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The ASPP proteins complex and cooperate with p300 to modulate the transcriptional activity of p53

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ABSTRACT

Understanding how p53 is able to specifically respond to particular stress signals and regulate many different signalling pathways remains a challenge. Several studies have demonstrated that p53's interactions with different protein partners are essential for it to be able to coordinate specific responses. In particular, the apoptotic pathway is regulated by p53 in cooperation with the Apoptosis Stimulating Proteins of p53 (ASPP) proteins. In this study, we showed that the ASPP proteins are able to bind and cooperate with p300, a well defined co-factor of p53, to selectively regulate p53's transcriptional activity on promoters such as p53-inducible gene 3 but not on p21waf1. This is the first demonstration that the ASPPs can function together with p300 in regulating the transcriptional activity of p53.

Structured summary of protein interactions:
ASPP2 physically interacts with **p300** and **p53** by anti bait coimmunoprecipitation (View interaction)
iASPP physically interacts with **p300** by anti bait coimmunoprecipitation (View interaction)
iASPP physically interacts with **p300** and **p53** by anti bait coimmunoprecipitation (View interaction)
ASPP2 physically interacts with **p300** by anti bait coimmunoprecipitation (View interaction)
ASPP1 physically interacts with **p300** by anti bait coimmunoprecipitation (View interaction)
ASPP1 physically interacts with **p300** and **p53** by anti bait coimmunoprecipitation (View interaction)

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1. Introduction

First discovered 30 years ago, p53 is an evolutionarily conserved tumour suppressor protein that is absent or mutated in 50% of human cancers [1,2]. Primarily acting as a transcription factor, p53 has a wide range of different functions, and regulates many diverse signalling pathways in mammalian cells [3]. Indeed, the majority of the p53 mutations that are linked to cancer affect its DNA binding domain (80% of all mutations; www.p53.iarc.fr). A recent study, based on a combination of ChIP and paired-end diTag (PET) sequencing, predicted that p53 is able to bind to at least 542 different loci, highlighting the vast number of genes that it regulates [4]. One of the most challenging unresolved questions is, therefore, how does p53 decide to activate or repress one signalling pathway more than another? Current evidence suggests that p53 integrates stress signals via post-translational modifications, adapting its response by coordinating with different protein partners, thus enabling the p53-mediated stress response to be tissue specific [3,5].

Recent studies have emphasised the role of p53's co-factor proteins in coordinating its response to stress signals. One of the first p53 co-factors identified was p300, an acetyl transferase that generally enhances p53's transcriptional activity. More recently, the Apoptosis Stimulating Protein of p53 (ASPP) family of proteins was found to represent another type of co-factor that can selectively regulate p53's activity. The ASPP family is composed of three members: ASPP1 and ASPP2, which are p53 activators; and iASPP, one of p53's most conserved inhibitors [6–8]. The p53-dependent apoptotic response has been demonstrated to be modulated by the ASPPs, which have also been shown to be involved in tumourigenesis [9,10].

Acetylation of p53 has been reported to modulate its transcriptional activity [11,12]. In particular, p300 was reported to influence the stability of p53 once it is bound to DNA [13]. However, p300 does not exhibit any known promoter selectivity. Since the ASPP

Abbreviations: ASPP, Apoptosis Stimulating Protein of p53; Bax, Bcl-2-associated X protein; mdm2, murine double minute 2; PET, paired-end diTag; PIG3, p53-inducible gene 3

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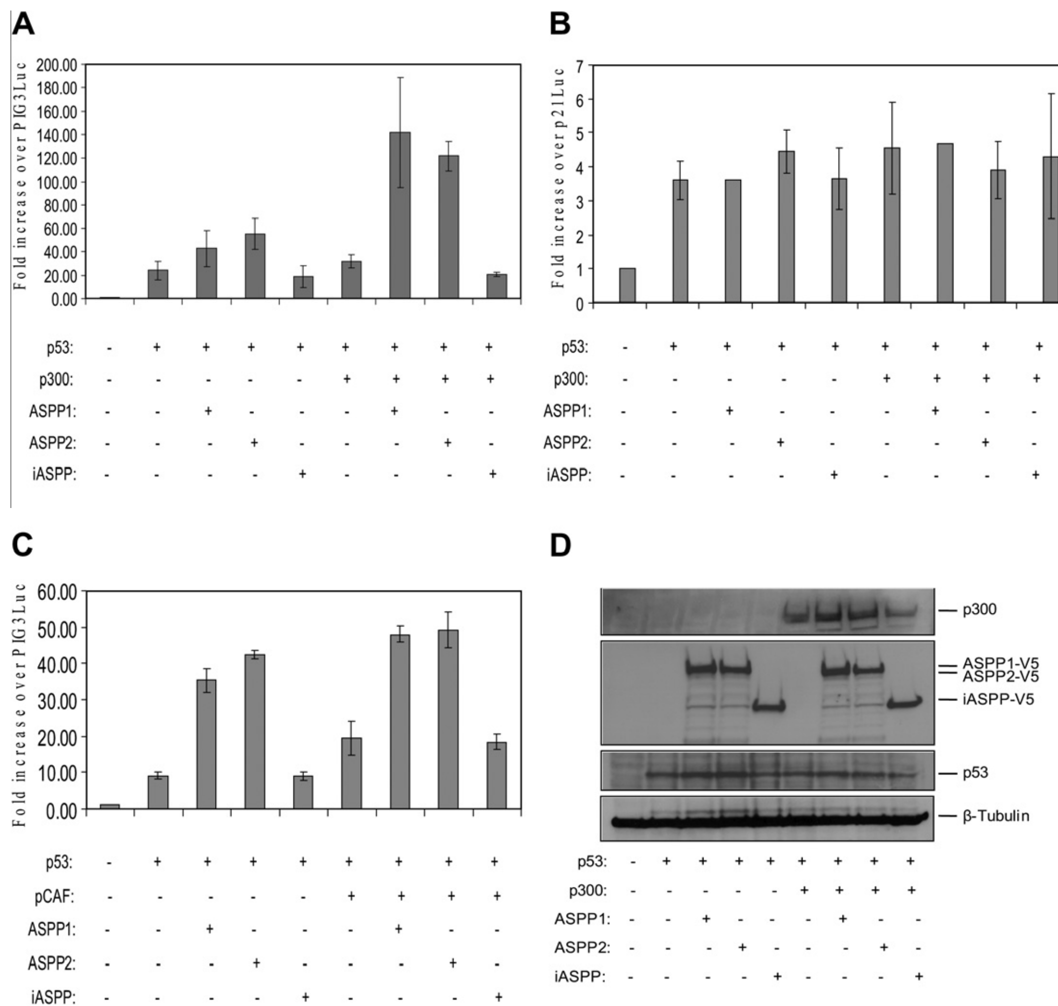

Fig. 1. The ASPP proteins synergise with p53 and p300 to activate expression of the PIG3 reporter gene in SaOS-2 cells. All transfections were done in duplicate. Cells were harvested 48 h after transfection for luciferase assay with the PIG3Luc 17mer reporter (A and C) or with the p21Luc reporter (B). Reporter values (control) were set to 1 to allow estimation of the effects of p300 and the ASPP proteins. (A) Representative of the average of five independent experiments with standard deviation indicated. (B and C) Represent the average of three independent experiments with standard deviation indicated. (D) Analysis of total cell lysate after luciferase assay by Western blot; equal amounts of protein were loaded onto gels and blotted for p300 (RW128), V5 (for the detection of the ASPP proteins), p53 (DO-1) and β-tubulin (loading control).

proteins are able to selectively affect the transcriptional activity of p53 on promoters like Bcl-2-associated X protein (Bax) and p53-inducible gene 3 (PIG3), but not on p21waf1 and murine double minute 2 (mdm2), we investigate here whether the ASPP family can cooperate with p300 to selectively regulate the activity of p53.

2. Materials and methods

2.1. Cell lines, antibodies, plasmids and reagents

H1299, SaOS-2 and U2OS cells were cultured in DMEM (Lonza) supplemented with 2 mM L-glutamine (Gibco), 200 units/ml penicillin/streptomycin (Gibco) and 10% (v/v) foetal calf serum, in flasks maintained in an incubator at 37 °C in the presence of 10% CO_2. Antibodies were purchased for β-tubulin (ab6046, Abcam), p300 (RW128, Upstate), V5 (MCA1360, Serotec) and Ku80 (ab3107, Abcam). Antibodies for p53 (DO-1), ASPP1 (LX54.2 and N8), ASPP2 (LX54.10 and BP77) and iASPP (LX49.3 and N1) were from ascites. Wild type pcDNA3.1(-)/p53, pcDNA3.1/ASPP1, pcDNA3.1/ASPP2 and pcDNA3.1/iASPP were from Samuels-Lev et al. pcDNA3.1/p300 and pcDNA3.1/pCAF were gifts from Prof. W. Gu. The PIG3Luc 17mer reporter gene and the p21Luc reporter gene were gifts from Prof. M. Dobbelstein and Prof. B. Vogelstein, respectively. The Renilla-TK-Luciferase (cat #E2241) and luciferase assay kit (cat #E1910) were purchased from Promega, UK.

2.2. Gene reporter assay

6×10^5 SaOS-2 cells were plated in 6 cm dishes and transfected with 50 ng pcDNA3.1/p53, 2 μg pcDNA3.1/p300, 2 μg pcDNA3.1/pCAF, 4 μg pcDNA3.1/ASPP1, 4 μg pcDNA3.1/ASPP2 and 250 ng pcDNA3.1/iASPP, as indicated using the $CaCl_2$ method [23]. All samples were co-transfected with 30 ng of Renilla-TK-Luciferase, and 1 μg of PIG3Luc 17mer reporter or 1 μg of p21Luc reporter. All transfections were done in duplicate. Cells were harvested 48 h after transfection to perform a luciferase assay following the manufacturer's instructions (Promega).

2.3. Immunoblotting and immunoprecipitation

For immunoblotting, cells were washed three times with 1× cold PBS and lysed with NET/NP-40 (150 mM NaCl, 50 mM Tris–

HCl, pH 8.0, 1 mM EDTA, 1% (v/v) NP-40 supplemented with 1× protease inhibitor (Complete, Roche). The cells were scraped with a sterile disposable cell scraper (Greiner), transferred to an eppendorf tube, centrifuged at 14 000 rpm, at 4 °C for 30 min and blotted as described in Gillotin et al. (2010). For immunoprecipitation (IP), 1000 μg of cell lysate was precleared with 30 μl of protein G Sepharose (50% slurry in PBS) for 30–60 min at 4 °C on an eppendorf rotating wheel. The lysate was centrifuged at 2500 rpm for 2 min and the supernatant removed to a fresh tube. One to four microlitres of antibody (ASPP1, N8; ASPP2, BP77; iASPP, N1) and 30 μl protein G Sepharose (50% slurry in PBS) was then added to the pre-cleared lysate. The mixture was left on an eppendorf rotating wheel overnight at 4 °C. Immunocomplexes were collected by centrifugation at 2500 rpm for 3 min and the supernatant discarded. Beads were washed with three successive changes of lysis buffer. After removing as much residual supernatant as possible, IP beads were mixed with 30 μl of 5× sample buffer and heated at 95 °C for 5 min. The beads were then centrifuged at 14 000 rpm for 15 s and all or part of the sample loaded onto an SDS–polyacrylamide gel. The separated proteins were then subjected to Western analysis.

3. Results

To understand whether p300 can cooperate with the ASPP proteins in gene transcription, we performed reporter gene assays using the pro-apoptotic PIG3-Luciferase reporter which contains the PIG3 promoter, a gene identified in p53 induced apoptosis in SaOS-2 cells, a cell line in which p53 is deleted (Fig. 1A). As previously published, co-expressing p53 with either ASPP1 or ASPP2 enhanced the PIG-3 reporter activity over transfection of p53 alone [6]. Under the conditions used, iASPP had a moderate effect on the inhibition of p53, whereas p300 alone had little effect on the transcriptional activity of p53. When ASPP1 or ASPP2 were co-transfected with p53 and p300 simultaneously, we observed a synergistic effect between the three proteins in activating the PIG3 reporter gene over the co-transfection of ASPP1 or ASPP2 with p53. In contrast, however, co-transfection of iASPP and p300 maintained the modest inhibitory effect of iASPP and did not increase it.

To investigate whether the ASPP proteins could maintain their ability to selectively enhance p53's transcriptional activity in the presence of p300, we tested the effect of the ASPP proteins and p300 on the p21waf1-Luciferase reporter which contains the p21 promoter, a cyclin dependent kinase inhibitor (Fig. 1B). In agreement with our current working model [6], we were unable to observe any synergy between ASPP1, ASPP2, p300 and p53 in transactivating the p21 reporter gene. Next, we tested whether the synergistic effect between p300 and ASPP1 or ASPP2 could be generalised to other acetyl-transferases. The p300-related acetyltransferase pCAF was used in a similar assay, using the PIG3-Luciferase reporter gene (Fig. 1C). Unlike p300, ASPP1 and ASPP2 were not able to cooperate with pCAF, suggesting a specific level of regulation between the ASPP proteins and p300. The observed differences in the activity of the reporter genes were not due to differences in the expression levels of p53 (confirmed by Western analysis) (Fig. 1D). All these results suggest that ASPP1 and ASPP2 can cooperate with p300 to enhance the transcriptional activity of p53, and that this synergy is promoter specific.

The binding between p53 and p300 is well established, and p300 binding sites have been reported at both the N- and C-termini of p53 [14]. To investigate how the ASPP proteins and p300 may cooperate, we investigated whether the ASPP proteins reside in the same protein complex as p300 and p53. We used U2OS cells expressing wild type p53, and compared them to H1299 cells which do not express p53, untreated or treated with doxorubicin to stimulate p53. ASPP1, ASPP2 and iASPP were immunoprecipitated (Fig. 2) and p300 and p53 visualised by Western blot. The

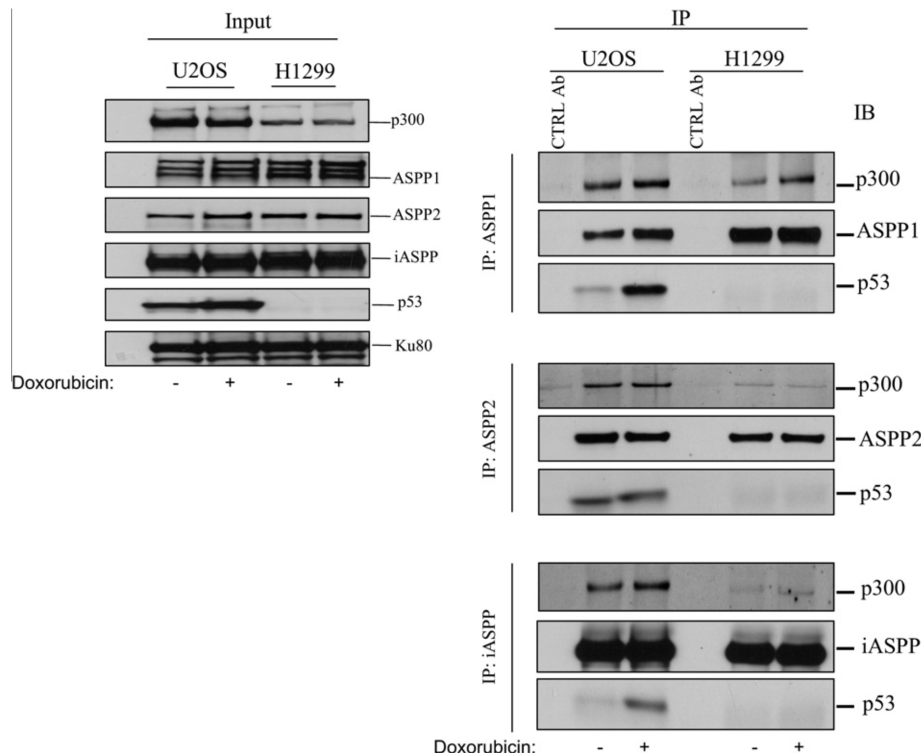

Fig. 2. Co-immunoprecipitation of the ASPP proteins. H1299 and U2OS cells were left untreated or treated with doxorubicin (2 μM) overnight. Expression levels of p300 (RW128), ASPP1 (LX54.2), ASPP2 (LX54.10), iASPP (LX49.3), p53 (DO-1) and Ku80 (loading control) (left panel) were determined by Western blot as described. Immunocomplexes were probed for p300 (RW128), ASPP1 (LX54.2) and p53 (DO-1) (top right panel), for p300 (RW128), ASPP2 (LX54.10) and p53 (DO-1) (middle right panel) and for p300 (RW128), iASPP (LX49.3) and p53 (DO-1) (bottom right panel).

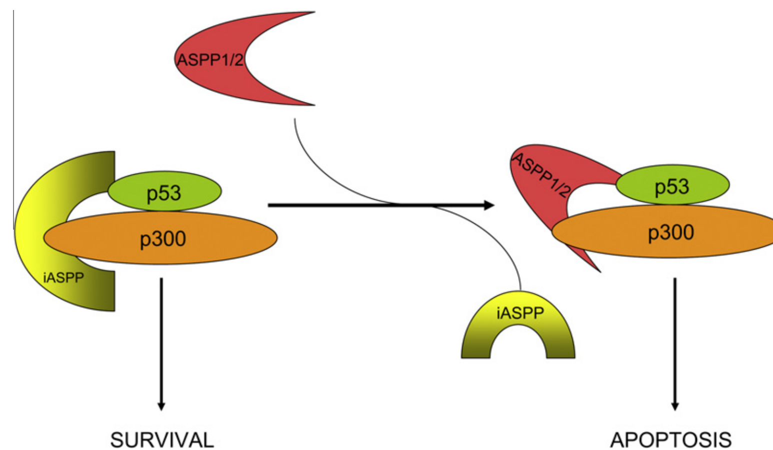

Fig. 3. Model for the cooperation between p53, the ASPP proteins and p300. Interaction of iASPP with the complex p53/p300 would favour cell survival, whereas interaction with ASPP1 or ASPP2 would favour cell death.

three ASPP proteins were each able to pull down p300 and p53 together in U2OS cells, with or without DNA damage. Importantly, ASPP proteins were also found to complex with p300 in H1299 cells that are null for p53. These results suggest that the ASPPs may cooperate with p300 to regulate the transcriptional activity of p53, by forming an ASPP/p300/p53 complex in cells. However, p53 is not absolutely required for ASPP to bind p300.

4. Discussion

Taken together, the results shown in this study suggest that ASPP1 and ASPP2 are able to bind and cooperate synergistically with p300 to enhance p53's transcriptional activity. Interestingly, this synergistic effect of ASPP1 or ASPP2/p300 on the transcriptional activity of p53 maintains the ASPPs' promoter selectivity. Under the same conditions, the interaction between p300 and iASPP had less of an effect on iASPP's inhibitory activity on p53 transcription.

While ASPP2 has been shown to localise on promoters such as Bax in vivo [6,8], a biochemical study using purified recombinant proteins containing the DNA binding domain of p53 and the C-terminus of ASPP2 showed that the co-localisation of p53 and ASPP2 on DNA is mutually exclusive [15]. Precisely how the ASPP proteins are involved in p53 target promoter selectivity remains unknown. However, it is known that p53 requires the two other p53 family members, p63 and p73, to induce apoptosis [16]. In addition, ASPP1 and ASPP2 are common activators of the three p53 family members [17], binding directly to their DNA binding domains [15,18,19]. p300's acetylation activity has previously been shown to be more active on p53 when p53 is bound to DNA [13,20]. The majority of the known apoptosis-related target genes of p53 have weak p53 binding sites, in contrast to promoters such as p21 and MDM2, which bind p53 with high affinity [21]. It is interesting to note that all of the promoters that the ASPP family is able to enhance are p53 target promoters, that have weak p53 affinity binding sites. Thus, it is possible that ASPP1 and ASPP2 may help to recruit p300 to such weak affinity sites, and consequently increase the activation of p53 in collaboration with p63 and p73. In contrast, iASPP preferentially binds to p53's proline rich region while ASPP1 and ASPP2 mostly bind to p53's DNA binding domain [22]. Moreover, p53's proline rich region is required for p300 to acetylate and stabilize p53 when bound to DNA [13], which suggests that iASPP and p300 may compete against each other to influence p53's activity when it is bound to chromatin. This supports the data presented here, and may explain why iASPP's inhibition of p53 does not seem to be modified by p300, despite the fact that they are able to bind to each other in the presence or absence of p53 (Fig. 3). Future studies are required to illustrate whether p63 and/or p73 are required for the ASPP proteins to modulate p53's transcriptional activity. In particular, an investigation of the interactions between p63 and p73 with iASPP may prove to be relevant to the design of new compounds for cancer therapy, which act by alleviating the inhibition of apoptosis.

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