ASPP Proteins Specifically Stimulate the Apoptotic Function of p53

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Summary

We identified a family of proteins termed ASPP. ASPP1 is a protein homologous to 53BP2, the C-terminal half of ASPP2. ASPP proteins interact with p53 and specifically enhance p53-induced apoptosis but not cell cycle arrest. Inhibition of endogenous ASPP function suppresses the apoptotic function of endogenous p53 in response to apoptotic stimuli. ASPP enhance the DNA binding and transactivation function of p53 on the promoters of proapoptotic genes in vivo. Two tumor-derived p53 mutants with reduced apoptotic function were defective in cooperating with ASPP in apoptosis induction. The expression of ASPP is frequently down-regulated in human breast carcinomas expressing wild-type p53 but not mutant p53. Therefore, ASPP regulate the tumor suppression function of p53 in vivo.

Introduction

Although p53 is one of the most frequently mutated genes found in human cancer, the mutation frequency varies among different tumor types: 70% in human lung cancer, 30% in breast cancer, and 5% in leukemia (see p53 mutation database at http://perso.curie.fr/Thierry.Soussi). It remains unclear why wild-type p53 fails to suppress tumor growth in the 70% of breast cancers that lack p53 mutations. In some cases, abnormalities of other tumor suppressor genes such as Rb, p16, BRCA1, or BRCA2 may allow the tumor cells to bypass the tumor suppression function of p53. Lack of costimulators or overexpression of inhibitors of p53 could also account for the loss of the tumor suppressor function of wild-type p53.

As a transcription factor, p53 transactivates and transrepresses many target genes that induce cell cycle arrest or apoptosis in response to DNA damage or cellular stress (Ko and Prives, 1996). The tumor suppression function of p53, however, is best linked to its ability to induce apoptosis. A new category of tumor-derived p53 mutants has been identified. They are defective in inducing apoptosis but are as active as wild-type p53 in inducing G1 arrest (Friedlander et al., 1996; Ludwig et al., 1996; Smith et al., 1999). They are also less able to transactivate promoters of proapoptotic genes such as Bax, PIG-3, and IGF-BP3 but competent to transactivate promoters of other p53 target genes such as mdm2 and p21waf1. These observations suggest that transactivation of genes such as Bax is important for the tumor suppression function of p53. However, how the promoter specificity of p53 is regulated remains unknown.

Two of the p53 mutants in this category were mutated at residue 181, namely 181L and 181C. Interestingly, residue 181 is one of the sites of p53 interaction with 53BP2 in the crystal structure (Gorina and Pavletich, 1996). 53BP2 (p53 binding protein 2) was initially discovered in a yeast two-hybrid assay (Iwabuchi et al., 1994). 53BP2 contains a proline-rich sequence, four ankyrin repeats, and an SH3 domain. The crystal structure of a complex of parts of p53 and 53BP2 revealed that the 53BP2 binding site on the central domain of p53 consists of evolutionarily conserved regions (Gorina and Pavletich, 1996). Individual alteration of six of the p53 residues most frequently mutated in human cancer causes loss of binding for 53BP2 and DNA (Thukral et al., 1994).

Subsequently, 53BP2 was also found to interact with other proteins, including Bcl-2 (Naumovski and Cleary, 1996), protein phosphatase 1 (Helps et al., 1995), p65 RelA (a component of NF-κB; Yang et al., 1999) and APCL (Nakagawa et al., 2000). A longer version of 53BP2 was isolated and named Bbp (Bcl-2 binding protein). Based on in vitro translation data, Bbp was predicted to consist of 1005 amino acids. In addition to the three functional domains of 53BP2, the N terminus of Bbp contains an α-helical domain (Naumovski and Cleary, 1996). Although the precise interaction sites between p53 and 53BP2 have been identified in vitro, little is known about the physiological consequences of the interaction. Interaction with the C-terminal fragment of 53BP2 inhibited the DNA binding activity of p53 in vitro (Iwabuchi et al., 1994). However, Bbp stimulated the transactivation function of p53 (Iwabuchi et al., 1998). The failure to produce stable clones expressing Bbp suggested that Bbp may suppress cell growth (Naumovski and Cleary, 1996), and an apoptotic function of Bbp was also reported (Yang et al., 1999). Contrary to the initial prediction based on the in vitro studies of 53BP2, it seems that Bbp functions as a growth suppressor rather than as an oncogene. What are the biological functions of the gene encoding for 53BP2/Bbp? Are there any related family members of 53BP2/Bbp, and how do they function in vivo?

Here, we show that 53BP2 and Bbp are both fragments of a protein we designate ASPP2. 53BP2 is the C-terminal 528 amino acid part of ASPP2 (amino acid residues 600–1128), Bbp is the C-terminal 1005 amino acid part of ASPP2 (residues 123–1128), and ASPP2 is the 1128 amino acid full-length polypeptide expressed in vivo. We also identify a related protein named ASPP1, and show that both ASPP1 and ASPP2 regulate the activities of p53.
Figure 1. p53 Interacts with ASPP1 and ASPP2 In Vivo

The transfected V5-tagged ASPP1 migrates similarly as endogenous ASPP1 in 293 cells (A). (B) shows the specificity of the anti-ASPP2 monoclonal antibody DX54.10. Lysates derived from 293-, JW2-, Tero-, and 53BP2-transfected Saos-2 cells were fractionated on an 8% SDS-PAGE gel in duplicate. The blot was cut in two. The presence of 53BP2 and endogenous ASPP2 in the indicated cell lysates was detected with DX54.10 depleted with GST (left panel) but not with GST-ASPP2 (right panel). (C) shows that the endogenous ASPP2 is larger than the transfected Bbp. The newly cloned ASPP2 cDNA comigrates with endogenous ASPP2 and is detected with DX54.10 (D) and the endogenous ASPP1 and
ASPP Stimulates the Apoptotic Function of p53

Results

Endogenous ASPP Interact with p53 In Vivo

We identified a novel protein family whose members include ASPP1 and ASPP2. ASPP stands for apoptosis stimulating protein of p53, and the name emphasizes the ankyrin repeats, SH3 domain, and proline-rich domains that characterize the family. The accession numbers for the nucleotide sequences are AJ318887 and AJ318888, respectively.

ASPP1 was identified in a genome database search for proteins with homology to the previously isolated protein called 53BP2/Bbp and encodes a protein of 1090 amino acids. ASPP2 encodes a protein of 1128 amino acids, compared with the 1005 amino acids reported previously for the Bbp clone. The greatest similarity between ASPP1 and ASPP2 was found in the N- and C-terminal parts of the protein (data not shown). The p53 contact residues identified in 53BP2 are conserved between ASPP1 and ASPP2, suggesting that ASPP1 may also interact with p53.

To understand the function of ASPP1 and ASPP2, we used GST fusion proteins to generate antibodies. The expression of endogenous ASPP1 and ASPP2 was studied in the kidney epithelium cell line 293, since the mRNA levels of ASPP1 and ASPP2 are relatively high in kidney (data not shown). The anti-ASPP1 antibody YX.7 immunoprecipitated a specific protein close to the predicted molecular weight, and this protein migrated almost identically to transfected epitope-tagged ASPP1. The transfected ASPP1 expression was revealed more clearly by the antibody V5, and the slight broadening of the ASPP1 band in the upper panel is thought to be due to detection of both endogenous ASPP1 and the slightly longer V5-tagged protein (Figure 1A).

The expression of endogenous ASPP2 was also examined in the 293 cell line. Also included were cell lines JW2, Tero, and Saos-2 derived from colorectal cancer, teratocarcinoma, and osteosarcoma, respectively. A specific protein band was recognized by the anti-ASPP2 antibody DX54.10 precleared with GST beads but not with GST-ASPP2 (residues 691–1128) beads (Figure 1B). The expression level of ASPP2 differs among these cell lines, but the endogenous ASPP2 protein migrates the same in all the cell lines used.

The discrepancy between the migration of endogenous ASPP2 and transfected Bbp was first detected in the cell lysates derived from Saos-2 cells transfected with either a control vector or an expression plasmid for Bbp. The immunoprecipitated Bbp was then detected with rabbit anti-ASPP2 antibody, pAbASPP2/77. Bbp was specifically immunoprecipitated by the monoclonal antibody DX54.10 but not by the control antibody pAb423 (Figure 1C). A computer database comparison study revealed that nt 412–nt 543 in the published Bbp sequence was derived from an Alu repeat sequence that contains a stop codon in-frame with Bbp, which does not exist in any of the EST clones homologous to ASPP2. Sequencing of the original clone derived from the cDNA library confirmed the absence of the Alu sequence (data not shown). ASPP2 expressed from the non-Alu-containing cDNA migrated with the same mobility as endogenous ASPP2 (Figure 1D). Moreover, we noticed that the homology between ASPP1 and ASPP2 starts with the sequence MMPM. Thus, both ASPP1 and ASPP2 may use one of these methionines as the start site for translation, and the ASPP2 protein contains 1128 amino acids, which would account for the larger size of the endogenous protein seen in Figure 1C.

p53 and 53BP2 interact directly in vitro (Iwabuchi et al., 1994), but it was not known whether a similar interaction takes place in vivo with the full-length ASPP2. ASPP1 and ASPP2 share high sequence homology in the region essential for interaction with p53, and the previously identified specific p53 contact residues on ASPP2 are also conserved in ASPP1. Thus, ASPP1 and ASPP2 may interact with p53. The protein level of p53 is generally low in cells expressing wild-type p53 but increases in response to DNA damage. We UV-irradiated MCF-7 cells and immunoprecipitated ASPP1 and ASPP2. Western blot analysis of the immunocomplexes showed that p53 interacts with ASPP1 and ASPP2 (Figure 1E). The control antibody pAb423 (an antibody to SV40 large T antigen) did not immunoprecipitate either p53 or ASPP proteins, indicating that the interaction is specific. We estimate that in normal growing MCF-7 cells, at least 4.1% and 1.5% of p53 is in complex with ASPP1 and ASPP2, respectively. Interestingly, in response to UV irradiation, there is a 3.4-fold increase in the amount of p53 in complex with ASPP2 (5.5% of p53 is now in complex with ASPP2). Under the same conditions, the amount of p53 binding to ASPP1 did not increase. About 2% of p53 was found in complex with ASPP1. Without the complete depletion of individual ASPP proteins, the absolute amount of p53 in complex with ASPP1 or ASPP2 is underestimated. However, the 3.4-fold increase in p53/ASPP2 complex detected in UV-irradiated MCF-7 cells in comparison to that of untreated MCF-7 cells suggests a role for ASPP2 in regulating the activities of p53 in response to UV irradiation.

ASPP Specifically Stimulate the Apoptotic Function of p53

As the tumor suppression function of p53 is tightly linked to its ability to induce apoptosis, we studied the effect of ASPP1 and ASPP2 on the apoptotic function of p53. Saos-2 cells were transfected with a p53 expression plasmid or a control plasmid together with cell surface marker CD20. FACS analysis showed that the expression of p53 caused about 17% apoptosis among the transfected cell population (Figures 2A and 2B). Expression of ASPP1 or ASPP2 in the absence of p53 caused a

ASPP2 complex with p53 in MCF-7 cells (E). The expression level of p53 is shown in the far right panel of (E). Mouse antibodies YX-7 or DX54.10 were used to immunoprecipitate ASPP1 and ASPP2, respectively. Control mouse antibodies were pAb423 or a nonrelated mouse serum labeled as control Ab. The expression of ASPP1, ASPP2, and p53 were detected with rabbit antibodies ASPP1.88 (A and E), ASPP2/77, or CM1 (E), respectively. For (A), the same blot was stripped and reprobed with antibody V5 to detect the transfected epitope-tagged ASPP1 (lower panel).
Molecular Cell

784

Figure 2. FACS Analysis Shows that ASPP Specifically Stimulate the Apoptotic Function of p53 in Saos-2 Cells

The percentage of apoptotic cells was measured by the accumulation of cells with a sub-G1 DNA content (the cells in M1 region as labeled) (A). The bar graphs in (B)–(E) represent the percentage of apoptotic cells transfected with expressing plasmids as indicated. Mean values were derived from three independent experiments.

small fraction of the cells to die by apoptosis. In contrast, coexpression of p53 with ASPP1 or ASPP2 caused 50% of the transfected cells to die of apoptosis (Figure 2B). ASPP1 and ASPP2 seem to specifically enhance the apoptotic function of p53, since they failed to show any significant effect on the percentage of cells arrested at G1 (Figure 2C).

In addition to p53, ASPP2 also interacts with Bcl-2. Hence, ASPP2 may stimulate the apoptotic function of p53 by preventing Bcl-2 from inhibiting p53-induced apoptosis. The effects of ASPP1 and ASPP2 on the apoptotic function of E2F1 and Bax were compared to that of p53. In agreement with previous reports, Bcl-2 inhibited apoptosis induced by the expression of p53 and E2F1 in Saos-2 cells. However, the expression of ASPP1 or ASPP2 only stimulated the apoptotic function of p53, not of E2F1 (Figure 2D). Moreover, the apoptosis induced by the expression of Bax was also unaffected by the coexpression of ASPP1 or ASPP2 (Figure 2E). These results suggest that ASPP1 and ASPP2 stimulate the apoptotic function of p53 specifically, rather than inhibit the antiapoptotic function of Bcl-2.

Endogenous ASPP Promote the Apoptotic Function of p53 in Response to Apoptotic Stimuli

To study the roles that endogenous ASPP1 and ASPP2 play in regulating apoptosis induced by endogenous p53, we first introduced ASPP1 or ASPP2 into the wild-type p53-expressing cell lines U2OS and MCF-7. The expression of ASPP1 and ASPP2 induced apoptosis in these cells. The expression of the viral oncoprotein E6, derived from human papilloma virus that can bind and specifically target p53 for degradation, inhibited apoptosis, demonstrating that ASPP1 and ASPP2 induces apoptosis via endogenous p53 (Figure 3A).

To investigate the role of endogenous ASPP1 and ASPP2 in regulating the apoptotic function of endogenous p53 in response to DNA damage, we used an antisense approach. We cloned fragments from the 5' ends of ASPP1 and ASPP2 cDNAs into a mammalian expression vector in antisense orientation. The ability of the antisense RNA to specifically inhibit the protein synthesis of ASPP1 and ASPP2 was confirmed in vitro (data not shown). Expression of antisense ASPP1 only inhibited apoptosis induced by ASPP1 but not by ASPP2. Similarly, expression of antisense ASPP2 only inhibited apoptosis induced by ASPP2 but not by ASPP1. The specific activity of antisense ASPP1 and ASPP2 was further supported by the observation that coexpression of antisense ASPP1 or ASPP2 did not influence the apoptosis mediated by Bax under the same conditions (Figure 3B). Hence, the antisense RNA of ASPP1 and ASPP2 allowed us to study the effects of endogenous ASPP1 and ASPP2 on endogenous p53-induced apoptosis. U2OS
Figure 3. Endogenous ASPP Stimulate the Apoptotic Function of p53 In Vivo

U2OS and MCF-7 cells were transfected with plasmids as indicated together with a cell surface marker CD20 (A–C). The transfected cells were gated and analyzed by FACS as described in Figure 2A. The plasmids expressing antisense RNA of ASPP1 and ASPP2 are labeled as α-ASPP1 and α-ASPP2, respectively. The viral oncoprotein E6 of human HPV16 is indicated as E6. In (C), U2OS and MCF-7 cells were transfected with plasmids as indicated first and then incubated with medium containing cisplatin at concentrations of 5 and 3 μg/ml, respectively. Thirty hours later, cells were harvested and analyzed.
Figure 4. ASPP Specifically Stimulate the DNA Binding and Transactivation Function of p53 on Promoters of Proapoptotic Genes Such as Bax and PIG-3

In (A), ASPP2-inducible U2OS cells were treated as indicated and the cell lysates were immunoprecipitated (IP) with various antibodies (pAb421 for p53, DX5410 for ASPP2, and V5 as the control). The presence of promoter sequences of Bax and p21waf1 was detected by PCR (upper panel). Genomic DNA was used as a positive control for PCR (gDNA), and bar graphs show the fold increase of PCR products detected. The
and MCF-7 cells were transfected with various expression plasmids prior to treatment with cisplatin. FACS analysis showed that around 20%–30% of control transfected cells undergo apoptosis. Expression of E6 halved this percentage, indicating that cisplatin can induce apoptosis through both p53-dependent and -independent pathways in these cells. Expression of antisense RNA of ASPP1 or ASPP2 inhibited cisplatin-induced apoptosis to the same extent as E6 (Figure 3C). This suggests that endogenous ASPP1 and ASPP2 play important roles in regulating the apoptotic function of p53 in response to apoptotic stimuli such as cisplatin.

ASPP Stimulate the DNA Binding and Transactivation Function of p53

To understand how ASPP proteins specifically stimulate the apoptotic function of p53, we studied the effect of ASPP on the DNA binding and transactivation function of p53. U2OS cells were used to construct a stable cell line in which the expression of ASPP2 is regulated by promoter in a p53-dependent manner. Neither ASPP1 nor ASPP2 stimulated the transactivation function of E2F1 or p65 on various reporters as indicated (F and G). The expression levels of various responsive luciferase reporter plasmids were 3xwt-luc, cyclin A-luc, and Bmyb-luc. Kb-luc is a reporter for p65. The fold increase in the transactivation activity by either ASPP1 or ASPP2 on various p53 reporters is shown in (C) and was obtained as 1. Upon the expression of ASPP2, the DNA binding activity of p53 on the Bax promoter was increased by 3-fold. No increase in p53 binding to the control promoter sequence was detected in the immunoprecipitates derived from ASPP2-expressing cells with untreated cells used to reflect the increased DNA binding activity of p53 in vivo.

expression of p53, ASPP2, and PCNA is shown in the middle lower panel labeled as “lysate.” IP Western blotting shows the amounts of p53 immunoprecipitated by pAb421 and used in the CHIP assay (middle lower panel labeled as “IP”). (B) shows that ASPP enhance the Bax promoter activity in a p53-dependent manner as they failed to stimulate the activity of p53 binding site-deleted Bax promoter (Mut Bax-luc). The fold increase in p53 transactivation activity by either ASPP1 or ASPP2 on various p53 reporters is shown in (C) and was obtained as following: activity of p53 + ASPP/activity of p53 alone. The coexpression of the transfected ASPP or p53 and endogenous Bax or mdm2 was examined in U2OS cells by double immunofluorescence labeling as indicated (E). The bar graphs show the number of cells coexpressing ASPP and endogenous Bax in 200 U2OS or MCF-7 cells transfected either with vector alone or ASPP plasmids (D).

ASPP do not stimulate the transactivation function of E2F1 or p65 on various reporters as indicated (F and G). The expression levels of various transfected proteins were detected using antibodies V5, DX.5410, DO.1, and C-20 (one for E2F1 and one for p65). The p53-responsive luciferase reporter plasmids were Bax-luc ( – 315– + 56), Mut Bax-luc ( – 76– + 51), PIG-3-luc, p21waf1-luc, PG-luc, cyclin G-luc, and mdm2-luc. The E2F1-responsive luciferase reporter plasmids were 3xwt-luc, cyclin A-luc, and Bmyb-luc. Kb-luc is a reporter for p65. The fold increase in the transactivation function of E2F1 and p65 by ASPP was obtained in a similar way as for p53, namely the activity of E2F1 or p65 + ASPP/ activity of E2F1 or p65 alone. The mean values were derived from at least three independent experiments.
Figure 5. The N Terminus of ASPP Is Required for Their Full Activity and the C-Terminal Half of ASPP2 (i.e., 53BP2) Acts as a Dominant-Negative Mutant of ASPP2

The bar graphs (A–D) show the effect of ASPP1, ASPP2, and their mutants KIAA0771 and Bbp on the apoptotic (A and B) and transactivation function of p53 (C and D) in transfected Saos-2 cells as indicated. Lower panels of (A)–(D) show the expression levels of ASPP1, ASPP2, KIAA0771, and Bbp detected with antibodies V5, DX.5410, and DO.I. 53BP2 can prevent ASPP2 from stimulating the apoptotic (E) and transactivation (F) functions of p53. Increasing amounts of 53BP2 can also prevent ASPP2 from binding to p53 (G). The bar graph represents the mean value of at least two independent experiments.

The bar graphs (A–D) show the effect of ASPP1, ASPP2, and their mutants KIAA0771 and Bbp on the apoptotic (A and B) and transactivation function of p53 (C and D) in transfected Saos-2 cells as indicated. Lower panels of (A)–(D) show the expression levels of ASPP1, ASPP2, KIAA0771, and Bbp detected with antibodies V5, DX.5410, and DO.I. 53BP2 can prevent ASPP2 from stimulating the apoptotic (E) and transactivation (F) functions of p53. Increasing amounts of 53BP2 can also prevent ASPP2 from binding to p53 (G). The bar graph represents the mean value of at least two independent experiments.
known to interact with relA p65, a component of NF-κB transcription factor, the transactivation function of p65 was also not affected by the coexpression of ASPP1 or ASPP2 (Figure 4G). These suggest that ASPP1 and ASPP2 are specific stimulators of p53.

**Full Activities of ASPP Require Intact Protein**
The EST clone KIAA0771 and Bbp are shorter forms of ASPP1 and ASPP2. The products of EST clone KIAA0771 and Bbp lack the N-terminal 143 and 123 amino acids, respectively. The regions missing in KIAA0771 and Bbp exhibit high sequence similarity between ASPP1 and ASPP2. If the N termini of ASPP1 and ASPP2 were important for their function as stimulators of p53, both KIAA0771 and Bbp would suffer from similar defects in their activity. Both KIAA0771 and ASPP1 were coexpressed with p53, and the apoptotic function of p53 was measured by FACS analysis. Comparing the ability of ASPP1 and KIAA0771 to stimulate the apoptotic function of p53, full-length ASPP1 is clearly more active than its mutant KIAA0771. A barely detectable amount of ASPP1 was able to show a clear enhancement of the apoptotic function of p53 despite the fact that the expression level of KIAA0771 was 5- to 10-fold higher than that of ASPP1 (Figure 5A, lower panel). Similar results were obtained with ASPP2 and its shorter form Bbp (Figure 5B). The low level of enhancement in this particular experiment was due to the low-level expression of ASPP2. Moreover, KIAA0771 and Bbp were less active in the stimulation of the transactivation function of p53 when compared to full-length ASPP1 and ASPP2, respectively (Figures 5C and 5D). Together with the observation that both Bbp and KIAA0771 bind p53 to a similar extent as full-length ASPP (data not shown), these results suggest that binding alone is not sufficient for ASPP to stimulate the transactivation function of p53. Therefore, KIAA0771 and Bbp, the two short forms of ASPP, are not able to stimulate the transactivation function of p53 as effectively as full-length ASPP proteins, indicating that full-length ASPP proteins are required for maximal activity.

**53BP2 May Be a Dominant-Negative Mutant of ASPP2**
The enhancement of the transactivation and apoptotic function of p53 by ASPP1 and ASPP2 were contrary to the previous in vitro observation that 53BP2 can inhibit the DNA binding activity of p53 (Iwabuchi et al., 1994). Expression of 53BP2 did not significantly enhance the apoptotic function of p53 (Figure 5E). When 53BP2 was coexpressed with p53 and full-length ASPP2, the percentage of apoptotic cells was reduced from 50% to 30%. Coexpression of 53BP2 also abolished the enhancement of the transactivation function of p53 by ASPP2 (Figure 5F). When the p53/ASPP2 lysates were incubated with increasing amounts of 53BP2, the amount of ASPP2 that coimmunoprecipitated with p53 was reduced (Figure 5G), suggesting that 53BP2 may compete with ASPP2 for interaction with p53. 53BP2 may thus act as a dominant-negative inhibitor of ASPP2.

**ASPP Do Not Stimulate the Activities of Tumor-Derived p53 Mutants 181L and 181C**
Since the interaction of ASPP and p53 specifically enhances the apoptotic function of p53, the reduced apoptotic function of two tumor-derived p53 mutants 181L and 181C may be due to a defect in their ability to cooperate with ASPP. Consistent with this, coexpression of ASPP1 or ASPP2 failed to enhance the apoptotic function of 181L and 181C even though coexpression of ASPP enhanced the apoptotic function of wild-type p53 (Figure 6A). The expression of ASPP1 and ASPP2 also failed to stimulate the transactivation function of 181C and 181L on the Bax promoter (Figure 6B). The failure to stimulate the activities of 181 mutants of p53 was not due to the lack of protein expression of ASPP1 and ASPP2 (Figure 6C).

Residue 181 of p53 is one of the sites involved in protein interaction with 53BP2 in vitro, and thus the ability of 181C and 181L to interact with ASPP1 and ASPP2 in vitro was analyzed by immunoprecipitation. Wild-type p53 specifically immunoprecipitated 35S-methionine-labeled ASPP1 and ASPP2. Under the same conditions, less ASPP1 was coimmunoprecipitated with 181L and 181C. However, the ability of ASPP2 to interact with wild-type and mutant p53 was very similar (Figure 6D). The binding data suggested that the interaction between p53 and ASPP1 was more sensitive to the 181 mutations than that with ASPP2. The failure of ASPP1 and ASPP2 to stimulate the transactivation function of the two p53 mutants on proapoptotic genes may explain why the two p53 mutants were defective in inducing apoptosis, and why mutation of this site of p53 may have been selected in some human tumors.

**Expression of ASPP1 and ASPP2 Is Frequently Downregulated in Human Breast Carcinomas**
All the identified 53BP2 residues that contact p53 have been found to be mutated in human tumors (Gorina and Pavletich, 1996), consistent with the observation that ASPP proteins promote the apoptotic function of p53. Hence, it might be expected that the expression levels of ASPP1 and ASPP2 would be downregulated in human tumors expressing wild-type p53. We used semiquantitative RT-PCR technique to study the expression levels of both ASPP1 and ASPP2 in a panel of paired normal and tumor RNA samples derived from 58 breast cancer patients. Forty of these breast carcinomas express wild-type p53 while the other 18 express mutant p53. Among the 40 carcinoma samples with wild-type p53, reduced expression levels of ASPP1 and ASPP2 were detected in 24 and 9 samples, respectively. Eight of nine tumors with reduced expression of ASPP2 also showed reduced expression of ASPP1. In the 40 cases, the proportion of samples with significantly reduced (greater than 75% reduction in signal) or lack of expression of ASPP1 was higher than for ASPP2 (60% and 22.5%, respectively; Table 1A). Among the 18 pairs of matched normal and mutant p53-expressing tumor samples, three and two tumors, respectively, showed reduced levels of expression of ASPP1 and ASPP2 (16% and 11%, respectively; Table 1B). As the expression levels of ASPP1 and ASPP2 were compared between normal cells and carcinomas derived from the same individuals, we conclude that...
Figure 6. ASPP Failed to Stimulate the Activities of p53 Mutants 181L and 181C, and Expression of ASPP Is Frequently Downregulated in Wild-Type p53-Expressing Human Breast Tumors

ASPP failed to stimulate the activities of two tumor-derived p53 mutants, 181C and 181L (A–C). The p53 mutants 181L and 181C bind to ASPP1 less effectively as compared to wild-type p53 (D). The mRNA expression level of ASPP1 and ASPP2 is downregulated in some of the human breast tumors (E). The effect of ASPP on the apoptotic (A) and transactivation (B) function of wild-type and mutant p53 was compared as indicated. The fold activation of p53 or its mutants by ASPP was obtained as follows: activity of p53 or p53 mutants + ASPP/activity of p53 or p53 mutant alone. In (C), the protein expression of ASPP1, ASPP2, p53, and p53 mutants was detected with antibodies V5, DX.5410, and DO.1, respectively. The in vitro translated ASPP were labeled with 35S-methionine but p53 and its mutants were not labeled. p53 was immunoprecipitated with DO.13 (mouse), and the antibody CM1 was used to detect p53 in (D) (right panel). The mRNA expression of ASPP1 and ASPP2 was detected using RT-PCR, and the PCR products were loaded on a 1% agarose gel and transferred to a nylon membrane. The ASPP1 and ASPP2 cDNAs were used as probes to hybridize with PCR products to verify their identity. The intensity of the hybridized signal was quantitated by means of a Phosphoimager. The normal and tumor RNA samples were labeled as N and T, respectively.
Table 1. Frequent Downregulation of ASPP in Wild-Type but Not Mutant p53-Expressing Human Breast Tumors

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Summary of mRNA expression of ASPP in the normal and tumor-matched breast carcinoma RNAs expressing wild-type or mutant p53 ([A] and [B], respectively). Down arrows indicate the reduced expression of ASPP1 or ASPP2. “+” signs indicate the expression of ASPP without obvious alteration.

there is a selective advantage for tumor cells to lose the expression of ASPP1 and ASPP2. The data are consistent with the notion that the ASPP family of proteins has an important tumor-suppressing role in human breast carcinomas.

Discussion

We present here the identification and functional analysis of a family of proteins, ASPP1 and ASPP2. ASPP proteins specifically enhance the apoptotic function of p53 by stimulating the DNA binding and transactivation function of p53 on the promoters of proapoptotic genes in vivo. The high percentage of wild-type p53-expressing breast tumors with altered expression patterns of ASPP suggests an important tumor suppression function for ASPP family members in the development of human cancer.

ASPP Proteins Specifically Regulate the Apoptotic Function of p53

The effects of ASPP1 and ASPP2 on the apoptotic and transactivation functions of p53 are very specific. Expression of ASPP did not have a significant effect on the number of cells arrested in G1 by p53. Expression of ASPP also failed to enhance the ability of p53 to transactivate the p21waf1 promoter in our assay system. However, Bbp was shown recently to enhance the transactivation function of p53 on the p21waf1 promoter (Iwabuchi et al., 1998). There are two p53 binding sites in the promoter of p21waf1. They resemble two classes of p53 binding sites: high-affinity (mdm2-like) and low-affinity (Bax-like) sites. The transactivation function of the p21waf1 promoter is mainly due to the high-affinity site (Resnick-Silverman et al., 1998), which may explain why we failed to see the stimulatory effect of ASPP on p21waf1. We therefore cannot rule out the possibility that under certain conditions, ASPP proteins may alter the expression level of p21waf1 through the low-affinity site in its promoter. The most dramatic effect of ASPP was, however, clearly seen on the promoters with low-affinity p53 binding sites that are often found in the promoters of proapoptotic genes like Bax and PIG-3. The ability of p53 to bind and transactivate these genes is weak, possibly making them dependent on activators such as ASPP.

Precisely how ASPP proteins alter the promoter specificity of p53 remains to be determined. Our studies suggested that ASPP proteins do not bind DNA and transactivate promoters independently of p53 and its family members (this study and data not shown). Interestingly, ASPP bind to the central region of p53 that is conformationally sensitive. Hence, the p53/ASPP complex may alter the protein conformation of p53 and enhance the binding activity of p53 to the promoters of Bax or PIG-3 but not mdm2 or p21waf1. However, binding to p53 is not sufficient for ASPP to alter the promoter specificity of p53. Intact ASPP proteins are required. Even the N-terminal 143 or 123 residues of ASPP1 and ASPP2 are needed for ASPP to show the maximal stimulation of the transactivation function of p53. This suggests that interaction with other unknown cellular proteins through the N terminus may be required for ASPP to stimulate the activity of p53. All the interacting proteins identified so far bind to the ankyrin repeats and SH3 domain of ASPP located at the C terminus of the protein (Helps et al., 1995; Nakagawa et al., 2000; Naumovski and Cleary, 1996; Yang et al., 1999). In addition, the N-terminal region of ASPP may be required to regulate their cellular distribution. Both ASPP1 and ASPP2 are predominantly cytoplasmic, but small amounts of ASPP1 and ASPP2 are detected in the nucleus of transfected cells when
examined by confocal microscopy. Coexpression of ASPP proteins and endogenous Bax correlated with nuclear expression of ASPP (Figure 4). However, the nuclear localization of ASPP is negatively regulated by its N terminus. The removal of the N-terminal half of ASPP2 allowed the remaining C-terminal part of ASPP2 to be expressed in the nucleus exclusively. Further studies showed that the ankyrin repeats of ASPP2 (residues 918–1017) contain a nuclear localization signal (Sachdev et al., 1998). Therefore, it is likely that modifications at the N-terminal half of ASPP proteins could regulate the cellular distribution and activities of ASPP, which could ultimately influence the transactivation and apoptotic function of p53.

ASPP: A Family of Proteins Important for the Development of Human Cancer

The failure of ASPP to enhance the apoptotic function of the two tumor-derived p53 mutants may explain why these two mutants were selected in the development of human cancer. Although residue 181 is not a hot spot for p53 mutation in human cancer, among the 28 BRCA tumors examined, two contained the 181C mutation (Crook et al., 1998). This frequency is much higher than that seen in sporadic cancers. In the database of p53 mutations in human tumors, the mutation rates of other p53 contact sites for ASPP are similar. It is therefore possible that the inability to bind ASPP may characterize a new group of p53 mutants that are selected in the development of human cancer. Future studies will demonstrate whether all the contact sites of ASPP on p53 are equally important in regulating the apoptotic function of p53.

Most p53 mutations occur in high-grade (grade III) breast carcinomas. All the carcinomas studied here are well-differentiated grade I and grade II tumors with low mitotic index and express wild-type p53, arguing for an early p53-dependent tumor suppressor function for ASPP. The frequent downregulation of ASPP expression agrees with one recent report showing a 5-fold reduction of ASPP2 expression in breast carcinoma cells compared to that seen in normal tissues from the same patient (Sgroi et al., 1999). Alteration of the ASPP2 locus at chromosome 1q42 was observed in 28% of breast cancer cases (Gendler et al., 1990). The expression level of ASPP2 has also been linked to cellular sensitivity to chemotherapy drugs (Mori et al., 2000). Among the tumor cell lines studied, the most sensitive cell lines expressed ASPP2 at the highest level.

Due to the relatively limited number of tumor samples, the precise frequency of altered ASPP1/2 expression is not clear. Nevertheless, some features of the expression patterns of ASPP1 and ASPP2 emerged in the 58 pairs of normal and tumor RNAs tested (see Tables 1A and 1B). The number of tumors having reduced expression of ASPP proteins that are wild-type for p53 is much higher than that with mutant p53. Downregulation of ASPP1 is much more frequent than that of ASPP2. In a small number of tumors, the expression levels of ASPP1 or ASPP2 are higher than their normal controls. Whether these tumors express a mutated version of ASPP or inhibitor of ASPP is currently under investigation.

Upstream regulators of ASPP have not yet been identified. From all the experimental assays performed so far, we have not been able to distinguish significant functional differences between ASPP1 and ASPP2. However, the p53 mutants 181C and 181L only showed reduced affinity to ASPP1 but not ASPP2. More p53 is in complex with ASPP2 but not with ASPP1, in response to UV-irradiated MCF-7 cells. These results suggest a functional difference between ASPP1 and ASPP2. The expression level of ASPP2 is subject to stress signals (Lopez et al., 2000). Therefore, it is possible that there is a functional autoregulation loop between p53 and ASPP2. Whether ASPP1 is subject to a similar regulation is currently under investigation. Since the expression level of ASPP2 can be regulated by p53, it is possible that genetic change in ASPP2 genes may not occur frequently. In agreement with this view, a recent study has reported that the genomic alteration of ASPP2 is a rather rare event in the tumor cell lines examined (Mori et al., 2000). Knowing that the apoptotic function of p53 is specifically and highly regulated by ASPP family members in vivo, strategies to stimulate the activities of ASPP may allow us to treat some of the human tumors effectively, by reactivating the apoptotic function of wild-type p53 in these tumor cells.

Experimental Procedures

Identification and Cloning of ASPP1

Using the Bbp sequence to carry out a Blast search, an EST clone named KIAA0771 was identified as the homolog of S3BP2. The EST clone KIAA0771 was subcloned into the pcDNA3 vector in-frame with the epitope of the antibody V5 tagged to its C terminus. A primer to the N terminus of KIAA0771 was synthesized (5’-CCGTCGGA TCCTTCAGTCTGCACCGGC-3’) and used to carry out PCR with a RACE cDNA library from human heart according to the manufacturer (Clontech). The PCR product was cloned and sequenced to confirm the overlapping sequence of KIAA0771. It also allowed the identification of A6265004 as the overlapping EST clone of KIAA0771. Using the MfeI site located at the 5’ end of KIAA0771 and the 3’ end overlapping region of A6265004, we joined the two sequences together to generate the longer cDNA sequence for ASPP1.

Cell Culture, Antibodies, and Plasmids

Cells were grown in DMEM plus 10% FCS. Antibodies DO-1, DO-13 (mouse monoclonal), and CM-1 (rabbit) are specific to p53. The V5 protein was used. This region of ASPP1 differs substantially in sequence from ASPP2. After immunization, the serum was tested for the inability to bind ASPP may characterize a new group of p53 mutants that are selected in the development of human cancer. Future studies will demonstrate whether all the contact sites of ASPP on p53 are equally important in regulating the apoptotic function of p53.

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ASPP Stimulates the Apoptotic Function of p53

793

(relative to the initial ATG): −74 to 923 and −253 to 839 for ASPP1 and ASPP2, respectively. The amplified segments were purified and ligated into the pcDNA3.1/V5-His TOPO vector (Invitrogen) to produce the plasmids containing antisense DNA of ASPP1 and ASPP2.

Chromatin Immunoprecipitation Assays

The assays were carried out as described (Szak et al., 2001). Briefly, culture medium was removed from cell plates and replaced with a 1% formaldehyde solution in PBS for 10 min. A 1 mM glycine solution in PBS was added for a final concentration of 0.125 M for 15 min. Cells were washed with PBS and scraped in 600 μl lucerase reporter lysate (LRL) buffer (Promega). Cell suspensions were then sonicated with a Heat Systems sonicator (six bursts of 15 s each) on ice and then cleared by centrifugation at 20,000 g for 10 min. Super- natants were precleared with 10 μl protein A Sepharose beads for 1 hr at 4°C and immunoprecipitated with 10 μl of protein. Beads were prebound to the relevant antibody overnight at 4°C. Beads were washed twice with LRL buffer plus 0.1% Nonidet P-40, then twice with LRL buffer with 150 mM NaCl and then with 500 mM NaCl. They were finally resuspended in 200 μl crosslink reversal buffer (12.5 mM Tris, 10% [i-mercaptoethanol], and 4% SDS [pH 6.8]) and incubated for 30 min at 95°C. The suspensions were later extracted with phenol/chloroform and the DNA was precipitated and washed with ethanol, dried, and dissolved in 20 μl TE. PCR for the Bax and p21waf1 promoter regions was performed under the same conditions (40 cycles of 30 min melting at 95°C, 1 min annealing at 63°C, and 1 min extension at 72°C) with the primers listed below. Bax: forward, 5'-CCCGGAAATTCGACGTAAGCAGCC-3'; backward, 5'-AGCATGGTCCTCGGAGGAGGTATAGTG-3'; p21waf1: forward, 5'-GGGAGGAAGGGAGGTGAGGGACAGGA-3'; backward, 5'-GGCGCTTGCACGAGGTCTCGTTG-3'.

DNA Transfection and Transactivation Assays

For the transfection assay, 5 × 104 Saos-2 cells were plated 24 hr prior to transfection in 6 cm dishes. Various combinations of plasmid DNA were transfected as indicated. All reporter assays contained 1 μg of reporter plasmid. Fifty to seventy-five nanograms of wild-type or mutant p53, 181C, or p53181L, 100 ng of plasmid expressing E2F1, 100 ng of plasmid expressing p65, 5 μg of either KIAA0771 or ASPP1 dishes were UV irradiated with 10 J/m². The presence of equivalent amounts of amplification products was verified for each sample by amplification of the plasmids containing antisense DNA of ASPP1 and ASPP2.

Flow Cytometry

For FACS analysis, 104 Saos-2 cells were plated 24–48 hr prior to transfection in 10 cm plates. The transfections consisted of 2 μg of CD20, 1 μg of either p53 or p53(181C) or p53(181L), 10 μg of ASPP1 and ASPP2, 2 μg of Bax, 4–8 μg of KIAA0771, 8–12 μg of Bbp, 10 μg of S3BP2, 2 μg of p21waf1, 5 μg of Bcl-2, and 15 μg of antisense ASPP1 and ASPP2 plasmid as indicated. The G1 block, 0.25 μg of p53 was used. Thirty-six hours after transfection, both attached and floating cells were harvested using 4 mM EDTA/PBS and stained with FITC-conjugated anti-CD20 antibody. The cells were then fixed and stained with propidium iodide. The DNA content of all the cells expressing CD20 was analyzed using a flow cytometer (Becton Dickinson) as described (Hsieh et al., 1997).

Protein Biochemistry

For Western blotting, cells were lysed in either NP40 lysis buffer (1% Nonidet P40, 50 mM Tris [pH 8.0], 150 mM NaCl, and 1 mM EDTA) or LRL buffer. Between 15 and 100 μg of extract was mixed with 5× sample buffer and loaded on SDS-PAGE gels. The gels were wet transferred onto Protran nitrocellulose membrane and the resulting blots were blocked and incubated with primary antibody and subsequently incubated with the appropriate secondary HRP-conjugated antibody (Dako). After washing, the blot was exposed to hyperfilm following the use of ECL substrate solution (Amersham Life Science).

For immunoprecipitation, cells were lysed and precleared with protein G beads for 1 hr at 4°C. One thousand micrograms of the extract was incubated with antibody prebound to protein G beads for 4 hr at 4°C. The beads were washed twice with NP40 lysis buffer and twice with NET buffer (50 mM Tris [pH 8.0], 150 mM NaCl, and 1 mM EDTA). The IP beads were mixed with 5× sample buffer and loaded onto an SDS-PAGE gel.

In Vitro Translation and In Vitro Immunoprecipitation

ASPP1, ASPP2, and p53 were in vitro translated and labeled with [-35S]-methionine using the TNT T7 quick-coupled transcription/translation system (Promega). Five to ten micrograms of p53 lysate (unlabeled) was incubated with 15 μl samples of the lysates containing the appropriate in vitro translated protein products as indicated. For the experiments shown in Figure 5G, 15, 30, and 45 μl of in vitro translated lysates of S3BP2 were added in addition to p53 and ASPP2. The proteins were incubated together at 30°C for 1 hr. Two hundred microliters of PBS was then added to the mixture of proteins and incubated at 4°C for 1 hr on a rotating wheel. The anti-p53 antibody DO-13 immobilized on protein G beads was added to the binding reactions and incubated on a rotating wheel at 4°C for 6–16 hr. The beads were then washed with PBS or NET. The bound proteins were released in SDS gel sample buffer and analyzed by 10% SDS-polyacrylamide gel electrophoresis (PAGE). Rabbit anti-p53 antibody CM1 was used to detect the presence of p53.

Cell Staining

Cells were grown in 30 mm dishes and fixed with 4% paraformalde- hyde for 15 min and then washed with PBS. 0.2% Triton X-100 in PBS was used to permeabilize the cells for 2 min. Cells were incubated with primary antibody for 3 hr and then with TRITC- or FITC-conjugated either anti-rabbit or anti-mouse antibodies for 1 hr. The antibodies used for detection were: ASPP1, VS antibody; ASPP2, DX.5410; p53, DO1; Bax, N-20; mdm2, C-18 and N-20.

UV Irradiation

Cells were plated at the required density 24 hr before irradiation. Prior to irradiation, the media from the dishes was removed and the dishes were UV irradiated with 10 J/m². The medium was replaced and the cells were returned to the incubator for the required length of time as indicated.

RNA Expression Level Determination by RT-PCR

Breast carcinomas and matched normal breast tissues were har- vested at operation, collected into liquid nitrogen, and stored until extraction of nucleic acids. The presence of at least 50% of tumor cells in each cancer was determined by histological analysis prior to isolation of RNA. Cancers containing a lower proportion of tumor cells were not analyzed further. RNA was isolated using phenol/ guanidinium (RNXazol B) according to the manufacturer’s instruc- tions (Biogeness). The presence of equivalent amounts of amplifi- able cDNA was verified for each sample by amplification of β-actin under linear cycling conditions. p53 status was analyzed by SSCP/ sequencing analysis. The expression of ASPP was determined using 3 μg of total RNA, which was subjected to reverse transcription using the Stratagene system. PCR was then performed for 28 cycles using primers specific to ASPP1 and ASPP2. In preliminary experi- ments, PCR was optimized to ensure that cycle numbers were in the linear portions of the amplification curve for each reaction. Amplified products were resolved on 1.2% agarose gels, transferred to nylon membrane, and probed with radiolabeled cDNAs specific for ASPP1 and ASPP2. Tumors showing a greater than 75% reduction in expression relative to normal tissue as assessed by phospho- imaging were scored as having reduced expression.

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References


Accession Numbers

The GenBank accession numbers for the ASPP1 and ASPP2 sequences reported in this paper are AJ318887 and AJ318888, respectively.