Tumor suppressor p53 induces the cellular response to DNA damage mainly by regulating expression of its downstream target genes. The human securin is an anaphase inhibitor, preventing premature chromosome separation through inhibition of separase activity. It is also known as the product of the human pituitary tumor-transforming gene, pttg, a proto-oncogene. Here we report that the expression of human securin is suppressed in cells treated with the DNA-damaging drugs doxorubicin and bleomycin. This suppression requires functional p53. Analysis of the human securin promoter reveals that DNA-binding sites for Sp1 and NF-Y are both required for activation of securin expression; however, only the NF-Y site is essential for the suppression by p53. Our study indicates that securin is a p53 target gene and may play a role in p53-mediated cellular response to DNA damage.

The securin proteins are a family of functional homologues, including the Pimples protein in Drosophila, Cut2 in fission yeast, Pds1 in budding yeast, and vSecurin in vertebrates (1–4). Securins play a critical role in regulating the separation of sister chromatids during mitosis. They prevent premature chromosome separation through inhibition of separase activity by forming tight securin/separase complexes. At anaphase securins are degraded, releasing separases, which facilitate the dissociation of sister chromatids by cleaving the cohesin complex (5). Deletion of securins has been found to cause abnormal chromosome segregation in fission yeast and human (6, 7).

Interestingly, the budding yeast Saccharomyces cerevisiae lacking Pds1 fails to arrest when it is treated with ionizing radiation (8, 9). In addition, ionizing radiation induces Pds1 phosphorylation at multiple locations, and this phosphorylation is thought to stabilize Pds1 and is essential for mitotic arrest (10, 11). These data indicate that Pds1 is involved in DNA damage checkpoints in budding yeast and human (6, 7).

DNA damage checkpoints are mechanisms that arrest the cell cycle and repair the damaged DNA by regulating expression of relevant genes (12). They are critical in maintaining genomic stability, which is vital to the health and survival of the cell. Genetic instability is believed to be an essential factor in the evolution of cancer in humans (13). One of the most important proteins controlling cellular responses to DNA damage in mammals is the tumor suppressor p53 (14, 15). By regulating expression of its downstream targets, p53 induces cell cycle arrest, promotes apoptosis, and facilitates DNA repair (14, 16).

To investigate whether vSecurin plays a role in the cellular response to DNA damage as its homologue Pds1 does in yeast, we examined its expression in several human cancer cell lines after treatment with the chemotherapeutic drugs doxorubicin (Dox) and bleomycin (Blm), both of which induce strand breaks in DNA (17, 18). Although the treated cells were mainly arrested at G2 phase, we found that the securin protein levels were dramatically reduced by Dox and Blm treatment. This drug-induced suppression of securin was strictly dependent on the presence of functional p53. Further studies on the human securin promoter revealed that activation of p53 by DNA damage reduced DNA binding of the transcription factor NF-Y to the securin promoter, resulting in repression of transcription. Our data clearly indicate that securin is a downstream target of p53 and suggest that suppression of securin plays an important role in cellular response to DNA damage.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The wild-type p53 (pCMVp53wt) and dominant negative mutant p53 (pCMVp53ΔN) expression vectors were obtained from Clontech Laboratories (Palo Alto, CA). A DNA fragment encoding the influenza hemagglutinin (HA) epitope was fused to the 5′-end of the p53ΔN coding region using PCR to generate a HA-tagged p53ΔN expression construct, pCMVHAp53ΔN. The control plasmid pCMVβ expressing β-galactosidase was obtained from Clontech. A murine p19ARF expression construct (pCDNAp19ARF) was kindly provided by R. A. DePinho. For p710Luc, a DNA fragment containing the human securin promoter region from −710 to +45 was isolated from p(−711/+201)Luc and subsequently subcloned into pGL3-basic vector (Promega, Madison, WI). The site-specific mutations in Sp1-binding sites and CCAAT boxes were generated by PCR-based site-directed mutagenesis. For each site-specific mutation, a pair of primers was designed to carry the desired mismatched nucleotides according to the promoter sequences (see below), and the reverse primer was phosphorylated at its 5′-end. PCR was performed with p710Luc as the template using a Takara LA Taq™ Polymerase kit (Panvera, Madison, WI) under the...
condition of 94°C for 1 min, 56°C for 1 min, and 68°C for 8 min for a total of 21 cycles. The PCR products were polished with Pfu DNA polymerase (Promega), treated with DpnI purified by agarose gel electrophoresis, and self-ligated. The authentication of the generated plasmids was confirmed by DNA sequencing. The promoters with desired mutations were then subcloned into pGL3-basic. The PCR primers are as follows (the underlined nucleotides were mismatched): CGTGGCCGCGAGTTGTGGT (forward), pGGGCCGCGAGTTGTGGTCAACAGTAGACC (reverse) for pSp1-1mt; pGGAGAGCCGAAATTGGCCGAAGGT (forward), pCATGCTGCGAGGCCCACTCGTGTGGT (reverse) for pSp1-2mt; pATGCCAGGGGCTGGGCGAGGCACCACTCGTGTGGT (forward) and pGGCTTCTGGTCAACAGTAGACC (reverse) for pCAT-1mt; pCGGCTTCTGGTCAACAGTAGACC (forward), pGGAGAGCCGAAATTGGCCGAAGGT (reverse) for pCAT-3mt; pACGTGGGGGCGGAGTTGTGGT (forward), pCGGCTTCTGGTCAACAGTAGACC (reverse) for pCAT-3mt; pACGTGGGGGCGGAGTTGTGGT (forward), pCGGCTTCTGGTCAACAGTAGACC (reverse) for pCAT-3mt; pACGTGGGGGCGGAGTTGTGGT (forward), pCGGCTTCTGGTCAACAGTAGACC (reverse) for pCAT-3mt; pACGTGGGGGCGGAGTTGTGGT (forward), pCGGCTTCTGGTCAACAGTAGACC (reverse) for pCAT-3mt. For pSp1-3mt with mutation of Sp1-2 and Sp1-3, a PCR was performed using primers ATGCCTCGGCGAGCAAGGAGCCAATAGGGCCCAAGT (forward) and pGGTCAAGCAGATATAGGGACCAATAGGGCCCAAGT (reverse) for pSp1-3mt; pACGTGGGGGCGGAGTTGTGGT (forward), pCGGCTTCTGGTCAACAGTAGACC (reverse) for pCAT-1mt; pACGTGGGGGCGGAGTTGTGGT (forward), pCGGCTTCTGGTCAACAGTAGACC (reverse) for pCAT-3mt; pACGTGGGGGCGGAGTTGTGGT (forward), pCGGCTTCTGGTCAACAGTAGACC (reverse) for pCAT-3mt; pACGTGGGGGCGGAGTTGTGGT (forward), pCGGCTTCTGGTCAACAGTAGACC (reverse) for pCAT-3mt; pACGTGGGGGCGGAGTTGTGGT (forward), pCGGCTTCTGGTCAACAGTAGACC (reverse) for pCAT-3mt; pACGTGGGGGCGGAGTTGTGGT (forward), pCGGCTTCTGGTCAACAGTAGACC (reverse) for pCAT-3mt; pACGTGGGGGCGGAGTTGTGGT (forward), pCGGCTTCTGGTCAACAGTAGACC (reverse) for pCAT-3mt; pACGTGGGGGCGGAGTTGTGGT (forward), pCGGCTTCTGGTCAACAGTAGACC (reverse) for pCAT-3mt; pACGTGGGGGCGGAGTTGTGGT (forward), pCGGCTTCTGGTCAACAGTAGACC (reverse) for pCAT-3mt.

Cell Culture, Transfection, and Luciferase Assay—HCT116, U2OS, Saos2, and DLD-1 were obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained according to the ATCC instructions. For transfection, U2OS and HCT116 were seeded into 12-well tissue culture plates and transfected using TransIT-LT1 transfection reagent according to the manufacturer’s instruction (Panvera). For each well, a total of 1 μg of plasmid DNA containing 0.2 μg of promoter construct, 0.2 μg of pCMVβ, and the others as indicated, plus 3 μl of TransIT-LT1 reagent were used. Twenty hours after transfection, the cells were harvested along with 1 μl of lysis buffer and the 5 μl of the lysates was analyzed on 12% SDS-PAGE. After electrophoresis, the membrane was probed with a rabbit anti-human securin antibody (Novus Biologicals), NF-YB (Rockland, Gilbertsville, PA), NF-I (Santa Cruz Biotechnology), and exposed to Kodak BioMax film. To quantify the protein-DNA complex, the radioactivity from the dried gel was recorded with a Storm PhosphorImager system (Amersham Biosciences).

RESULTS

DNA-damaging Agents Induce Suppression of Securin Expression—To study the regulation of securn in DNA damage, we treated the human osteosarcoma cell line U2OS and the human colorectal cancer cell line HCT116, both containing functional p53, with Dox and Blm. The treated cells were arrested at both G1 and G2 phases of the cell cycle, with most cells arrested at G2 (Fig. 1A), indicating that these cells contain functional cell cycle checkpoints. It has been reported that the expression of securin in cycle cells is cell cycle dependent. Its level is low in G1 phase, increases as cells enter S, and peaks in G2/M (21). In U2OS and HCT116, the securin protein was readily detectable in the untreated cycling cells by Western blotting (Fig. 1B). As a control, we treated cells with the anti-microtubule drug nocodazole and found the majority of cells were arrested at M phase (Fig. 1A and data not shown). In agreement with the previous findings (21), a strong band with a higher apparent molecular mass than that in the untreated cycling cells was observed in cells treated with nocodazole (Fig. 1B). This band is presumably the phosphorylated form of the securin protein (21). Surprisingly, the securin level was significantly lower in the Dox- and Blm-treated U2OS and HCT116 cells, despite that the majority of the treated cells were arrested at G2 (Fig. 1B). In contrast, Dox and Blm treatment suppressed p53 expression in cells treated with DNA-damaging drugs. Because p53 plays a critical role in regulating cellular response to DNA damages, we next examined securin expression in p53-deficient human osteosarcoma Saos2 and colorectal cancer DLD-1 cells. When these cells were treated with Dox and Blm, only G2 arrest was observed (Fig. 1C), consistent with the previous finding that p53 is required for DNA damage-induced G2 arrest (22). Interestingly, no securin suppression was observed in the drug-treated Saos2 and DLD-1 cells. Instead, a higher level of securin protein was found in the drug-treated cells compared with the untreated cycling cells (Fig. 1D), indicating that the DNA damage-induced suppression of securin is p53-dependent.

We next examined the securin RNA transcripts in cells treated with Dox and Blm. The mRNA was readily detected in all untreated cells (Fig. 2, A–C). However, the mRNA levels were profoundly diminished in U2OS and HCT116 cells treated with drugs (Fig. 2, A and B). In contrast, Dox and Blm treatment did not change the mRNA level in p53-deficient DLD-1 cells (Fig. 2C). These data indicate that the DNA damage-
p53 Suppresses Securin Expression

Fig. 1. DNA-damaging agents induce growth arrest and suppress securin expression in cancer cells containing wt p53. Human cancer cells were treated with Dox and Blm for 36 h. As controls, cells were also treated with nocodazole (Noc) alone or with Noc plus Dox and Blm as indicated. Cells without any treatment are presented as cycling cells. Cell cycle arrest was determined by FACS as previously described (19), and securin expression was measured by Western blotting as described under “Experimental Procedures.” A, FACS data for the wt p53-containing U2OS and HCT116 cells showing growth arrest, mainly at G2 phase. B, securin protein levels in U2OS and HCT116. C, FACS data for the p53-deficient Saos2 and DLD-1 cells. D, securin protein levels in Saos2 and DLD-1.

Mediated by p53

To investigate how p53 was involved in the DNA damage-induced suppression of securin, we analyzed the transcriptional repression of the securin promoter by DNA-damaging agents using a luciferase reporter construct. When p-710Luc was transiently transfected into U2OS and Saos2 cells, the wild-type (wt) p53 has a very short half-life due to its rapid degradation mediated by Mdm2 (24, 25). Expression of p19Arf blocks Mdm2 function, thereby inducing p53 accumulation (26, 27). In p53-proficient U2OS cells, co-transfection of a p19Arf expression vector, pcDNAp19Arf, significantly suppressed luciferase expression from p-710Luc in a dose-dependent manner (Fig. 3B). In contrast, no significant drug-induced suppression of the luciferase activity was observed in p53-deficient Saos2 and DLD-1 cells (Fig. 3A). These data indicate that the p-710Luc responds to DNA damage agents in a similar way as the endogenous securin gene does.

In agreement with the previous findings that DNA-damaging agents induce p53 accumulation (23), p53 levels were significantly elevated in both U2OS and HCT116 cells after treated with Dox and Blm (Data not shown). Considering the fact that Dox and Blm only reduced securin expression in p53-proficient cells, but not in p53-deficient cells (Fig. 1), we reasoned that transcriptional repression of the securin promoter by DNA-damaging agents may be attributed to the accumulation of p53. To test this possibility, we examined the effects of p19Arf on luciferase expression from p-710Luc in U2OS and Saos2 cells. The wild-type (wt) p53 has a very short half-life due to its rapid degradation mediated by Mdm2 (24, 25). Expression of p19Arf blocks Mdm2 function, thereby inducing p53 accumulation (26, 27). In p53-proficient U2OS cells, co-transfection of a p19Arf expression vector, pcDNAp19Arf, significantly suppressed luciferase expression from p-710Luc in a dose-dependent manner (Fig. 3B). In contrast, transfection of pcDNAp19Arf had no effect on p-710Luc in p53-deficient Saos2 cells (Fig. 3B), suggesting that activation of p53 is required for suppressing transcriptional activation of the securin promoter. We next tested whether the direct introduction of wt p53 would suppress securin promoter activity. The promoter construct p-710Luc was co-transfected with wt p53 or mutant p53 (p53mt135) expression constructs (pCMVp53wt and pCMVHAp53mt135, respectively) into U2OS and Saos2 cells. p53mt135 is a dominant negative mutant containing a single amino acid mutation, changing Cys-135 to Tyr, within its DNA binding domain (28). Expression of the exogenous wt p53 suppressed luciferase expression in both cell lines, whereas expression of the mutant p53 failed to do so (Fig. 3C), indicating that p53 is directly involved in repression of the securin promoter.

To further confirm that suppression of the securin promoter during drug treatment was caused by p53 activation, we co-transfected p-710Luc with pCMVHAp53mt135 into U2OS cells and treated the cells with Dox. In the absence of p53mt135, the expression of luciferase was suppressed significantly by Dox. As the amount of pCMVHAp53mt135 increased, the degree in
p53 Suppresses Securin Expression

Dox suppression of the luciferase expression was gradually reduced (Fig. 3D). When a sufficient amount of HA-p53 wt or mt135 construct was included, the securin promoter activity was completely restored from the Dox suppression (Fig. 3D). Because the p53 wt or mt135 alone did not activate p-710Luc in p53-deficient Saos2 cells (Fig. 3C), restoration of the securin promoter activity from Dox suppression by p53 wt or mt135 is, therefore, due to the neutralization of the wt p53 activated by drug treatment. Taken together, these data convincingly demonstrate that DNA damage agent-induced suppression of securin expression is mediated by p53.

Suppression of Securin Expression by p53 Is Mediated via the CCAAT Sites Overlapping the Major Start Site on the Securin Promoter—The promoter sequence of securin between −710 and +45 consists of four Sp1-binding sites and four CCAAT boxes (Fig. 4A). It has been shown that p53 inhibits transcription from the human telomerase reverse transcriptase promoter and the SV40 promoter by repression of Sp1 DNA binding (29, 30). In addition, p53 suppresses expression of several genes through CCAAT sites on their promoters (31–33). Therefore, it is possible that transcription factors interacting with Sp1 sites and CCAAT boxes are involved in the p53-mediated repression of the securin promoter. To explore this possibility, we mutated each of the Sp1-binding sites by changing the consensus sequence GGCGG to GATGG and tested how these mutations affected securin promoter activity in U2OS cells. Mutation of each Sp1-binding site individually did not significantly change activity of the promoter (pSp1–1mt, pSp1–2mt, pSp1–3mt, and pSp1–4mt in Fig. 4B); however, when two or more Sp1 sites were mutated simultaneously, the securin promoter activity was dramatically decreased (pSp1–23mt, pSp1–123mt, and pSp1–Allmt in Fig. 4B). Then, we tested the activities of these mutant promoter constructs in response to Dox treatment. Interestingly, Dox treatment suppressed the activity of all these constructs by ~90%, very similar to the Dox-mediated suppression of the wt promoter construct p-710Luc (Fig. 4B). These results indicate that the transcription factor Sp1 is essential for basal transcription activity of the securin promoter but not required in p53-mediated repression of the promoter.

Next, we investigated whether CCAAT boxes are required for p53-mediated repression of the securin promoter. We mutated the CCAAT box by changing two or more nucleotides in the consensus core sequence of CCAAT (see “Experimental Procedures”). The mutated promoters were inserted into pGL3-Basic and tested for their transcription activities. It is necessary to point out that two CCAAT boxes are found overlapping the major transcription initiation site, the forward CCAAT-3 and CCAAT-4 (Fig. 4A). The CCAAT-3 and CCAAT-4 overlap each other; therefore, changing the two nucleotides shared by them in the core region mutates both CCAAT boxes. Thus, they were considered as one CCAAT box represented by CCAAT-3, and the promoter construct pCAT-3mt actually contained both mutated CCAAT-3 and CCAAT-4. As shown in Fig. 4C, mutation of CCAAT-1 and -2 did not affect the promoter activity in U2OS cells (Fig. 4C, pCAT-1mt and pCAT-2mt). However, mutation of CCAAT-3 reduced the promoter activity by ~90% compared with that of the wt p-710Luc in U2OS cells (Fig. 4C, pCAT-3mt). More interestingly, Dox treatment only slightly reduced activity of pCAT-3mt, while it significantly suppressed activity of pCAT-1mt and pCAT-2mt (Fig. 4C). In addition, when the CCAAT-3 site was mutated in pSp1–Allmt,
the generated pSp1-All-CAT-3mt had an activity similar to that of the promoter-less vector pGL3-Basic, which was much lower than that of pSp1-Allmt (Fig. 4C). Furthermore, Dox treatment failed to suppress the promoter activity significantly from pSp1-All-CAT-3mt. These data clearly demonstrate that the CCAAT boxes overlapping the start site, represented as CCAAT-3, are required not only for transcription activation, but also for Dox-induced suppression of the securin promoter, whereas CCAAT-1 and -2 sites are dispensable for both activities. A similar result was also observed in HCT116 cells (data not shown). To further examine whether CCAAT-3 was critical for p53-mediated repression of the securin promoter, pCAT-3mt and pSp1-All-CAT-3mt were co-transfected with the pCMVp53wt expression construct into U2OS cells. As expected, the wt p53 only slightly reduced luciferase expression from the promoters with CCAAT-3 mutations, whereas it significantly reduced expression of luciferase from their corresponding control promoters, p-710Luc and pSp1-Allmt (Fig. 4D), demonstrating that CCAAT-3 is indeed the site through which p53 represses the securin promoter.

NF-Y Complexes Bound to CCAAT-3 Were Reduced by Dox Treatment—To elucidate the mechanism by which p53 represses the securin promoter, we performed EMSA to identify the proteins binding to the CCAAT-3 site. A 30-bp oligomer, equivalent to the DNA sequence from position –17 to +13 of the human securin promoter containing CCAAT-3, was synthesized and named as CCAAT-3wt. As a control, a similar oligomer, named as CCAAT-3mt, was also generated to contain a
mutated CCAAT-3 by changing sequences of CCAAT to CCAACG (Fig. 5A). CCAAT-3wt was labeled with $^{32}$P and incubated with nuclear extracts isolated from untreated U2OS cells or cells treated with Dox. The protein-DNA complexes were analyzed by non-denaturing polyacrylamide gels. A high molecular weight band was detected in the lane with nuclear extract from untreated U2OS cells (Fig. 5B, lane 2). This band diminished when the nuclear extract was preincubated with excessive unlabeled CCAAT-3wt (Fig. 5B, lanes 3 and 4), whereas preincubation with the same amount of the unlabeled CCAAT-3mt did not affect the band (Fig. 5B, lanes 5 and 6). This indicates that the protein-DNA complex formed with this oligomer is CCAAT-3 sequence-specific. The complexes were also found with nuclear extracts from the Dox-treated cells (Fig. 5B, lane 7); however, the intensity of the band was much lower than that with nuclear extract of the untreated cells (Fig. 5B, comparing lanes 2 and 7). This suggests that Dox treatment reduces binding of the nuclear proteins to this site.

Several transcription factors are capable of binding to DNA containing the CCAAT sequence, such as C/EBP, CBP/NF-1, and CBF/NF-Y (34). A careful comparison between the consensus binding sequences of each CCAAT-binding protein with the sequences around the CCAAT-3 in the human securin promoter revealed that the sequence of this region matched the binding site for NF-Y (CCAAT) (34) as well as the half site for NF-1 (GCCAA) (35). NF-Y is a complex of three subunits, NF-YA, NF-YB, and NF-YC (36). To identify which transcription factor forms complexes with CCAAT-3, we incubated nuclear extracts with antibodies specifically against NF-1, NF-YA, NF-YB, and NF-YC, respectively, before the labeled probe was added. These bands were supershifted by NF-YA antibody (Fig. 5D, lane 3), suggesting that Dox treatment does not reduce NF-Y binding to CCAAT-3 in Saos2 cells. To quantify DNA binding of NF-Y, we performed EMSA with nuclear extracts from p53-deficient Saos2 cells. A specific high molecular weight band was found in both lanes with nuclear extracts from untreated and Dox-treated cells (Fig. 5D, lanes 2 and 5). The bands were supershifted by NF-YA antibody (Fig. 5D, lanes 3 and 6), indicating that it is also the transcription factor NF-Y that binds to CCAAT-3 in Saos2 cells. However, there was no significant difference in the intensities of the band between lanes with nuclear extracts from the untreated and Dox-treated cells (Fig. 5D), suggesting that Dox treatment does not reduce NF-Y binding to CCAAT-3 in Saos2 cells.
cells. However, Dox treatment did not have any effect on the complex formation in Saos2 cells (Fig. 6B). These data indicate that repression of the securin promoter by the DNA-damaging agent Dox is attributed to the decline in NF-Y binding to its DNA sites, which requires the presence of functional p53.

One of the possible mechanisms leading to the reduction of NF-Y DNA binding is that expression of one or more NF-Y subunits may be decreased in Dox-treated U2OS cells. To explore this possibility, we prepared whole cell lysate from U2OS cells with or without Dox treatment and examined the expression of each NF-Y subunit by Western blotting. We did not detect any changes in protein level for any of the NF-Y subunits in cells with Dox treatment compared with those found in cells without Dox treatment (data not shown), indicating that NF-Y expression is not affected by the elevated level of p53. Another possibility is that p53 may interact with NF-Y and prevent it from binding to DNA, similar to the previously reported suppression of human hsp70 promoter by p53 (31). To investigate this possibility, we performed EMSA with U2OS nuclear extract preincubated with purified recombinant wt p53 protein or the same amount of BSA (Fig. 7, upper panel). The samples from the duplicate reactions were resolved by 10% SDS-PAGE, which was later stained with Coomassie Blue to demonstrate that equal amounts of nuclear extracts were used in each reaction (lower panel). The arrows indicate the positions of BSA and wt p53.

**Fig. 6.** DNA-damaging drugs induce decline in NF-Y DNA binding. A, EMSA reactions using nuclear extracts from U2OS and Saos2 with or without Dox treatment were prepared in duplicate. After incubated with the γ-32P-labeled CCAATwt, the DNA-protein complexes in one set of the reactions were resolved by 5% native polyacrylamide gel (upper panel). The samples from the duplicate reactions were resolved by 10% SDS-PAGE, which was later stained with Coomassie Blue to demonstrate that equal amounts of nuclear extracts were used in each reaction (lower panel). B, the NF-Y-DNA complexes from U2OS and Saos2 with or without Dox treatment were quantified by a PhosphorImager. The reading from the Dox-treated nuclear extracts was compared with that from its untreated counterpart as 100.

**Fig. 7.** Recombinant wt p53 inhibits NF-Y DNA binding in vitro. EMSA reactions using U2OS nuclear extracts were prepared in duplicate. The nuclear extract (2 μg) was preincubated with recombinant wt p53 (2 μg) or BSA (2 μg) before the labeled CCAATwt was added. The DNA-protein complexes in one set of the reactions were resolved by 5% native polyacrylamide gel (upper panel). The samples from the duplicate reactions were resolved by 10% SDS-PAGE, which was later stained with Coomassie Blue to demonstrate that equal amounts of nuclear extracts were used in each reaction (lower panel). The arrows indicate the positions of BSA and wt p53.
DISCUSSION

DNA damage activates p53, which induces cell cycle arrest, allowing for repair of the damage. If the damage is beyond repair, p53 promotes programmed cell death. p53 functions mainly through inducing or inhibiting expression of its target genes (16, 37, 38). For example, p53 causes cell cycle arrest at G1 by stimulating expression of the CDK inhibitor p21, while p53-induced G2 arrest is mediated by inducing expression of 14-3-3, Gadd45, p21, and Reprimo, as well as inhibiting Cdc2 and cyclin B1 (39). In this study, we showed that DNA damage induced by Dox and Blm suppressed expression of securin in the presence of functional p53. We further demonstrated that activation of p53 alone is sufficient to cause repression of the securin promoter. Finally, we provided evidence demonstrating that p53 suppresses securin expression by reducing the binding of the transcription factor NF-Y to its promoter. Our data indicate that human securin is a p53 target gene, which is suppressed in response to DNA damage.

Transcription factor NF-Y has been shown to play an important role in regulating expression and mediating p53-repression of several cell cycle-regulated genes, such as cyclin A, cyclin B1, cyclin B2, cdc2, and cdc25C (32, 33, 40, 41). The promoter of human securin contains four CCAAT boxes, which are the DNA-binding sites for NF-Y. Interestingly, our data show that not all of these sites are involved in activation and p53-mediated repression of the promoter. When CCAAT-1 and -2 are mutated, neither the transcription activity, nor the p53-mediated repression of the promoter is significantly affected. However, when the overlapped CCAAT-3 and -4 (represented as CCAAT-3 in this study) are mutated, both promoter activity and the p53-mediated transcription repression are severely compromised. This selective utilization of NF-Y-binding sites for the regulation of securin promoter may be attributed to the location and the surrounding DNA sequences of each individual site. For example, CCAAT-1 is located 320 bp away from the major transcription start site, and there are no other transcription factor-binding sites nearby. CCAAT-2 is in between two Sp1 sites. It could be important in activating the securin promoter. However, CCAAT-3 overlaps the major start site and has three Sp1-binding sites juxtaposed at its upstream. This unique setting may make CCAAT-3 much more prominent in activating transcription from the promoter and undermines the potential role of CCAAT-2 in regulating the securin promoter. The promoter of human securin is TATA-less and contains no apparent initiator element. The NF-Y has been shown to activate transcription by recruiting the basal transcription factor TFIID (42) and co-activators P/CAF and p300 (43, 44). In addition, functional interaction between NF-Y and Sp1 is necessary in regulating expression of many genes, such as human A-myb (45), p27kip (46), type A natriuretic peptide receptor (47), metalloproteinase-2 inhibitor (48), and cystathionine β-synthase-1b (49). Therefore, it is likely that the CCAAT-3-bound NF-Y plays a critical role in transcription initiation of the securin promoter, whereas the Sp1 functions as an activator when bound to the upstream sequences near the CCAAT-3. The binding of Sp1 to the nearby sequence may stabilize the DNA binding of NF-Y by direct physical interaction (50, 51) or through interaction with the same co-activator, such as p300 (52, 53). This is consistent with our finding that both Sp1 and NF-Y are required for the optimal activation of the securin promoter. It has been shown that Sp1 interacts with TFIID and is involved in transcription initiation of tumor necrosis factor α-responsive gene (54, 55). Thus, Sp1 may become the major player activating transcription initiation in the absence of CCAAT-3, which may be responsible for activities of the promoter with mutated CCAAT-3. The initiation site activated by Sp1 may or may not be the same location as that activated by the CCAAT-3-bound NF-Y. Nevertheless, it is interesting to notice that this transcription activation mediated by Sp1 in the absence of CCAAT-3 is resistant to the p53-mediated repression. This is not due to the low promoter activity, because the promoter with all Sp1 sites mutated is still sensitive to p53 expression despite that the promoter activity is even lower than that lacking CCAAT-3. These results suggest that p53 represses the human securin promoter by affecting the transcription initiation through the CCAAT-3-bound NF-Y. Taken together, we propose a mechanism by which DNA damage induces the suppression of human securin expression. The p53 activated by DNA damages interacts with transcription factor NF-Y, preventing it from binding to the DNA overlapping the transcription start site on human securin gene promoter. This reduces transcription initiation from the promoter, thus leading to the expression suppression of human securin.

The human securin expression is cell cycle-regulated, which is low in G1 phase and starts to accumulate in S phase (21). The chromosomes are duplicated during S phase of the cell cycle. To ensure the correct distribution of the newly duplicated chromosomes to daughter cells, the sister chromatids have to be held together until the cell cycle reaches anaphase when they are separated. As the securin binds to separate and inhibits its function, the accumulation of securin during S phase may be necessary to prevent premature separation of the newly synthesized sister chromatids. Thus, it is conceivable that a high level of Pds1, a homologue of human securin, is essential for mitotic arrest in the budding yeast in response to DNA damage (10, 11). However, our data demonstrate that DNA damage results in suppression of securin in human cells containing functional p53. Therefore, this suggests that in vertebrates other mechanisms must exist to hold the sister chromatids together during G2 arrest. Several studies have indicated that securin binding is not the only mechanism that regulates sister chromatid separation. For example, deletion of securin does not affect the cell cycle progression of the mouse embryonic stem cells and human HCT116 (6, 56). Furthermore, the high activity of Cdc2 has been shown to inhibit anaphase independent of securin (57).

Numerous studies indicate that the vSecurin is a multifunctional protein. In addition to anaphase inhibition, it is also known as the product of pttg, which is a proto-oncogene (4, 20, 58). It stimulates expression of c-myc (59), promotes angiogenesis (60), and transforms NIH3T3 cells (20, 58). Recently, securin has been shown to form complexes with the Ku protein (61). The Ku protein is the regulatory subunit of the DNA-dependent protein kinase (DNA-PK), an essential component in the repair of DNA double-strand breaks (62, 63). In addition, Ku has also been indicated in maintaining the stability of telomeres (64, 65). Therefore, the suppression of securin by p53 may lead to the inhibition of its functions related to the Ku protein. For example, Dox and Blm have been known to cause DNA double-strand breaks (17, 18). The main mechanism for DNA double-strand break repair in mammalian cells is non-homologous recombination end-joining, in which Ku plays an essential role by activating DNA-PK. It has been reported that the Ku protein dissociates from securin in the presence of sonicated DNA (61), suggesting that Ku is released from the securin binding in the presence of double-strand breaks. Considering that the securin functions as an inhibitory protein when it binds to securin, we postulate that securin may act as an inhibitor of Ku, thereby to prevent formation of DNA-PK holoenzyme in the absence of DNA double-strand breaks and/or inhibit other Ku-dependent functions. High levels of securin expression may lead to the depletion of the function of Ku.
proteins by securin binding, therefore adversely affecting the repair process of the damaged DNA. We hypothesized that DNA damages activate p53, which in turn suppresses securin expression and decreases its cellular concentration. Consequently, more Ku proteins free of securin are available for DNA repair or other Ku-dependent functions, such as the protection of the telomere. It will be important to clarify the physiological significance of securin suppression by p53 to fully appreciate the role of this multifunctional protein in cell cycle control, DNA damage repair, and malignant transformation.

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