

Inhibitory Member of the Apoptosis-stimulating Proteins of the p53 Family (iASPP) Interacts with Protein Phosphatase 1 via a Noncanonical Binding Motif*

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Background: Regulatory subunits confer substrate specificity to protein phosphatases.

Results: iASPP, unlike ASPP2, interacts with protein phosphatase 1 (PP1) via a noncanonical binding motif within its SH3 domain.

Conclusion: iASPP is a new PP1-binding partner.

Significance: The identification of all ASPP family members as PP1-binding partners extends our understanding of how PP1 activity may be regulated *in vivo*.

Although kinase mutations have been identified in various human diseases, much less is known about protein phosphatases. Here, we show that all apoptosis-stimulating proteins of p53 (ASPP) family members can bind protein phosphatase 1 (PP1) via two distinct interacting motifs. ASPP2 interacts with PP1 through an RVXF PP1 binding motif, whereas the inhibitory member of the ASPP family (iASPP) interacts with PP1 via a noncanonical motif (RNYF) that is located within its Src homology 3 domain (SH3). Phe-815 is crucial in mediating iASPP/PP1 interaction, and iASPP(F815A) fails to inhibit the transcriptional and apoptotic function of p53. This study identifies iASPP as a new binding partner of PP1, interacting through a noncanonical PP1 binding motif.

More than 400 Ser/Thr kinases have been identified in the human genome, many of which are implicated in various human diseases (1, 2). In contrast, fewer genes are known that encode Ser/Thr phosphatase catalytic subunits. For example, the two major Ser/Thr phosphatases, protein phosphatase 1 and 2A (PP1 and PP2A),⁴ are encoded by only five catalytic subunit genes in mammals (3). The majority of Ser/Thr protein phosphatases, including PP1, do not recognize consensus

motifs within their substrates. Instead, the substrate selectivity of PP1 is largely conferred by its specific regulatory subunits, which are extremely varied. Thus, their association with PP1 catalytic subunits may result in >100 different PP1 enzymes, providing the needed diversity to counterbalance the Ser/Thr kinases. The identification and characterization of PP1 regulatory subunits are, therefore, essential to our understanding of Ser/Thr phosphorylation and its importance in human physiology.

Many PP1 regulatory subunits, including ASPP2, interact with PP1 via a common RVXF motif (4, 5). A shorter fragment of ASPP2, corresponding to the last 529 amino acids in its C terminus, was found to interact with PP1 in a yeast two-hybrid screen using the catalytic subunit of PP1_{γ1} as bait (6). The interaction of the ASPP2 C-terminal fragment with PP1 was further characterized and was shown to be more stable at high salt concentrations than its interaction with p53. Because both PP1 and p53 compete for interaction with ASPP2 at its C terminus, their binding to ASPP2 is mutually exclusive (7). Interestingly, a 32-residue peptide of ASPP2 that contains its RVXF motif can indeed interact with PP1. Furthermore, the ASPP2 peptide is able to disrupt the binding of PP1 to its regulatory subunits M₁₁₀ and G_L (4). Functionally, an RVXF-containing ASPP2 fragment was able to modulate the activity of PP1 by eliminating its effect on glycogen phosphorylase, but not on myosin-P light chains (7). Together, these results suggest that ASPP2 may be a regulatory subunit for PP1 *in vitro*. However, it remains unknown whether ASPP2 can interact with PP1 as a full-length protein *in vivo*.

ASPP2 belongs to the ASPP family of proteins (ankyrin repeat-, SH3 domain- and proline-rich region-containing proteins) which consists of three members: ASPP1, ASPP2, and iASPP (8). They exhibit a high degree of homology in their C termini, which contain their signature sequences of ankyrin repeats, an SH3 domain, and a proline-rich region. They have mainly been studied in the context of their ability to interact with, and to regulate, the apoptotic function of p53 and its fam-

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⁴ The abbreviations used are: PP1 and PP2A, protein phosphatase 1 and 2A, respectively; ASPP, apoptosis-stimulating protein of p53; iASPP, inhibitory ASPP; SH3, Src homology 3.

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ily members p63 and p73. ASPP1 and ASPP2 are transcriptional targets of E2F1 (9–11). When expressed, ASPP1 and ASPP2 promote, whereas iASPP inhibits, the activities of p53 and its siblings (12, 13). However, over the past few years, an increasing number of ASPP-binding partners such as Par3 have been identified, suggesting that the ASPP family of proteins has functions in addition to regulating the p53 family (8, 14, 15).

In this study, we confirm and demonstrate that all three ASPP family members can interact with PP1 *in vivo*. We also confirm that the RVXF motif of ASPP2 is responsible for its interaction with PP1. Importantly, we also observed strong binding between iASPP and PP1, even though iASPP does not contain a canonical RVXF motif. Detailed analysis revealed that iASPP contains a novel PP1 binding motif within its SH3 domain and that Phe-815 of iASPP is critical in mediating the iASPP/PP1 interaction and important for iASPP inhibition of p53 function.

EXPERIMENTAL PROCEDURES

Cell Culture, Antibodies, and Plasmids—The two osteosarcoma cell lines, Saos-2 and U2OS (p53-null and p53 wild type, respectively) were grown as described before (12). The following antibodies were used: anti-p53 (DO-1), anti-V5 (Invitrogen, Abcam), anti-PP1 α and anti-PP1 γ (Santa Cruz Biotechnology, sc-6108 and sc-6104). Rabbit polyclonal anti-ASPP1 (N8) and anti-ASPP2 (BP77) were raised against GST-hASPP1 (395–575) and GST-53BP2, respectively, as described previously (13). Rabbit polyclonal (B18P) and mouse monoclonal anti-iASPP (LX49.3) were described previously (16). The mouse monoclonal LX54.1 antibody was raised against His-hASPP1 (1–308) and can detect both ASPP2 and ASPP1. Its ability to cross-react with both proteins was established using *in vitro* translated ASPP1 and ASPP2, respectively (data not shown).

Full-length ASPP1, ASPP2, and iASPP were generated by PCR-directed cloning into pcDNA3.1/V5-His-TOPO (Invitrogen) unless otherwise stated. The following primers were used for the mutagenesis of the RVXF motif of ASPP2 and obtained: ASPP2(V922A, F924A), 5'-GCTCATGGAATGAGGGCGAAAGCCAACCCCTTGCTTTAC-3' and 5'-GTAAAGCAAGGGGTTGGCTTTCGCCCTCATTCCATGAGC-3'. PP1 α (101–334)-V5 and PP1 α (1–200)-V5 were generated by PCR from a PP1 α expression construct using the following pairs of primers: 5'-ATGTCCGACAGCGAG-AAGCTCAACCTG-3'/5'-CAGGCCCTGGTCAGGCACATCTGTGG-3' and 5'-ATGTTGGAGACCATCTGCCTGTGCTGG-3'/5'-TTTCTTGGCTTGGCGGAATTGCGG-3'. They were then cloned into pcDNA3.1/V5-His-TOPO according to the manufacturer's protocol. iASPP(1–478), iASPP(479–828), iASPP(479–728), iASPP(649–728), iASPP(649–828), and iASPP(729–828) were generated as described before (16). iASPP(479–814), iASPP(479–782), iASPP(F815A), iASPP(F818A), iASPP(V821A), iASPP(F818A, V821A), iASPP(479–828/N813A), iASPP(479–782/Y814A) and iASPP(479–782/N813A, Y814A) were cloned into pcDNA3.1/V5-His-TOPO.

Co-immunoprecipitation—Saos-2 or U2OS cells were harvested in NET buffer containing 1% Nonidet P-40 and supplemented with phosphatase and protease inhibitors (Roche Applied Science). Lysates were centrifuged at 14,000 rpm at

4 °C for 30 min before subjection to immunoprecipitation and SDS-PAGE/immunoblotting.

In Vitro Translation—*In vitro* translation was performed using the Promega TNT[®] T7 Quick-coupled Transcription/Translation system according to the manufacturer's protocol. Prior to co-immunoprecipitation, *in vitro* translated proteins were mixed as indicated in the figure and incubated at 30 °C for an additional 1 h.

Nickel Pulldown—A His-tagged purified fragment of iASPP corresponding to amino acids 625–828 (17), conjugated to nickel beads, was incubated with 1 μ g of purified PP1 β for 3 h at room temperature. The mix was then washed three to five times with 1 ml of NET buffer complemented with protease inhibitors (Roche Applied Science). Proteins bound to the beads were eluted with 5 \times Laemmli buffer. Nickel beads alone or conjugated to a purified His-tagged consensus ankyrin domain (18) were used as a negative control and subjected to the same experimental conditions. Purified PP1 β was obtained from Origene and detected by SDS-PAGE/immunoblotting using a mouse monoclonal anti-myc antibody (9E10).

Flow Cytometry—FACS analysis was performed as described before (12, 19).

Luciferase Reporter Assay—Transcriptional assays were performed in Saos-2 cells and were carried out as described previously (13). For each experiment, the total DNA in each condition was kept constant (4 μ g), with the addition of pcDNA3 vector when necessary. The following constructs were used at the given quantities: iASPPwt (300 ng, 500 ng, 1 μ g), iASPP(F815A) (300 ng, 500 ng, 1 μ g), p53 (50 ng) and the PIG3 luciferase reporter (1 μ g) (20). Each assay was normalized by the addition of 50 ng of thymidine kinase *Renilla* luciferase in each sample. Protein expression levels were verified by SDS-PAGE/immunoblotting using 60 μ g of luciferase lysate from each condition.

RESULTS

ASPP2 Binds the C Terminus of PP1 in Vivo via Its RVXF Motif—Although an ASPP2 peptide containing the RVXF motif was previously shown to interact with PP1 *in vitro*, it remained unclear whether the identified RVXF motif is exposed at the protein surface in full-length ASPP2 protein to mediate PP1 binding. It was also not known whether the ASPP2/PP1 interaction could occur in cells. To address these questions, we first mutated Val-922 and Phe-924 of the RVXF motif of ASPP2 to alanine to destroy the RVXF motif and generate the ASPP2 mutant, ASPP2(V922A, F924A) (Fig. 1A). Wild-type full-length ASPP2 and ASPP2(V922A, F924A) were transfected into Saos-2 cells to investigate their ability to interact with endogenous PP1 α . Because both wild-type ASPP2 and ASPP2(V922A, F924A) were V5-tagged, they were immunoprecipitated using an anti-V5 antibody. The results in Fig. 1B show that full-length ASPP2 is able to co-immunoprecipitate with PP1 α in cells, confirming the previous results obtained with the C terminus of ASPP2, 53BP2. Interestingly, ASPP2(V922A, F924A), failed to co-immunoprecipitate with PP1 α , demonstrating that the RVXF motif of ASPP2 is required for its interaction with PP1 α *in vivo*.

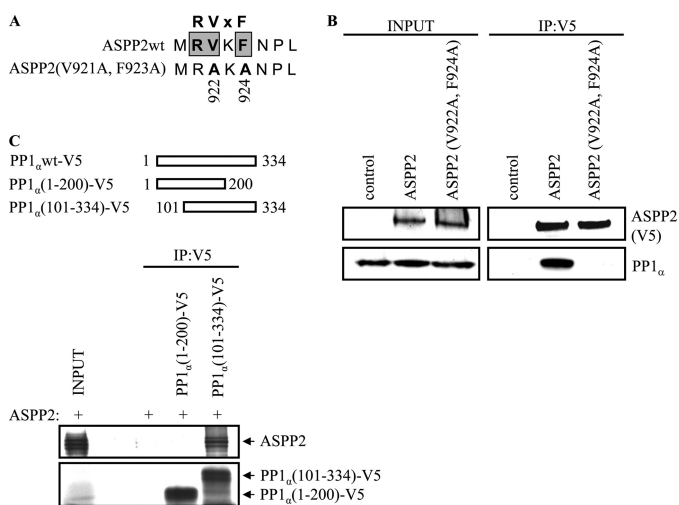


FIGURE 1. Full-length ASPP2 interacts with PP1 through the RVXF motif. *A* and *B*, mutation of the RVXF PP1 binding motif of ASPP2 (*A*) prevents its interaction with PP1 (*B*). *A*, Val-922 and Phe-924 of the RVXF motif of ASPP2 were mutated to alanine. *B*, wild-type ASPP2 and ASPP2(V922A, F924A) were expressed in Saos-2 cells and immunoprecipitated with an anti-V5 antibody to investigate their ability to interact with endogenous PP1 α . *C*, the ability of *in vitro* translated PP1 α (1-200)-V5 and PP1 α (101-334)-V5 to interact with ASPP2 was tested by co-immunoprecipitation. For this particular experiment, ASPP2 cloned in pcDNA3 was used (13).

The ability of full-length ASPP2 to interact with PP1 α was further confirmed using *in vitro* translated proteins (Fig. 1C). In this system, the ASPP2 binding region of PP1 α was mapped using two PP1 fragments, PP1 α (1-200 amino acids) and PP1 α (101-334 amino acids), truncated in their C and N termini, respectively. Interestingly, PP1 α (101-334) but not PP1 α (1-200) was able to co-immunoprecipitate with ASPP2, suggesting that the C-terminal 134 amino acids of PP1 are required to interact with ASPP2.

iASPP Binds PP1 in the Absence of an RVXF Motif—All three ASPP family members have high sequence similarity in their C termini where the RVXF motif of ASPP2 is located. Interestingly, however, the RVXF motif only exists in ASPP1 and ASPP2, and not in iASPP (Fig. 2A), suggesting that unlike ASPP2, iASPP may not be able to interact with PP1. To test this, we transfected V5-tagged ASPP1, ASPP2, or iASPP into Saos-2 cells, and the ability of all three ASPPs to interact with endogenous PP1 was examined. The results in Fig. 2B show that all three ASPP family members are able to co-immunoprecipitate with endogenous PP1 α as well as PP1 γ . The ability of iASPP to co-immunoprecipitate with PP1 was unexpected because it does not contain a recognizable RVXF motif. Moreover, because Saos-2 cells are p53-null, this result also shows that all ASPP proteins are able to interact with PP1 independently of p53. The ability of all three endogenous ASPP family members to interact with endogenous PP1 was also confirmed in U2OS cells (Fig. 2C). It is important to note that both exogenous and endogenous iASPP were able to bind PP1 as effectively as ASPP1 or ASPP2 (Fig. 2, B and C). Altogether, these results demonstrate that iASPP can interact with PP1 despite not having a classical RVXF motif in its sequence.

Identification of a New PP1 Binding Motif in iASPP—To identify the PP1 binding site on iASPP, we first tested whether iASPP interacts with PP1 through its N- or C-terminal regions.

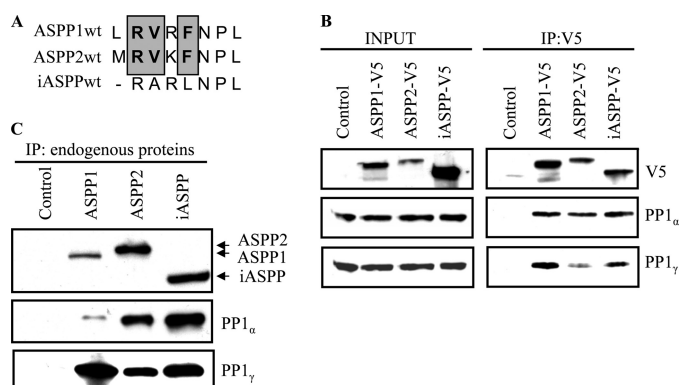


FIGURE 2. All three ASPP family members bind to PP1 despite iASPP lacking the conserved RVXF PP1 binding motif. *A*, alignment of ASPP1, ASPP2, and iASPP protein sequences reveals that the RVXF motif of ASPP2 is conserved in ASPP1 but not iASPP, where a RARL sequence is found instead. *B* and *C*, all three ASPP family members interact with PP1. *B*, V5-tagged ASPP1, ASPP2, and iASPP were expressed in Saos-2 cells and immunoprecipitated (*IP*) with an anti-V5 antibody, and the ability of endogenous PP1 α and PP1 γ to co-immunoprecipitate with them was investigated by SDS-PAGE/immunoblotting. *C*, endogenous ASPP family members were immunoprecipitated using U2OS cell lysates to test their ability to interact with endogenous PP1 α and PP1 γ .

Two iASPP constructs, iASPP(1-478) and iASPP(479-828), were used to investigate their ability to interact with PP1 α in Saos-2 cells. PP1 α was able to co-immunoprecipitate with iASPP(479-828) but not iASPP(1-478), demonstrating that iASPP interacts with PP1 through its C-terminal region (Fig. 3A). Because iASPP does not contain a classical RVXF motif, it was critical to establish whether its interaction with PP1 was direct or not. To address this question, we performed a nickel pull-down assay using a purified His-tagged C-terminal fragment of iASPP (residues 625-828) (17). This fragment conjugated to nickel beads was able to pull down pure PP1 β . In contrast, nickel beads alone or a consensus ankyrin domain conjugated to nickel beads were unable to interact with PP1, demonstrating that the C terminus of iASPP can directly and specifically interact with PP1 *in vitro* (Fig. 3B).

To determine further which region of iASPP(479-828) mediates the interaction between iASPP and PP1, a series of four iASPP truncation mutants were made as shown in the upper panel of Fig. 3C. Interestingly, iASPP(649-728), a fragment that lacks the SH3 domain but contains the ankyrin repeat domains of iASPP, failed to co-immunoprecipitate with PP1 α , suggesting that the SH3 domain of iASPP may be involved in mediating its binding to PP1. Furthermore, iASPP(479-728), a fragment that contains the RARL sequence and the ankyrin repeats, also failed to co-immunoprecipitate with PP1 α , demonstrating that the RARL sequence is not required for iASPP to bind PP1 *in vivo*. In contrast, iASPP(649-828) and iASPP(728-828) both co-immunoprecipitated with PP1 α , supporting the notion that the SH3 domain of iASPP is likely to be involved in mediating its interaction with PP1. To test this further, two additional iASPP mutants were constructed, iASPP(479-782) and iASPP(479-814). The results in Fig. 3D show that none of these constructs was able to interact with PP1 α , indicating that the PP1 binding region of iASPP is contained within the last 15 amino acids of the protein (amino acids 814-828).

iASPP Binds to PP1 via a Noncanonical Motif

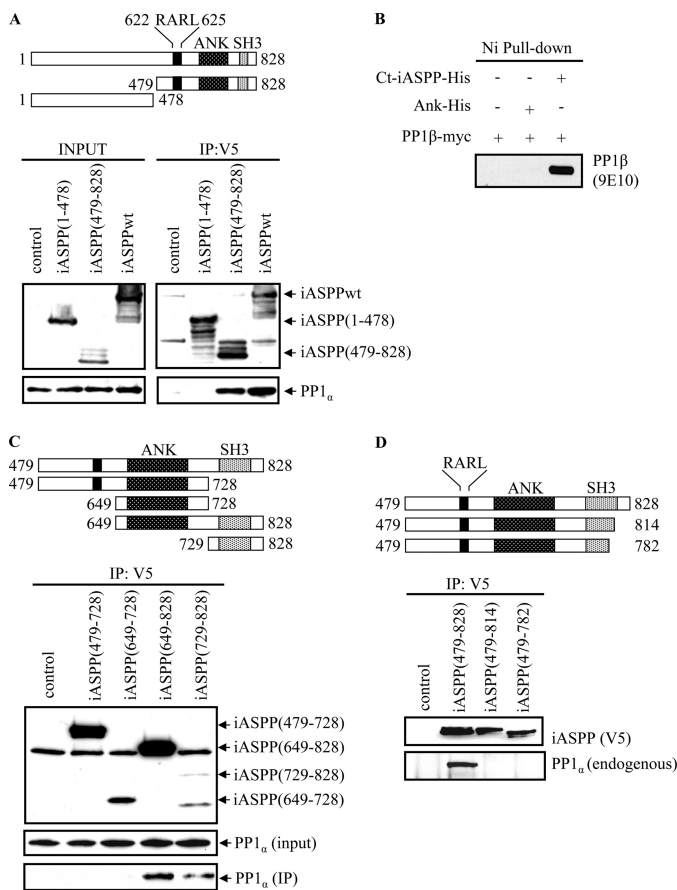


FIGURE 3. The extreme C terminus of iASPP is responsible for its interaction with PP1. *A*, iASPP interacts with PP1 through its C-terminal half, which contains the RARL domain and the signature domains of the ASPP family members. Two V5-tagged constructs corresponding to the N-terminal (amino acids 1–478) and the C-terminal (amino acids 479–828) portions of iASPP were designed (upper panel). The fragments were expressed in Saos-2 cells and immunoprecipitated (IP) with an anti-V5 antibody to test their ability to interact with PP1_α (lower panel). *B*, iASPP interacts directly with PP1. A His-tagged purified C-terminal construct of iASPP was used in a nickel pull-down assay with pure PP1β to demonstrate their direct interaction. Nickel beads alone or conjugated to a His-tagged consensus ankyrin domain were used as negative controls. *C*, iASPP interacts with PP1 through a region found in its N terminus. Various V5-tagged iASPP fragments were made from iASPP(479–828) (upper panel) and expressed in Saos-2 cells, to delineate which region of iASPP binds to PP1 (lower panel). *D*, the last 15 amino acids in the C terminus of iASPP are responsible for its binding to PP1. Two additional iASPP deletion mutants, iASPP(479–814) and iASPP(479–782), were designed from the C-terminal half of iASPP and expressed in Saos-2 cells to determine more accurately which region of iASPP is required for its interaction with PP1.

To identify the amino acids responsible for the binding of iASPP to PP1 within this sequence, we looked for potential, partial, or noncanonical RVXF motifs within the last 15 amino acids of iASPP that could not be found in ASPP1 and ASPP2 (Fig. 4A). Several potential partial RVXF motifs within this region were mutated, generating the following iASPP mutants: iASPP(V821A), iASPP(F818A), iASPP(F815A), and iASPP(F818A,V821A) (Fig. 4B). Interestingly, when transfected in U2OS cells, all mutants except iASPP(F815A) were able to co-immunoprecipitate with PP1_α (Fig. 4C and data not shown). These results identified Phe-815 at the extreme C terminus of iASPP, within its SH3 domain, as a crucial residue for the iASPP/PP1 interaction. Furthermore, Phe-815 is unique to the iASPP SH3 domain and is conserved in *Xenopus laevis*,

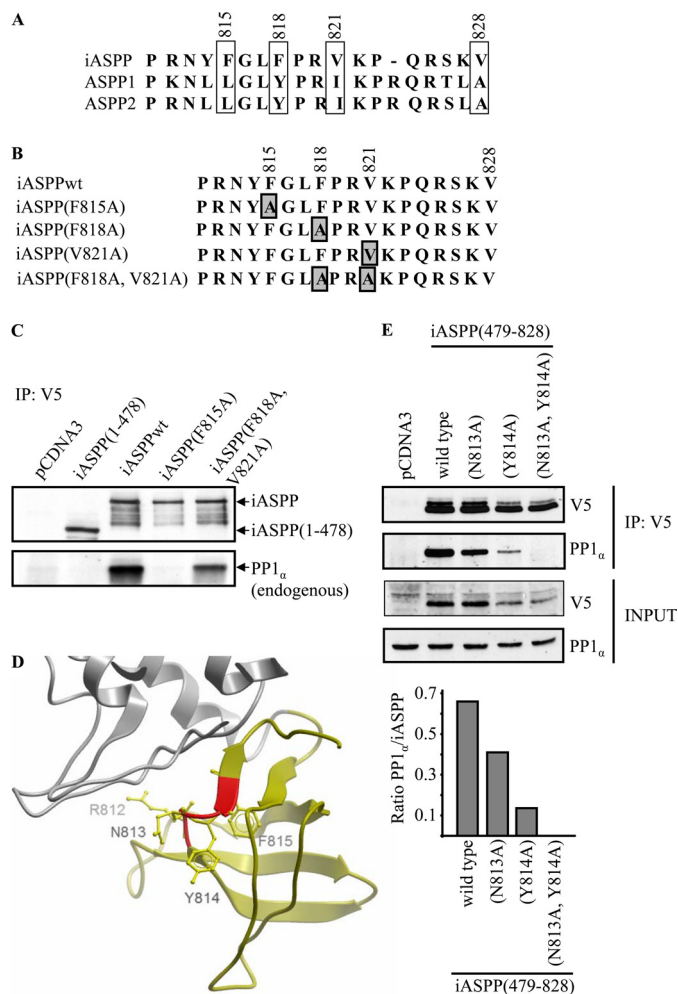


FIGURE 4. Identification of a noncanonical PP1 binding motif in iASPP. *A*, alignment of ASPP1, ASPP2, and iASPP reveals several potential partial RVXF motifs in the extreme C terminus of iASPP that are not conserved in ASPP1 and ASPP2 (boxed amino acids). Numbers indicate amino acid position in the iASPP sequence. *B*, iASPP point mutations were designed in the potential partial RVXF motifs found at the extreme C terminus of iASPP: iASPP(F815A), iASPP(F818A), iASPP(V821A), and iASPP(F818A,V821A). *C*, the ability of those iASPP mutants to interact with endogenous PP1 was tested in U2OS cells by immunoprecipitation with an anti-V5 antibody. pcDNA3 and iASPP(1–478) were transfected in U2OS cells as two separate negative controls, and wild-type iASPP was used as a positive control. *D*, three-dimensional view to show where the RNYF motif is located in the iASPP SH3 domain. The RNYF motif is marked in red (in the ribbon), with the residue side chains shown as sticks. Note that Asn-813 and Tyr-814 are on the surface of the SH3 domain, whereas Arg-812 and Phe-815 are buried within the structure. *E*, Asn-813 and Tyr-814 of iASPP are required for its interaction with PP1. Following transfection of U2OS cells with the indicated iASPP constructs, immunoprecipitation (IP) with a V5 antibody was performed to test their ability to interact with endogenous PP1_α. ImageJ software was used to quantify the amounts of immunoprecipitated PP1_α and the various iASPP constructs. The ratios between co-immunoprecipitated PP1_α and iASPP constructs are represented on a graph for this particular experiment.

underlying its importance (data not shown). Phe-815, being part of a RNYF motif that resembles the classical RVXF motif, suggests that this motif may be a novel noncanonical PP1 binding site. However, further examination of the RNYF motif within the structure of the SH3 domain of iASPP revealed that, whereas Arg-812 and Phe-815 are buried, Asn-813 and Tyr-814 are available at the surface, suggesting that Tyr-814 and Asn-813 may be the contact residues that mediate the interaction between the SH3 domain of iASPP and PP1 (Fig. 4D).

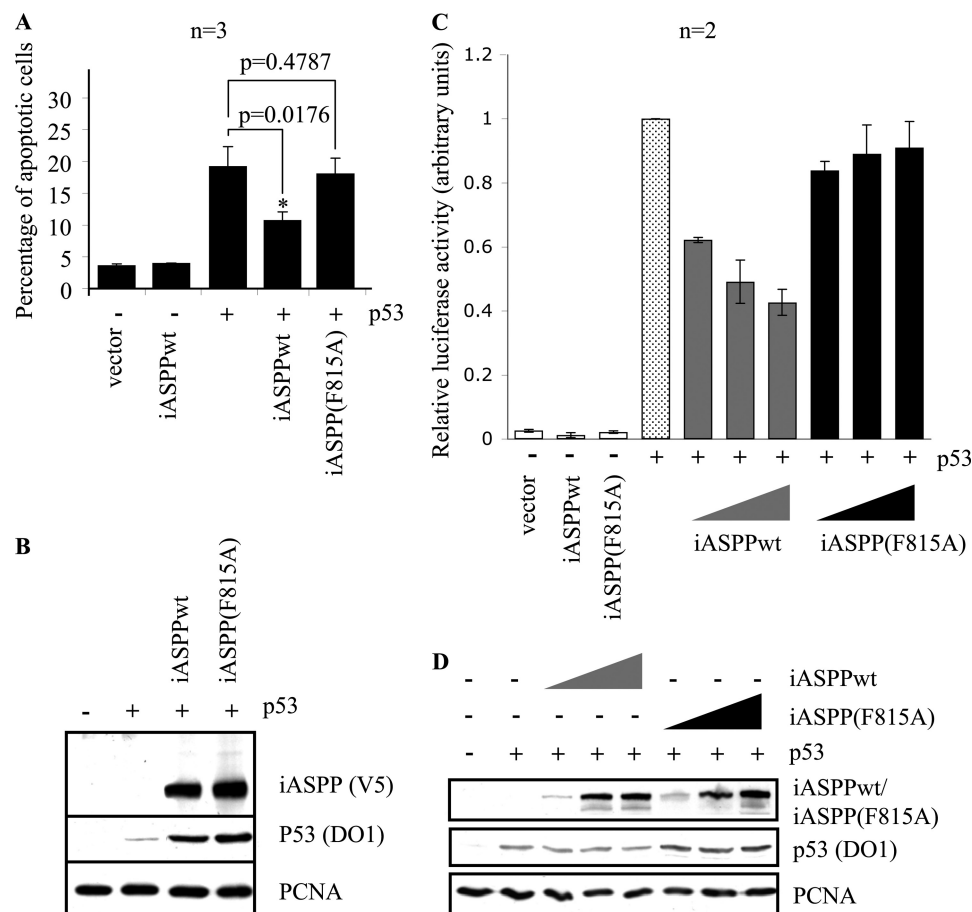


FIGURE 5. The interaction between iASPP and PP1 is crucial for the inhibitory function of iASPP in p53-mediated apoptosis. *A*, ability of wild-type iASPP and iASPP(F815A) to inhibit p53-mediated apoptosis as analyzed by flow cytometry. The *bar graph* represents the percentage of apoptotic Saos-2 cells for each condition. Indicated *p* values were calculated from three independent experiments using a paired *t* test. *B*, analysis of protein expression by SDS-PAGE/immunoblotting in cell lysates obtained from Saos-2 cells transfected with the indicated expression constructs. PCNA expression levels were used as a loading control. *C* and *D*, ability of wild-type iASPP and iASPP(F815A) to inhibit p53-mediated apoptosis investigated in a luciferase PIG3 reporter assay (*C*). Values were obtained from two independent experiments. iASPP, iASPP(F815A), and p53 expression levels in lysates obtained from this experiment were analyzed by SDS-PAGE/immunoblotting (*D*). PCNA levels were analyzed as a loading control.

To test this hypothesis, we mutated Asn-813 and Tyr-814 alone or together to alanine in iASPP(479–828) to generate the mutants iASPP(479–828/N813A), iASPP(479–828/Y814A), and iASPP(479–828/N813A,Y814A), respectively. The three mutants were transfected into U2OS cells, and their ability to bind to endogenous PP1 $_{\alpha}$ was tested (Fig. 4E). Mutating either Asn-813 or Tyr-814 to alanine resulted in decreased interaction with PP1 $_{\alpha}$, although a more pronounced defect was observed with the Y814A mutant. Importantly, when both Asn-813 and Tyr-814 were mutated to alanine, the interaction with PP1 $_{\alpha}$ was entirely lost (Fig. 4E). These results illustrate that both Asn-813 and Tyr-814 are important for the iASPP/PP1 interaction and further indicate that RNYF may represent a new noncanonical PP1 binding motif.

PP1-binding Defective iASPP Is Unable to Inhibit p53 Function—iASPP has been shown to inhibit p53-mediated apoptosis by specifically inhibiting its ability to induce the transcription of proapoptotic genes (12). Importantly, Tyr-814 of iASPP was previously identified as a key residue that mediates iASPP/p53 interaction through the proline-rich region of p53 (21). Because the identified PP1 binding residue Phe-815 is adjacent to Tyr-814, it is possible that Phe-

815 is also required for iASPP to inhibit p53. To test this hypothesis, we analyzed the impact of overexpressing wild-type iASPP and iASPP(F815A) on p53-mediated apoptosis by flow cytometry (Fig. 5A). As previously shown, wild-type iASPP was able to decrease p53-mediated apoptosis. In contrast, iASPP(F815A) failed to inhibit p53-mediated apoptosis under the same conditions. Importantly, p53 protein levels remained the same in the presence of either wild-type iASPP or iASPP(F815A), indicating that the inhibition of p53-mediated apoptosis seen in the presence of iASPP takes place at the transcriptional level (Fig. 5B). This was further supported by evidence obtained from a PIG3-luciferase reporter assay showing that, in contrast to wild-type iASPP, increasing amounts of iASPP(F815A) were not able to inhibit the induction of the reporter by p53 (Fig. 5C). Again, this effect was not a result of variations in p53 protein levels (Fig. 5D). Together, our data show that Phe-815 is required for the inhibitory function of iASPP. Because Phe-815 is critical in mediating iASPP/PP1 binding, it is possible that the interaction between iASPP and PP1 may be required for iASPP to inhibit p53-mediated apoptosis.

DISCUSSION

In this study, we have shown that the three members of the ASPP family, ASPP1, ASPP2, and iASPP, can bind the catalytic subunit of PP1 in cells. ASPP1 and ASPP2 both contain a conserved classical RVXF PP1 binding motif, and our data have confirmed that this domain is required for full-length ASPP2 to interact with PP1 because, when it was mutated, ASPP2 and PP1 could no longer interact.

Conversely, iASPP does not contain a classical RVXF motif. Instead, it is replaced by a RARL sequence as suggested by sequence alignment with ASPP1 and ASPP2. Our data show that this RARL sequence is not sufficient for iASPP to bind to endogenous PP1 because several iASPP constructs containing the RARL motif, such as iASPP(479–728), iASPP(479–814), and iASPP(479–782), were unable to interact with endogenous PP1_α. This suggests that the RARL sequence, at least in the context of full-length iASPP protein, cannot be the main motif mediating the interaction between iASPP and PP1. Furthermore, our results clearly demonstrate the importance of the extreme C-terminal part of iASPP for its interaction with endogenous PP1 because all fragments lacking the C terminus of iASPP, including a fragment lacking as little as 15 amino acid residues, are unable to bind to PP1. This strongly suggests that the major PP1-binding element of iASPP is located in its extreme C terminus.

Detailed analysis of the last 15 amino acid residues of iASPP identified Phe-815 as being critical for the iASPP/PP1 interaction. This particular phenylalanine is part of a RNYF motif and does not correspond to any of the refined consensus sequences of the RVXF motif (22), suggesting that such a motif may constitute a new noncanonical PP1 binding motif. Interestingly, Phe-815 is part of the iASPP SH3 domain and is adjacent to two of the most conserved interacting residues of SH3 domains, Asn and Tyr (21). Those two residues, in contrast to Arg-812 and Phe-815, are available at the surface of the SH3 domain. Mutation studies demonstrated that these two residues are also important for the iASPP/PP1 interaction. Together, the results suggest that the RNYF motif is required for iASPP to interact with PP1.

It seems that Phe-815 is unique to iASPP and only changed later during evolution because it is not present in *Caenorhabditis elegans* (17). Additionally, Phe-815 does not exist in ASPP1, ASPP2, or in any other SH3 domains (21). However, in iASPP, Phe-815 is conserved at least from *Xenopus* to humans. Thus, one can speculate that the iASPP/PP1 interaction provides iASPP with a more selective advantage. This hypothesis is supported by the observation that the C-terminal region of PP1 contains a putative type II SH3 domain binding motif, PXXPXR, and that this region is conserved among various protein phosphatase 1 isoforms.⁵ Future studies are needed to test whether the RNYF motif of iASPP can indeed interact with the PXXP motif found in the C terminus of PP1.

Having identified the three ASPP family members as PP1-binding partners in cells, future challenges will be to demonstrate the biological significance of the ASPP/PP1 interaction *in vivo*. Although we observed that iASPP(F815A) is unable to

bind PP1 and also failed to inhibit p53-mediated apoptosis, it is impossible for us to attribute this defect to PP1 binding. This is due to the fact that the adjacent residue of Phe-815, Tyr-814, was previously identified to be a crucial residue mediating the iASPP interaction with, and the regulation of, codon 72-polymorphic p53 (21). Mutating Phe-815 to alanine might, therefore, indirectly affect the ability of Y814 of iASPP to interact with the p53 proline-rich region. Regardless of how iASPP(F815A) failed to inhibit p53 function, the identification of iASPP as a novel binding partner of PP1 through a noncanonical binding site broadens the spectrum of potential PP1 regulatory proteins. The identification of all three ASPP family members as PP1-binding partners *in vivo* also extends our understanding of how PP1 activity may be regulated *in vivo*.

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