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High-Mobility Group A1 Proteins Regulate p53-Mediated Transcription of Bcl-2 Gene

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Abstract

We have previously described a mechanism through which the high-mobility group A1 (HMGA1) proteins inhibit p53-mediated apoptosis by delocalizing the p53 proapoptotic activator homeodomain-interacting protein kinase 2 from the nucleus to the cytoplasm. By this mechanism, HMGA1 modulates the transcription of p53 target genes such as Mdm2, p21^{waf1}, and Bax, inhibiting apoptosis. Here, we report that HMGA1 antagonizes the p53-mediated transcriptional repression of another apoptosis-related gene, Bcl-2, suggesting a novel mechanism by which HMGA1 counteracts apoptosis. Moreover, HMGA1 overexpression promotes the reduction of Brn-3a binding to the Bcl-2 promoter, thereby blocking the Brn-3a corepressor function on Bcl-2 expression following p53 activation. Consistently, a significant direct correlation between HMGA1 and Bcl-2 overexpression has been observed in human breast carcinomas harboring wild-type p53. Therefore, this study suggests a novel mechanism, based on Bcl-2 induction, by which HMGA1 overexpression contributes to the escape from apoptosis leading to neoplastic transformation. Cancer Res; 70(13); OF1–10. ©2010 AACR.

Introduction

The high-mobility group A (HMGA) family is comprised of three proteins: HMGA1a, HMGA1b, and HMGA2. They are encoded by two distinct genes, HMGA1a and HMGA1b proteins being products of the same gene generated through alternative splicing (1). These proteins bind the minor groove of AT-rich DNA sequences through their DNA-binding domain, the so-called “AT-hooks.” HMGA proteins do not have transcriptional activity per se; however, by interacting with the transcription machinery, they alter the chromatin structure and thereby regulate the transcriptional activity of several genes (2, 3). In normal cells and adult tissues, the levels of HMGA proteins are low or absent (4). In contrast, in neoplastically transformed cells as well as in embryonic cells, the constitutive expression of HMGA proteins is exceptionally high (5). Their overexpression is mainly associated with a highly malignant phenotype, also representing a poor prognostic index because HMGA overexpression often correlates with the presence of metastasis as well as reduced survival (6). Moreover, both in vitro and in vivo studies have established the oncogenic role of HMGA genes (7–11).

Recently, we have shown a novel mechanism, based on HMGA1-p53 interaction, by which HMGA1 proteins have a role in the process of carcinogenesis. In fact, HMGA1 binds p53 and interferes with the p53-mediated transcription of Bax, p21^{waf1}, and Mdm2, leading to a reduction of p53-dependent apoptosis (12). HMGA1 is also able to interfere with the apoptotic function of p53 by another mechanism that involves the p53 proapoptotic activator homeodomain-interacting protein kinase 2 (HIPK2), which binds to and phosphorylates p53 on Ser^{156} leading to apoptosis (13, 14). Indeed, HMGA1 overexpression promotes HIPK2 relocalization from the nucleus to the cytoplasm, inhibiting p53 apoptotic function, whereas HIPK2 overexpression re-established HIPK2 nuclear localization and sensitivity to apoptosis (15). Consistently, a strong correlation among HMGA1 overexpression, HIPK2 cytoplasmic localization, and a low spontaneous apoptosis index was observed in wild-type p53-expressing human breast carcinomas (15).

On the basis of these data, we looked for other genes regulated by HMGA1 proteins among p53 target genes involved in cell apoptosis. In particular, we have focused our attention on B-cell lymphoma gene 2 (Bcl-2). Bcl-2 represents a good candidate as the target for HMGA1 proteins because its promoter region contains some AT-rich DNA sequences and its transcription is regulated by p53 (16, 17). The Bcl-2 protein exerts an antiapoptotic function: it inhibits the release of cytochrome c into the cytosol which, in turn, activates caspase-9 and caspase-3, leading to apoptosis (18). The Bcl-2 gene has been found to be overexpressed in different cancers, including B-cell lymphoma (19), melanoma (20), neuroblastoma...
(21), breast (22), prostate (23), ovarian (24), and lung carcinomas (25). Bcl-2 and its homologues might intervene in oncogenesis at different levels by favoring the persistence of malignant cells that, in normal circumstances, would be eliminated by apoptosis, and also by promoting resistance to conventional cancer treatments (26).

Here, we show that Bcl-2 is a gene regulated by the HMGA1-p53-HIPK2 complex and that HMGA1 is able to abolish the repression promoted by p53 on Bcl-2 expression. Subsequently, we looked for other components of this multiprotein complex, finding that Brn-3a belongs to this complex. The POU transcription factor Brn-3a strongly activates the expression of the Bcl-2 proto-oncogene, protecting neuronal cells from programmed cell death (16). Recent works show that Brn-3a expression is not restricted to neuronal cells, as its activity was also detected in cancer cells of nonneuronal origin (27). Latchman and colleagues showed that the transcriptional repression exerted by p53 on the Bcl-2 promoter is achieved through Brn-3a, suggesting that the two proteins could cooperate in the negative control of the expression of this gene (16). Finally, we show that HMGA1 overexpression reduces Brn-3a from its role as corepressor following p53 activation. In addition, validating our data by immunohistochemical analyses, we found a significant association between HMGA1 and Bcl-2 overexpression in 11 of 14 biotissue of human breast carcinomas carrying wild-type p53.

Materials and Methods

Cell culture, transfections, and transactivation assays

NMD7, MCF7, mouse embryonic fibroblasts, and SAOS-2 cells were maintained in DMEM with 10% FCS (Life Technologies; Invitrogen), glutamine, and antibiotics. FRTL5, FRTL5-KimSV, and FRTL5-KiMSV as HMGA1 cells were maintained in F12 with 5% calf serum (Life Technologies; Invitrogen), glutamine, and antibiotics. Cells were transfected with plasmids by LipofectAMINE plus reagent (Invitrogen) as suggested by the manufacturer. Cells were transiently transfected with previously described reporter vectors (12, 13, 16) and normalized with the use of a cotransfected β-galactosidase construct. Luciferase activity was analyzed by Dual-Light System (Applied Biosystems). For inhibition of HMGA1 expression, small interfering RNAs and corresponding scramble RNAs (Santa Cruz Biotechnology, Inc.) were used as suggested by the manufacturer.

Expression constructs

The pCAG-p53, pCMV-Hmga1b, pCEFL-HA-Hmga1b, pFLAG-HIPK2, pEGFP-HIPK2, pLTR-Brn-3a, and the Bcl-2 promoter-reporter constructs have been previously described (12, 13, 16).

Western blotting and coimmunoprecipitation

Protein extraction, Western blotting, and coimmunoprecipitation procedures were carried out as reported elsewhere (12). Differential nuclear/cytoplasmic cell lysates were obtained as previously reported (15). The antibodies used for Western blotting were as follows: anti-FLAG M5 (Sigma-Aldrich), anti-HA 12C5 (Roche), anti-p53 D01, anti–Brn-3a 14A6, anti-Sp1 H225, and anti–γ-tubulin C11 (Santa Cruz Biotechnology), anti–Bcl-2 50E3 (Cell Signaling), anti-HMGA1 are polyclonal antibodies raised against a synthetic peptide located in the NH2-terminal region and recognize both HMGA1a and HMGA1b isoforms (12), and anti-HIPK2 M01 (Abnova).

In vitro protein translation and electrophoretic mobility shift assay

DNA-binding assays with the recombinant protein were performed as previously described (12). The radiolabeled double-strand oligonucleotide corresponds to a region spanning from −526 to −477 nucleotides of the human Bcl-2 promoter region (16).

Cell viability and terminal nucleotidyl transferase–mediated nick end labeling assays

Cell viability and terminal nucleotidyl transferase–mediated nick end labeling (TUNEL) assays were carried out as reported elsewhere (12).

Reverse transcriptase-PCR

Total RNA was isolated using TRI-reagent solution (Sigma) according to the protocols of the manufacturer and treated with DNase (Invitrogen). Reverse transcription was performed according to standard procedures (Applied Biosystems). cDNA was amplified by PCR using the following primers: Mu-Bcl-2-up 5′-ggCTATggTgTgAgCg-3′, Mu-Bcl-2-dw 5′-gCagAgTCTTCCAgAgAc Ag-3′, Mu-Gadph-dw 5′-TggTgAaggTcggTgAAC-3′, and Mu-Gadph-dw 5′-ggAA-gAgTgAgTgTgAg-3′.

Indirect immunofluorescence

Indirect immunofluorescence was carried out as reported elsewhere (15).

Chromatin immunoprecipitation and reprecipitation

After transfection, chromatin samples were processed for chromatin immunoprecipitation (ChIP) and re-ChIP experiments as reported elsewhere (12). Samples were subjected to immunoprecipitation with the following antibodies: anti-p53 (Ab-7; Calbiochem), anti–Brn-3a (14A6; Santa Cruz Biotechnology), anti-HIPK2 (M01; Abnova), and anti-HMGA1 (12). Immunoprecipitated chromatin was amplified by PCR using the following primers: Hu-Bcl-2-pr-up 5′-gAagCagAgAgTTgAggtGgAg-3′, Hu-Bcl-2-pr-dw 5′-gcggTgTTgCTTTgCAGAcgagCc-3′, Hu-Gadph-pr-up 5′-gTgTTTTtCCCAggTtTTCAgTg-3′, Hu-Gadph-pr-dw 5′-TCTTCCAgTgTgTgAgCg-3′, Mu-Bcl-2-pr-up 5′-AcAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgAgA
Immunohistochemistry

Immunohistochemistry was carried out as reported elsewhere (15). HMGA1 and Bcl-2 proteins were considered overexpressed when more than 10% of the neoplastic cells presented a strong immunoreaction. p53 was considered positive (mutated) only when a distinct nuclear staining was observed in more than 10% of cancer cells.

Results

HMGA1 correlates with Bcl-2 protein expression

Looking for a possible correlation between HMGA1 and Bcl-2 protein expression, we analyzed their expression level in the MCF-7 breast cancer cell line stably transfected with HMGA1b. All the HMGA1-transfected MCF7 clones showed higher Bcl-2 protein levels than the untransfected cells (Fig. 1A). This correlation was then confirmed in other cell systems. Indeed, mouse embryonic fibroblasts null for the hmga1 gene express lower Bcl-2 mRNA and protein levels in comparison with the wild-type mouse embryonic fibroblasts (data not shown). Moreover, a rat thyroid cell line, transformed with the Kirsten sarcoma virus (FRTL5-KiMSV), expressing HMGA1 shows higher Bcl-2 protein levels in comparison with normal uninfected cells. Conversely, the suppression of HMGA1 protein expression in the FRTL5-KiMSV–infected cells, by an antisense construct for HMGA1, leads to a drastic reduction in Bcl-2 mRNA and protein levels (data not shown). To further support a causal correlation between HMGA1 and Bcl-2 expression, a neuronal cell line (ND7) transiently interfered for the expression of HMGA1 proteins showed a reduction in Bcl-2 protein levels compared with cells transfected with a scrambled oligonucleotide (Fig. 1B).

Because the interaction between HMGA1 and p53 modulates the transcription of p53 target genes, we investigated whether Bcl-2 was regulated by HMGA1-p53 interaction. Therefore, ND7 cells, endogenously expressing HMGA1 and p53, were cotransfected with expression vectors encoding p53 and/or HMGA1b. According to previous data (17), p53 reduces Bcl-2 transcription, whereas HMGA1 overexpression reverts the effects of p53 on Bcl-2 transcription, as shown by reverse transcription-PCR and Western blot analyses (Fig. 1C and D). In addition, preliminary data also suggest that HMGA1a isoform and HMGA2 overexpression are able to abolish p53 repression exerted on Bcl-2 gene transcription (data not shown).
HMGA1 proteins bind Bcl-2 promoter in vitro and in vivo

Subsequently, we investigated whether HMGA1 is able to bind the Bcl-2 gene-regulatory regions. As shown in Fig. 2A, using an electrophoretic mobility shift assay, we showed that His-HMGA1b recombinant protein was able to bind the region spanning from −526 to −477 nucleotides related to the transcription start site of the human Bcl-2 gene (16), which contains AT-rich putative HMGA1 binding sites and two p53 consensus sequences. Binding specificity was demonstrated by competition experiments showing loss of binding with the addition of a 200-fold excess of unlabeled Bcl-2 promoter oligonucleotide. Next, we evaluated whether HMGA1 proteins bind the human Bcl-2 promoter in vivo by performing ChIP assays. MCF7 cells were transfected with HMGA1b, crosslinked, and immunoprecipitated with anti-HMGA1 or anti-IgG antibodies as control. Immunoprecipitation of chromatin was then analyzed by semiquantitative PCR, using primers spanning the −676/−320 region of the Bcl-2 promoter. The Bcl-2 promoter region was amplified in both the MCF7 cells transfected with HMGA1b or the backbone vector because HMGA1 proteins are weakly expressed in MCF7 (Fig. 2B). However, the Bcl-2 promoter amplification was higher in HMGA1-transfected cells in comparison with the cells transfected with the empty vector. Conversely, no amplification was observed with anti-IgG precipitates and when primers for the control promoter GAPDH were used (Fig. 2B), demonstrating that the binding is specific for the Bcl-2 promoter. Analogous results were obtained by ChIP performed in untransfected and HMGA1-transfected murine ND7 cell lines (Fig. 2C). These results indicate that HMGA1 proteins are able to bind the Bcl-2 promoter in vitro and in vivo.

HMGA1 modulates p53-mediated transcriptional activity on Bcl-2 promoter

To verify the functional effects of HMGA1 on Bcl-2 promoter, we evaluated the activity of a reporter vector carrying the luciferase gene under the control of Bcl-2 promoter in ND7 cells transfected with HMGA1 or an empty vector. As expected, Bcl-2 expression was drastically reduced in cells overexpressing p53 compared with control cells (Fig. 2D). HMGA1 overexpression alone had no effect on Bcl-2 promoter activity, whereas the overexpression of both HMGA1 and p53 resulted in a drastic reduction of Bcl-2 promoter repression compared with cells transfected with p53 alone. Furthermore, when
HMGA1 protein expression was transiently blocked by small interfering RNA. Bcl-2 promoter activity was significantly reduced compared with untransfected cells. These results indicate that HMGA1 suppresses the inhibitory effect of p53 on Bcl-2 transcription.

**HMGA1 represses p53 apoptotic function mediated by Bcl-2 downregulation**

To evaluate the biological effects of HMGA1 on the regulation of Bcl-2 expression by p53, SAOS-2 cells, which express HMGA1 but are null for p53 expression, were transfected with a vector encoding p53, in the presence or absence of HMGA1 overexpression. As expected, exogenous p53 expression induced cell death as shown by trypan blue test (Fig. 3A). However, when HMGA1 was overexpressed together with p53, the percentage of dead cells was drastically reduced (Fig. 3A).

To verify the antiapoptotic activity of HMGA1 in a more physiologic context, we incubated the wt-p53-carrying ND7 cells with H2O2 to activate the endogenous p53 in the presence or absence of HMGA1 protein overexpression. As shown in Fig. 3B and C, HMGA1 overexpression significantly reduced the number of trypan blue or TUNEL-positive cells induced by H2O2. Taken together, these data indicate that HMGA1 could functionally interfere with the apoptotic function of p53 mediated by Bcl-2 downregulation.

**HMGA1 proteins bind to Brn-3a transcription factor**

The data shown above strongly suggest the existence of a multiprotein complex that regulates the transcription levels of Bcl-2 supported from our previous studies, which also showed that HMGA1 is able to constitute a complex with p53 and HIPK2 that regulates p53 apoptotic function (15). It has been previously shown that Brn-3a is a potent transcriptional regulator of the Bcl-2 promoter, able to bind both p53 and HIPK2 (28). Therefore, we investigated whether HMGA1 interacts with Brn-3a. ND7 cells were transiently cotransfected with hemagglutinin (HA)-tagged HMGA1b and Brn-3a expression vectors. Protein lysates were immunoprecipitated with anti–Brn-3a or anti-HA antibodies and immunoblotted with reciprocal antibodies (Fig. 3D and E). Coexpression of Brn-3a and HMGA1 resulted in the coimmunoprecipitation of the two proteins. Conversely, there was no coimmunoprecipitation when ND7 cells were transfected with HA-HMGA1b or Brn-3a expression vectors alone. Western blot analysis showed that the transfected cells expressed adequate levels of the Brn-3a and HA-HMGA1b proteins (Fig. 3F).

**The HMGA1-p53-Brn-3a-HIPK2 complex regulates Bcl-2 promoter activity**

The Brn-3a transcription factor has been shown to strongly activate the expression of Bcl-2 protecting cells from apoptosis. However, when p53 is overexpressed, Brn-3a acts as a corepressor sustaining the p53-repressive action on Bcl-2 promoter (16). Moreover, HIPK2 interacts with Brn-3a, promoting its binding to DNA and suppressing Brn-3a–dependent transcription of some Bcl-2 family members (28). In addition, our previous results indicate that HIPK2 reverts the inhibitory activity of HMGA1 on the p53-dependent promoters and leads to a complex interplay among p53, HIPK2, and HMGA1 in the regulation of p53 target gene expression (15).

Therefore, to better define the role of the HMGA1-p53-Brn-3a-HIPK2 multicomplex in the regulation of Bcl-2 transcription, we analyzed Bcl-2 promoter activity using a vector carrying the luciferase gene under the control of Bcl-2 promoter in the SAOS-2 cells transiently transfected with different combinations of plasmids expressing the proteins participating in the complex. As expected from previous data (16), p53 represses Bcl-2 transcription and this effect is increased by Brn-3a (Fig. 4A). When HMGA1 was co-transfected with p53 and Brn-3a, the repression on Bcl-2 promoter was antagonized. In particular, the repressive activity exerted by Brn-3a was abolished (Fig. 4A). To verify whether HIPK2 has a role in the transcriptional regulation of the Bcl-2 gene, HIPK2 was coexpressed with p53, HMGA1, and Brn-3a in ND7 cells. HIPK2 restores the transcriptional repression of Bcl-2 promoter exerted by p53 and Brn-3a counteracting HMGA1 effects (Fig. 4A).

**HMGA1 represses p53-Brn-3a–mediated apoptosis**

We have shown above that HMGA1 proteins are able to abolish the repression exerted by p53-Brn-3a cooperation on Bcl-2 promoter activity and that HIPK2 overexpression reverts the effects of HMGA1 on Bcl-2 transcription. Consequently, we analyzed the biological effects of these observations on cell death. To this aim, ND7 cells were transfected with a vector encoding Brn-3a alone or in combination with HMGA1, and incubated with H2O2 to activate the endogenous p53. As expected, the expression of both p53 and Brn-3a induced an increased apoptotic rate (Fig. 4B and C) likely due to repression of Bcl-2 promoter activity (16). HMGA1 overexpression impaired the cooperation between Brn-3a and p53, and an apoptotic rate comparable to that exerted by p53 alone was observed. In addition, HIPK2 overexpression inhibited the antiapoptotic activity of HMGA1 and induced a percentage of cell death comparable to that promoted by p53-Brn-3a cooperation (Fig. 4B and C).

**HMGA1 exerts its antiapoptotic function by promoting HIPK2 cytoplasmic delocalization in ND7 cells**

We have previously shown that HMGA1 inhibits p53-induced apoptosis by acting on HIPK2 delocalization from the nucleus to the cytoplasm (15). Therefore, we asked whether this mechanism also occurs following HMGA1 overexpression in ND7 cells. To this aim, we transfected ND7 cells with a vector encoding EGFP-HIPK2 protein to follow its localization by immunofluorescence. We observed a nuclear localization of HIPK2 in EGFP-HIPK2 transfected cells. Conversely, when HMGA1 was overexpressed, we observed a cytoplasmic localization of HMGA1 that was strongly associated with cytoplasmic relocation of EGFP-HIPK2 (Fig. 5A). HIPK2 delocalization was confirmed by Western blotting analysis on nuclear/cytoplasmic cellular extracts. Indeed, as shown in Fig. 5B, the amount of cytoplasmic HIPK2 was...
higher in HMGA1-overexpressing ND7 cells compared with control cells. Sp1 and γ-tubulin were used as markers of nuclear/cytoplasmic separation as well as loading controls (Fig. 5B). HIPK2 and HMGA1 protein levels from ND7 total cell extracts are shown in Fig. 5C.

**Figure 3.** HMGA1 interferes with the apoptotic activity of p53. A, p53-null SAOS-2 cells were transfected with the p53 vector in combination or not with the HMGA1b vector, and analyzed by trypan blue exclusion test. Expression of the indicated proteins was analyzed by Western blotting. The result of one of three experiments performed is reported. B, wt-p53-carrying ND7 cells were transfected with HMGA1b and then incubated or not with H2O2. The percentage of dead cells and the protein levels were analyzed as in A. C, ND7 cells were treated as in B and analyzed by TUNEL assay. The percentage of TUNEL-positive cells from one out of four representative experiments is reported. The expression of the indicated proteins was analyzed by Western blotting. HMGA1 interacts with Brn-3a in vivo. D, ND7 cells were transfected with HA-HMGA1b and Brn-3a vectors. Cellular extracts were prepared, and equal amounts of proteins were immunoprecipitated with anti–Brn-3a (D) or anti-HA (E) antibodies, and the immunocomplexes were analyzed by Western blotting using the reciprocal antibodies. F, cellular extracts used for immunoprecipitation experiments were analyzed by Western blotting.

**HMGA1 displaces HIPK2 and Brn-3a from Bcl-2 promoter**

We next investigated whether HMGA1 is part of the complex, including p53, Brn-3a, and HIPK2, that forms at the p53 binding sites of the Bcl-2 promoter in vivo by a combination of ChIP and re-ChIP analyses. ND7 cells were transfected
Figure 4. HMGA1, p53, Brn-3a, and HIPK2 coexpression regulates Bcl-2 gene transcription and apoptosis. 

A. The pCMV-p53 vector was transfected alone or with the indicated plasmids in SAOS-2 cells. All transfections were performed in duplicate; data are mean ± SD of five independent experiments. Empty vectors were used as a control. Western blot analyses of p53, HMGA1, Brn-3a, and FLAG-HIPK2 protein expression from one representative experiment (bottom). 

B. ND7 cells were transfected with HMGA1b, Brn-3a, HIPK2 vectors, or control vectors and then incubated or not with H2O2. Cells were collected and analyzed by trypan blue exclusion test. The results of one indicative experiment are reported. 

C. The same cells reported in B were analyzed by TUNEL assay. One representative experiment is reported. 

D. Expression of the indicated proteins was analyzed by Western blot. Expression of γ-tubulin shows equal loading of proteins.
with vectors encoding p53 and Brn-3a plus HMGA1 alone or in combination with HIPK2 (Fig. 6A). The crosslinked genomic DNAs were immunoprecipitated in two rounds with two specific antibodies directed against HMGA1, p53, Brn-3a, and HIPK2 (Fig. 6B). Re-ChIPped DNA was analyzed by PCR using Bcl-2 promoter–specific primers that encompass the p53 binding sites. As shown in Fig. 6B, HMGA1 is a component of the promoter-bound multimeric complex containing p53, Brn-3a, and HIPK2. The analysis of the subcomplexes following HMGA1 overexpression revealed the mechanisms whereby HMGA1 could abolish Bcl-2 repression exerted by p53 and Brn-3a. Indeed, HMGA1 overexpression leads to a decrease in the amount of HIPK2 on Bcl-2 promoter likely following HIPK2 delocalization from the nucleus to the cytoplasm. Moreover, the binding of Brn-3a to its DNA consensus was strongly reduced. These two effects were reversed by HIPK2 overexpression that reestablishes its nuclear localization, impaired by HMGA1, and its biological functions, i.e., the activation of p53 by Ser46 phosphorylation (13) and the increase of Brn-3a binding activity to its DNA consensus sequences (28). Therefore, these results show that HMGA1 impairs p53 proapoptotic activity by a significant reduction of HIPK2 and Brn-3a binding to the Bcl-2 promoter.

**Suppression of the p53-mediated transcription of Bcl-2 by HMGA1 may have a role in human carcinogenesis**

To verify whether HMGA1 also has a role in regulating Bcl-2 promoter activity in human cancer, we analyzed, by immunohistochemistry, Bcl-2 and HMGA1 protein expression in a panel of human breast carcinoma samples carrying a wild-type p53. As shown in Fig. 6C, 14 of 52 (27%) breast carcinomas analyzed carried a wild-type p53 and, interestingly, there was an increased expression of both HMGA1 and Bcl-2 genes in 11 of these 14 samples (78%). Therefore, in these cases, the apoptotic activity of p53 is likely impaired by HMGA1, which blocks its negative effect on Bcl-2 expression, thereby promoting the Bcl-2 antiapoptotic activity.

**Discussion**

Defective apoptosis represents a major causal factor in the development and progression of cancer. Moreover, the ability of tumor cells to evade the engagement of apoptosis could play a significant role in their resistance to conventional therapeutic regimens.

Recently, we reported that HMGA1, the aberrant expression of which is implicated in the process of carcinogenesis, binds to the p53 oncosuppressor and inhibits its apoptotic activity by a significant reduction of HIPK2 and Brn-3a binding to the Bcl-2 promoter. Moreover, the ability of tumor cells to evade the engagement of apoptosis could play a significant role in their resistance to conventional therapeutic regimens.
activity. This inhibition is associated with increased transcription of the p53 inhibitor Mdm2 and repression of the p53 effectors Bax and p21<sup>waf1</sup> (12). We have also shown that HMGA1 represses p53 apoptotic activity by promoting the cytoplasmic relocalization of the p53 proapoptotic activator HIPK2 (15). Here, we report that the HMGA1 antiapoptotic effect also occurs by deregulation of Bcl-2 transcription following a mechanism quite similar to those previously described (15). First, we have found a strong positive correlation between HMGA1 and Bcl-2 protein expression in several cell systems. Then, we investigated the molecular mechanisms which could account for this correlation. We have found that HMGA1 proteins are a component of a multiprotein complex including p53, HIPK2, and Brn-3a, which modulates Bcl-2 promoter activity. Moreover, we have shown that HMGA1 has a critical role in this complex. Indeed, HMGA1 overexpression impaired the transcriptional repression exerted by Brn-3a and p53 on the Bcl-2 promoter. Consistently, the transcriptional regulation of Bcl-2 supports the antiapoptotic effect of HMGA1 observed in the TUNEL assays. Finally, we have shown, using ChIP and re-ChIP assays, that HMGA1 overexpression drastically reduces the binding of HIPK2 and Brn-3a to the Bcl-2 promoter impairing p53 proapoptotic activity. This conclusion is supported by the reversion of the HMGA1-induced effect on the Bcl-2 promoter activity by the overexpression of HIPK2.

Therefore, HMGA1 overexpression counteracts p53-mediated downregulation of Bcl-2 transcription that results in a drastic inhibition of p53 apoptotic activity, which then contributes to the process of carcinogenesis. This effect has been previously described by us for other p53 target genes such as Bax and p21<sup>waf1</sup> (12).

On the basis of the data presented, it is reasonable to hypothesize that HMGA1 overexpression contributes to cancer progression through this novel mechanism, impairing p53 activity notwithstanding the absence of p53 gene mutations and/or deletions. This hypothesis seems validated by the finding of a significant correlation between HMGA1 and Bcl-2 overexpression in 11 out of 14 breast carcinoma cases harboring a p53 wild-type gene, indicating that in these tumors, there is an inactivation of the p53 apoptotic function mediated by HMGA1-dependent regulation of Bcl-2 promoter activity.

In conclusion, taken together, all the data reported here allow us to indicate a new mechanism by which HMGA1 overexpression, displacing HIPK2 and Brn-3a from the Bcl-2 promoter, impairs the apoptotic activity exerted by p53 on Bcl-2 expression, contributing to the progression step of carcinogenesis. This mechanism could also have a role in the resistance of malignant...
cells to chemotherapy and radiotherapy treatments because it is often dependent on Bcl-2 overexpression (24).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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