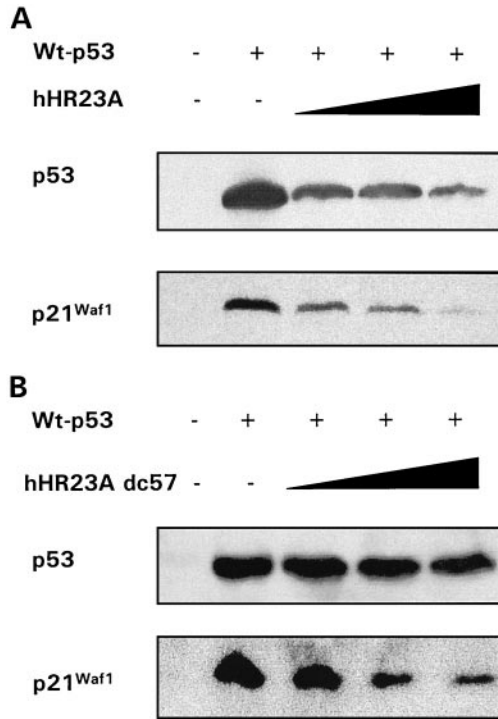


Fig. 5. Overexpression of hHR23A results in a reduced steady-state level of p53 and decreased p21^{waf1} protein. MDAH041 or SaoS2 cells (10^6 per 10-cm dish) were transfected individually or cotransfected with Wt-p53 expression vector (0.2 μ g) together with increasing amounts (0, 2, 4, 6, and 8 μ g) of expression vectors pCMV-hHR23A (A) and pCMV-hHR23A dc57 (B). The vector DNA was used to make up the total amount of transfected DNA to a constant 10 μ g/10-cm dish. In parallel experiments, linear dose responses for transfected gene expression were determined using pCMV-Tag2 control construct (0–10 μ g) and assaying for luciferase activity. After 48 h of transfection, cells were lysed, and the proteins (45 μ g) were processed by SDS-PAGE and Western blotting for p53 and p21^{waf1} proteins. The equal protein loading was confirmed by Coomassie Blue staining of gels. This figure is representative of at least three independent experiments.

target for hHR23A. We found that the inhibitory effect of hHR23A can be overcome by coexpression of p300, CBP, and p300 segments harboring the C/H1 domain. In support of these results, we next demonstrated the interaction between hHR23A and p300 in the yeast two-hybrid system and the GST pull-down assay. Finally, we examined the effects of overexpression of hHR23A on the steady-state levels of p53 and p21^{waf1}. To this end, our results demonstrated that by interacting with the p300/CBP C/H1 domain, overexpression of hHR23A inhibits p53 transcriptional activity, decreases the steady-state level of p53, and subsequently reduces the level of p21^{waf1}. Overexpressed hHR23A *per se* seems to titrate out p300/CBP and prevent it from efficiently potentiating p53-mediated transcriptional activation. However, this explanation cannot account for why overexpression of hHR23A also causes a decrease in the level of p53. Given the importance of interactions between the C/H1 domain of p300/CBP and p53 and MDM2 in p53 degradation, it seems that hHR23A also participates in such p53 degradation processes. Apparently, this possibility needs to be further addressed to rule out that hHR23A could affect p53 protein abundance at the mRNA transcription or translation level. It may be argued that to achieve the observed inhibitory effects, a large excess of hHR23A expression over p53 expression was used for cell transfections. This might very well be attributable to the relatively higher abundance of endogenous hHR23A within cells (31).

The major finding of this study that the down-regulation of p53 transcriptional activity by hHR23A occurs through p300/CBP supports the hypothesis that hHR23 proteins play a role in cell cycle control (22). In addition, the demonstrated interactions between p300/

CBP and hHR23A have clear implications in DNA repair. Future studies aimed at delineating the precise roles of hHR23 proteins in regulation of p53 activity should be useful in understanding molecular events in cross-talking between the p53 pathway and DNA repair processes. It will also be interesting to determine whether hHR23 proteins interact with p300/CBP to contribute in the processing of

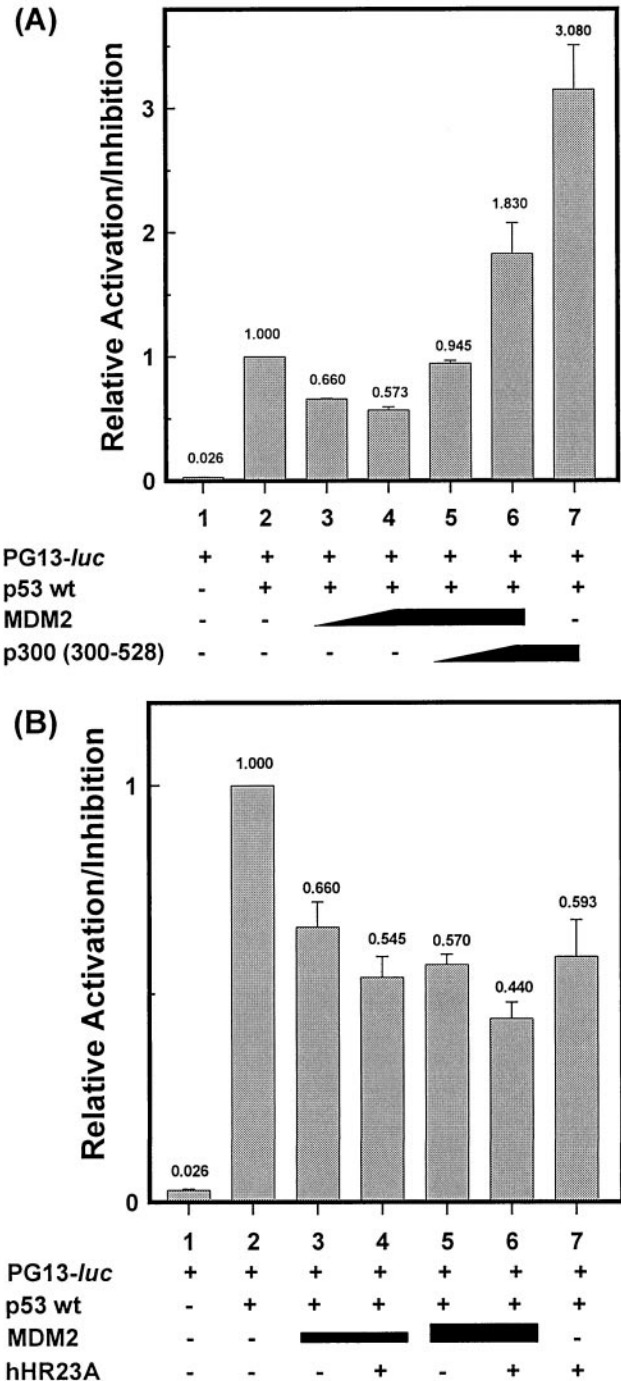


Fig. 6. Cooperative inhibition of p53 transcriptional activity by hHR23A and MDM2. A, MDAH041 cells (2×10^5) were transfected with p53 reporter PG13-luc (0.2 μ g), expression vectors for p53 (p53 wt; 2 ng) and MDM2 (0.1 and 0.2 μ g), or 0.2 μ g of MDM2 together with increasing amounts (0, 1.0, and 1.8 μ g) of p300 (300-528)-expressing vector. B, MDAH041 cells were transfected with p53 reporter PG13-luc (0.2 μ g), expression vectors for p53 (p53 wt; 2 ng) and MDM2 (0.1 or 0.2 μ g) alone or together with hHR23A-expressing vector (1.0 μ g). In these experiments, total DNA for each transfection was kept at 3.0 μ g by adding vector pcDNA3. Numbers shown are average values. Bars, SE.

DNA damage, particularly global genomic repair, via histone acetylation of the chromatin.

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