

The ASPP interaction network: electrostatic differentiation between pro- and anti-apoptotic proteins

Hadar Benyamini^a and Assaf Friedler^{a*}

The ASPP proteins are apoptosis regulators: ASPP1 and ASPP2 promote, while iASPP inhibits, apoptosis. The mechanism by which these different outcomes are achieved is still unknown. The C-terminal ankyrin repeats and SH3 domain (ANK-SH3) mediate the interactions of the ASPP proteins with major apoptosis regulators such as p53, Bcl-2, and NFκB. The structure of the complex between ASPP2_{ANK-SH3} and the core domain of p53 (p53CD) was previously determined. We have recently characterized the individual interactions of ASPP2_{ANK-SH3} with Bcl-2 and NFκB, as well as a regulatory intramolecular interaction with the proline rich domain of ASPP2. Here we compared the ASPP interactions at two levels: ASPP2_{ANK-SH3} with different proteins, and different ASPP family members with each protein partner. We found that the binding sites of ASPP2 to p53CD, Bcl-2, and NFκB are different, yet lie on the same face of ASPP2_{ANK-SH3}. The intramolecular binding site to the proline rich domain overlaps the three intermolecular binding sites. To reveal the basis of functional diversity in the ASPP family, we compared their protein-binding domains. A subset of surface-exposed residues differentiates ASPP1 and ASPP2 from iASPP: ASPP1/2 are more negatively charged in specific residues that contact positively charged residues of p53CD, Bcl-2, and NFκB. We also found a gain of positive charge at the non-protein binding face of ASPP1/2, suggesting a role in electrostatic direction towards the negatively charged protein binding face. The electrostatic differences in binding interfaces between the ASPP proteins may be one of the causes for their different function. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: ASPP; ASPP2; iASPP; p53; computational studies; interaction network; Bcl-2; NF-kappa-B

INTRODUCTION

The ASPP protein family plays a key role in apoptosis regulation, both in the p53 pathway and in the mitochondrial pathway (Samuels-Lev *et al.*, 2001; Kobayashi *et al.*, 2005). ASPP2 (600–1128) was initially identified as 53BP2 (p53-binding protein 2) in a yeast two-hybrid screen, using the p53 core domain (p53CD) as bait (Iwabuchi *et al.*, 1994). Another fragment of ASPP2 (123–1128) was discovered as binding Bcl-2 (Naumovski and Cleary, 1996). The full length ASPP2 protein was identified later (Samuels-Lev *et al.*, 2001), while its family member and close homolog ASPP1 was identified by homology search (Nagase *et al.*, 1998). The third member of the ASPP family, iASPP, was originally identified as an inhibitor of NFκB (Yang *et al.*, 1999a). ASPP1 and ASPP2 enhance, while iASPP inhibits, p53-dependent apoptosis (Samuels-Lev *et al.*, 2001; Bergamaschi *et al.*, 2003). iASPP is considered more ancient in evolution, since it is the only family member for which a homolog was found in *C. elegans* (Bergamaschi *et al.*, 2003).

All members of the ASPP family share sequence homology in the C-terminal part, which contains the proline rich region followed by four ankyrin repeats and an SH3 domain (Figure 1). ASPP1 and ASPP2 also share sequence homology in the N-terminus, containing a β-grasp ubiquitin-like fold, followed by a predicted α-helical domain (Figure 1).

ASPP2 is the most well-characterized member of the ASPP family. The C-terminal ankyrin repeats and SH3 domain (ANK-SH3) of ASPP2 interact with numerous proteins (reviewed in Rotem *et al.*, 2007). These include p53 and its family members (Samuels-Lev *et al.*, 2001; Robinson *et al.*, 2008), the anti-apoptotic protein Bcl-2 family members (Naumovski and Cleary, 1996; Katz

et al., 2008), the p65 subunit of the transcription factor NFκB (NFκB(p65), (Yang *et al.*, 1999b)), protein phosphatase 1 (Helms *et al.*, 1995), yes-associated protein (Españel and Sudol, 2001), and hepatitis C virus core protein (Cao *et al.*, 2004). The interaction with p53CD was also described for ASPP1 and iASPP (Patel *et al.*, 2008; Ahn *et al.*, 2009). The interaction with NFκB(p65) was also described for iASPP (Yang *et al.*, 1999a). Our previous studies revealed an intramolecular interaction between the ANK-SH3 domains and the proline rich domain of ASPP2. This interaction is suggested to regulate the ASPP2 intermolecular interactions (Rotem *et al.*, 2008). The ANK-SH3 domains of ASPP2 are highly negatively charged, with pI = 4.75.

Among the protein partners of the ASPP family, p53, Bcl-2, and NFκB are hubs of cellular apoptosis regulation. p53 is a transcription factor that activates cell cycle arrest or apoptosis in response to oncogenic stress (Vogelstein *et al.*, 2000). NFκB is a family of dimeric transcription factors, and the heterodimer of sub-units p65 and p50 represents the most abundant and best-known form of NFκB (Baldwin, 1996). When aberrantly regulated, NFκB is constitutively active, a phenomenon observed in various malignancies (reviewed in Basseres and Baldwin, 2006; Dutta *et al.*, 2006). Proteins from the Bcl-2 family play a major role in the mitochondrial death pathway.

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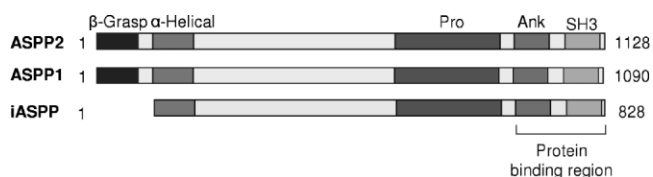


Figure 1. Domain organization of the ASPP family members. The ASPP family shares sequence homology in the C-terminal part containing the proline rich region (Pro), four ankyrin repeats (ANK), and Src homology 3 (SH3) domain. ASPP1 and ASPP2 also share sequence homology of β -grasp ubiquitin-like domain within their N-termini.

The Bcl-2 family consists of antiapoptotic members like Bcl-2 as well as proapoptotic members such as Bax. These proteins cooperate through dimerization to maintain the balance between cell death and survival (Adams and Cory, 1998). Because of their pivotal role in apoptosis regulation, p53, Bcl-2, and NF κ B are major targets in anti-cancer therapeutics.

The intermolecular interactions of ASPP2 with p53, Bcl-2, and NF κ B were structurally characterized. The structure of the complex between ASPP2_{ANK-SH3} and p53CD was solved using X-ray crystallography (Gorina and Pavletich, 1996). We have recently published experimentally supported 3D structural models for the complexes of ASPP2_{ANK-SH3} with Bcl-2 (Katz *et al.*, 2008) and of ASPP2_{ANK-SH3} with NF κ B(p65) (Benyamini *et al.*, 2009). The structural models were built based on the combination of peptide binding data, protein docking, and molecular dynamics techniques.

Here we examined the structures and sequences of the protein-binding region of the ASPP protein family, containing the ANK-SH3 domains, to elucidate the basis for the differences in their activity. We show that the binding sites of ASPP2 to p53, Bcl-2, and NF κ B(p65) are different but lie on the same molecule face, with the binding site to the ASPP2 proline rich domain overlapping these binding sites, in support of a regulatory role of the intramolecular interaction. Furthermore, we compared the ASPP family members and extracted the ANK-SH3 residues that have unique properties in ASPP1/2 compared to iASPP, which were found to be mostly surface exposed. The main difference between the ANK-SH3 domains of the ASPP family members is in their electrostatic features. Our results reveal a gain of negative charge in ASPP1 and ASPP2 compared to iASPP, in residues that bind positively charged residues in p53, Bcl-2, and NF κ B. Gain of positive charge is found in the non-binding molecule face, possibly providing electrostatic direction towards the negatively charged binding face.

RESULTS

ASPP2 uses different sites on the same molecule face to bind p53, Bcl-2, and NF κ B(p65)

A combined view on the solved structure of the complex ASPP2_{ANK-SH3}-p53CD (Gorina and Pavletich, 1996), and the structural models for the complexes of ASPP2_{ANK-SH3} with Bcl-2 (Katz *et al.*, 2008) and with NF κ B(p65) (Benyamini *et al.*, 2009), reveals that ASPP2 binds the three proteins using different sites that lie at the same face of the molecule (Figure 2). The intramolecular interaction of ASPP2_{ANK-SH3} with the proline rich domain (Rotem *et al.*, 2008) occurs via this face of the molecule as well (Figure 2), overlapping the binding sites for the other three proteins and supporting the role proposed for this domain in regulating the intermolecular interactions of ASPP2. This protein

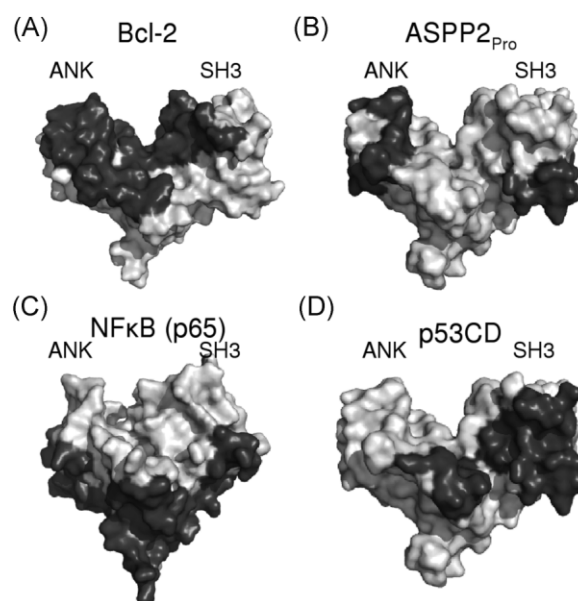


Figure 2. The binding regions of ASPP2_{ANK-SH3} to its partner proteins and the proline-rich domain. (A)–(D) ASPP2_{ANK-SH3} is colored light gray, the binding region to the ASPP2 partner proteins is colored in dark gray. In all cases the ankyrin repeats domain is at the left side and the SH3 domain is at the right side. The binding surfaces for p53, Bcl-2, and NF κ B were colored according to solved structures or previously published structural models: p53 core domain (Gorina and Pavletich, 1996), Bcl-2 (Katz *et al.*, 2008), and NF κ B(p65) (Benyamini *et al.*, 2009). The binding surface with ASPP2_{Pro} was deduced from peptide arrays binding results (Rotem *et al.*, 2008).

binding face is represented in Figure 2 by a view where the ankyrin repeats are on the left side and the SH3 domain is on the right side.

Sequence alignment reveals a subset of ASPP1/2 unique residues at the protein-binding region

We aligned the sequences of the protein-binding region (ANK-SH3 domains) of ASPP1, ASPP2, and iASPP (Figure 3). We extracted the significantly different residues between the anti-apoptotic iASPP and the pro-apoptotic ASPP1/ASPP2 and focused our analysis on amino acids that are ASPP1/2-unique: residues that have a certain property in ASPP1/2, and a different property in iASPP. The residue properties were classified as hydrophobic, aromatic, polar, negatively charged, or positively charged (for details see Methods section). For each of these positions, we examined whether a certain property is lost or gained in ASPP1/2 relative to iASPP, which is considered more ancient in evolution (Bergamaschi *et al.*, 2003). Such unique residues within the binding region (ANK-SH3) are potentially associated with the functional difference between the proteins since they may affect the affinities to other proteins and hence the pro- or anti-apoptotic activity. The set of 39 unique residues is detailed in Figure 3 and Table 1.

The main residue alterations are in surface exposed, charged residues

On the structure of ASPP2_{ANK-SH3} (PDB id 1ycs, chain B), 110 (57%) residues are exposed and 83 (43%) are buried. ASPP1/2 have 39 unique residues compared to iASPP. Within this subset, 34 (87%) are exposed, while 5 (13%) are buried (Table 1). Hence, residues that differentiate ASPP1/2 from iASPP are mainly surface exposed and may affect ligand binding.

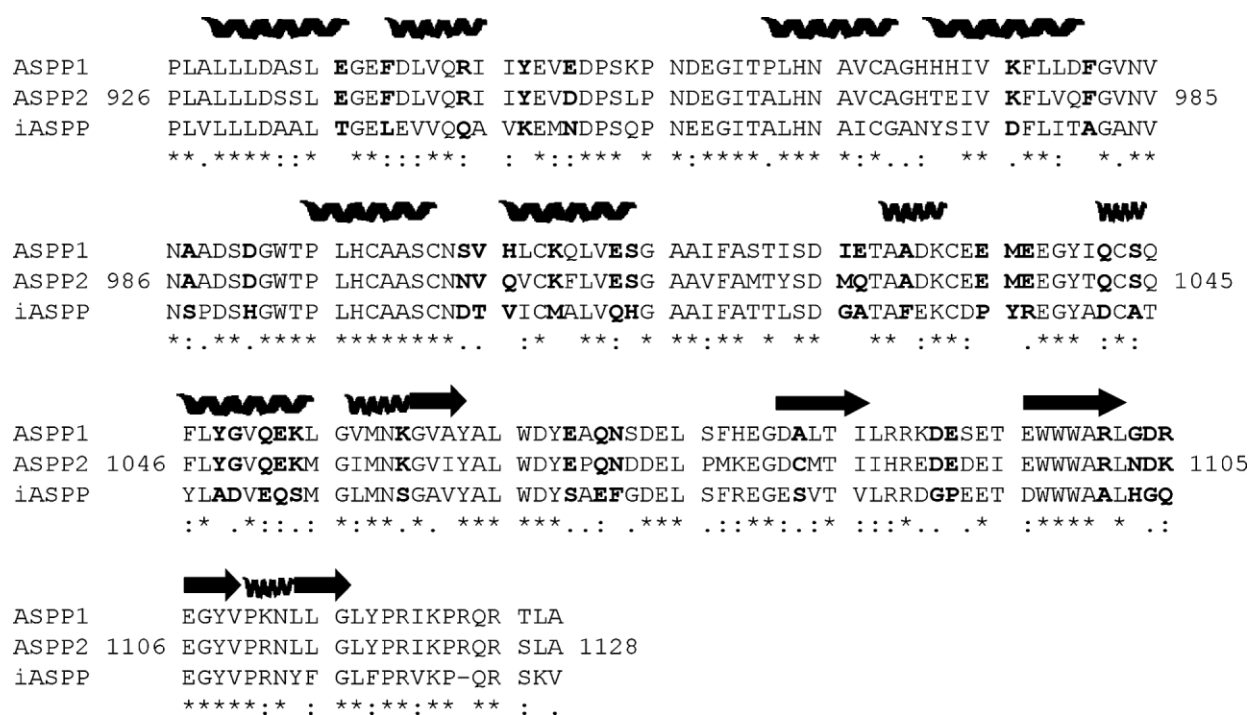


Figure 3. Sequence alignment of the ankyrin repeats and SH3 domains of ASPP1, ASPP2, and iASPP. Residues with an alteration of a chemical feature in ASPP1/2 relative to iASPP are bolded. α -helices of the ankyrin repeats domain are marked with springs, β sheets of the SH3 domain are marked with arrows.

Within the surface exposed ASPP1/2-unique residues, there are 30 cases of gain of a feature, and 29 cases of loss of a feature compared to iASPP. Obviously, many cases involve both loss and gain of a feature (Table 1). The distribution of features *gain* in ASPP1/2 is: aromatic (4), hydrophobic (5), negative (11), polar (4), and positive (7). The distribution in features *loss* in ASPP1/2 is: aromatic (6), hydrophobic (9), negative (6), polar (3), and positive (5). In the loss category there were three cases of Histidine, which was considered both aromatic and positively charged. The residues alterations distribution is depicted in Figure 4, showing a majority of electrostatics features changes, in particular gain of negative charge in ASPP1 and ASPP2 relative to iASPP.

How the residue alterations may affect the protein interactions of the ASPP family

We analyzed the potential effect of the residue alterations between ASPP1/2 and iASPP on their interactions with p53, Bcl-2, and NF κ B(p65). The binding interface of ASPP2 with p53 is known from the determined structure (Gorina and Pavletich, 1996). The binding interfaces with Bcl-2 and NF κ B(p65) are taken from the 3D structural models of the complexes (Katz *et al.*, 2008; Benyamini *et al.*, 2009). We looked for residues on ASPP2 that participate in protein binding and are within the ASPP1/2 unique subset, hence may be associated with the different functions of ASPP1/2 and iASPP. For example, gain of negative charge in a residue that is within interaction distance from a positively charged residue on a protein partner was considered a residue change that is potentially important for binding.

Residue alterations at the binding site to the p53CD

ASPP2_{ANK-SH3} interacts with two loops of p53CD using three stretches of residues (Gorina and Pavletich, 1996): (i) ASPP2

1021–1026, containing one ASPP1/2-unique residue with a mild gain of hydrophobicity (Ile and Met in ASPP1 and ASPP2, respectively, vs. Gly in iASPP). A peptide based on this sequence was previously shown not to bind p53CD independently, suggesting that it is less important for this interaction (Friedler *et al.*, 2002); (ii) ASPP2 1068–1075: in position 1072 ASPP1/2 have Asn while iASPP has a Phe. Asn1072 in ASPP2 makes a hydrogen bond with Asn247 of p53CD. In addition, a structural alignment of ASPP2_{ANK-SH3} with iASPP_{ANK-SH3} (PDB: 2vge, (Robinson *et al.*, 2008)) suggests that Phe in this position may cause a steric clash with p53CD. Hence, this change of residue in this position is likely to be functionally important; (iii) ASPP2 1091–1097: this peptide was previously shown to be sufficient for binding and stabilizing p53CD (Friedler *et al.*, 2002). It contains two adjacent negatively charged residues in ASPP1/ASPP2 (Asp-Glu, 1091–1092) that are absent in iASPP, with Gly-Pro instead. These residues in ASPP2 form electrostatic interactions with p53CD residues Arg280 and Arg273 according to the crystal structure of the complex (Figure 5A). The absence of negative charge in these residues on iASPP is likely to result in the observed lower affinity to p53CD (Ahn *et al.*, 2009). Overall, three residue alterations that are potentially important for binding were observed: one change of aromatic to polar residue and two adjacent residues with a gain of negative charge in ASPP2. The gain of negative charge is likely to increase the binding affinity of ASPP1/2, compared to the affinity of iASPP, to p53CD. Such tighter affinity of ASPP2 to p53CD was also found experimentally (Ahn *et al.*, 2009).

Residue alterations at the binding site to Bcl-2

ASPP2_{ANK-SH3} binds Bcl-2 at two sites (Katz *et al.*, 2008): (i) the BH4 motif, which is known to be sufficient for anti-apoptotic activity; (ii) a binding site for pro-apoptotic regulators. Examination of the 3D structural model of the complex between ASPP2_{ANK-SH3} and

Table 1. ASPP1/2 unique residues^a

ASPP2	iASPP	1,2, ^b	Gain ^c	Loss ^c
936	637	EET	negative	
939	640	FFL	aromatic	
944	645	RRQ	positive	
947	648	YYK	aromatic	positive
950	651	EDN	negative	
976	677	KKD	positive	negative
981	682	FFA	aromatic	
987 ^d	688	AAS	hydrophobic	polar
991	692	DDH	negative	positive/aromatic
1004	705	SND		negative
1005	706	VVT	hydrophobic	polar
1006	707	HQV	polar	hydrophobic
1009	710	KKM	positive	hydrophobic
1013	714	EEQ	negative	
1014	715	SSH		positive/aromatic
1026	727	IMG	hydrophobic	
1027	728	EQA	polar	hydrophobic
1030 ^d	731	AAF		aromatic
1035	736	EEP	negative	hydrophobic
1036	737	MMY		aromatic
1037	738	EER	negative	positive
1042	743	QQD		negative
1044 ^d	745	SSA	polar	hydrophobic
1048	749	YYA	aromatic	
1049 ^d	750	GGD	hydrophobic	negative
1051 ^d	752	QQE		negative
1052	753	EEQ	negative	
1053	754	KKS	positive	
1059	760	KKS	positive	
1069	770	EES	negative	
1071	772	QQE		negative
1072	773	NNF	polar	aromatic
1083	784	ACS	hydrophobic	polar
1091	792	DDG	negative	hydrophobic
1092	793	EEP	negative	hydrophobic
1101	802	RRA	positive	hydrophobic
1103	804	GNH		positive/aromatic
1104	805	DDG	negative	hydrophobic
1105	806	RKQ	positive	

^aThe table details the subset of ASPP1/2 unique residues. Residue properties assignment is detailed in Methods section.

^bLeft: the residue in ASPP1, middle: the residue in ASPP2, right: the residue in iASPP.

^cGain or loss of a feature refers to appearance of a certain feature in ASPP1/2 relative to iASPP and reflects the evolutionary process of the protein family.

^dBuried in ASPP2.

Bcl-2 revealed that nine residues within the ASPP2_{ANK-SH3} binding site belong to the ASPP1/2 unique subset. Among them, five harbor features alterations that are potentially important for binding, in positions 950, 991, 1014, 1069, and 1071 as detailed below. ASPP2 Asp950 is within interaction distance from Bcl-2 Arg26 and Arg164. While ASPP1 and ASPP2 have a negatively charged residue in this position, iASPP has an asparagine residue. ASPP2 Asp991 is within interaction distance from Bcl-2 Arg106. In this position, ASPP1/2 have an aspartic acid, while iASPP has a histidine, i.e. a gain of negative and loss of positive charge. ASPP2 Ser1014 is within interaction distance from Bcl-2 Arg26. In this

position iASPP has a histidine, which may contribute to repulsion from Bcl-2. ASPP2 Glu1069 interacts with four Bcl-2 positively charged residues: Arg11, Lys17, His20, and His29. In this position, iASPP has a serine while ASPP1/2 have glutamic acid. The gain of negative charge may thus increase the affinity of ASPP1/2 to Bcl-2. There may be a limited compensation by the loss of negative charge in the adjacent position 1071. However, this position is within interaction distance only from Lys17 and His29. Overall, the residue alterations involve mainly gain of negative charge of residues that interact with positively charged residues at the BH4 motif of Bcl-2 (Figure 5B). Similar to the interaction

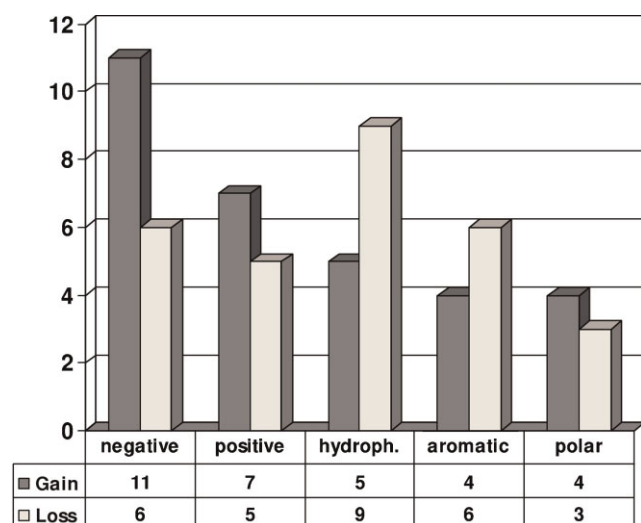


Figure 4. Distribution of features gain/loss on exposed residues. The most prevalent change of features is of electrostatic features, in particular presence of negative charge in ASPP1 and ASPP2 that is absent in iASPP.

with the p53CD, the residue alterations in the binding site of ASPP2_{ANK-SH3} with Bcl-2 suggest tighter binding affinity of ASPP1/2 to Bcl-2, compared to the affinity of iASPP to Bcl-2.

Residue alterations at the binding site to NFκB(p65)

ASPP2_{ANK-SH3} binds NFκB in two binding sites (Benyamini *et al.*, 2009): (i) residues 240–250, (ii) residues 290–313, containing the nuclear localization signal of NFκB. Examination of the 3D structural model of the ASPP2_{ANK-SH3}–NFκB(p65) complex revealed that 15 residues at the ASPP2_{ANK-SH3} binding site to NFκB(p65) belong to the ASPP1/2 unique residues subset. The altered residues within the NFκB(p65) binding site on ASPP2 are 936, 1004, 1026, 1027, 1030, 1035–1037, 1044, 1048, 1051–1053, and 1091–1092. Eight of these 15 alterations are potentially important for binding. Two residue alterations suggest better binding by iASPP: (i) ASPP2 Ser1004 displays negative charge loss, within interaction distance to R253 and R295 of NFκB(p65); (ii) Met1036 with loss of aromaticity, within interaction distance from Phe239 of NFκB(p65). Six out of the eight potentially significant alterations suggest tighter binding affinity of ASPP1/2 to NFκB(p65): (i) ASPP2 Glu936 with gain of negative charge, adjacent to the positively charged nuclear localization signal of NFκB(p65) at residues 301–304; (ii) Glu1035 with gain of negative charge, adjacent to Arg236 and Arg253; (iii) Ser1044 with loss of hydrophobicity, adjacent to Thr52, Thr55; (iv) Glu1052 with gain of negative charge, adjacent to Arg50 and Lys62; (v) Asp1091 with gain of negative charge, adjacent to Lys62; (vi) Glu1092 with gain of negative charge, adjacent to Lys62. Five of these six residue alterations involve gain of negative charge in positions that are within interaction distance from positively charged residues in NFκB(p65) (Figure 5C).

The common feature change at ASPP interaction sites is gain of negative charge

In summary, our results reveal a common feature change on protein-binding sites in the ANK-SH3 domains of ASPP proteins: In ASPP1/2 there is a gain of negative charge in residues that bind positively charged residues of the binding partners.

Energy assessment suggests tighter binding of ASPP1/2 over iASPP to p53 and Bcl-2

To assess whether ASPP1/2 bind tighter to the major apoptosis regulators, we used three different algorithms applied in the servers FastContact (Camacho and Zhang, 2005), CoilCheck (Alva *et al.*, 2008), and FireDock (Andrusier *et al.*, 2007). Details of the used protein structures are given in the Methods section. The energy assessment of the interactions of p53 or Bcl-2 with the ASPP family members is given in Table 2. For the interaction with p53, ASPP1 and ASPP2 have similar, lower assessed binding energies than iASPP. In one server (FireDock), the binding energy of p53CD–iASPP was assessed as positive. For the interaction with Bcl-2, ASPP1 and ASPP2 have similar assessed binding energies. The interaction with iASPP_{ANK-SH3} is assessed as having a positive energy, suggesting that this interaction is unlikely to occur. In one server (FastContact), the assessment of the suggested complex Bcl-2–iASPP_{ANK-SH3} failed to complete, also suggesting an unlikely protein complex. In summary, the energy assessment results for the interactions of ASPP proteins with p53CD and Bcl-2 suggest tighter affinity of ASPP1/2 over iASPP, in agreement with our predictions based on the amino acid differences of the ASPP proteins.

Surface distribution of electrostatic features changes is associated with location of protein-binding sites in ASPP

Gain of negatively charged residues is observed mainly on one face of the molecule, taking place in binding sites with the major apoptosis regulators p53, Bcl-2, and NFκB(p65) (Figure 6A). Gain of positive residues is found in seven residues: ASPP2 944, 976, 1001, 1005, 1009, 1053, and 1059 (Table 1). Six of these seven residues form a belt-like shape on the non-protein binding face of the molecule (Figure 6B). The positive patch at the non-binding face of the molecule may direct, by repulsion, binding partners to the negatively charged binding face of the molecule.

DISCUSSION

In the current study we examined the protein-binding regions of the ASPP family members to gain insight into the basis for the functional diversity of the family. We first compared between the known binding sites between ASPP2_{ANK-SH3} and the apoptosis regulators p53CD, Bcl-2, and NFκB(p65). We then proceeded to compare the full binding domains of the three ASPP family proteins. We found that ASPP2 binds different proteins using different sites that lie on the same face of the molecule. We further found a subset of ASPP1/2-unique residues that contains mostly surface exposed residues with the most prevalent features changes being of electrostatic features. Gain of negative charge in ASPP1/2 occurred mainly on the protein binding face of the molecule, and in residues that bind positively charged residues on the protein partners. The other, non-protein binding face of the molecule in ASPP1/2 displayed a belt-shaped cluster of positively charged residues that is absent in iASPP, possibly providing an electrostatic direction of protein partners toward the other, negatively charged face.

The ASPP interaction network

Structural information exists for some of the protein interactions of ASPP2: the structure of the complex with p53 CD was

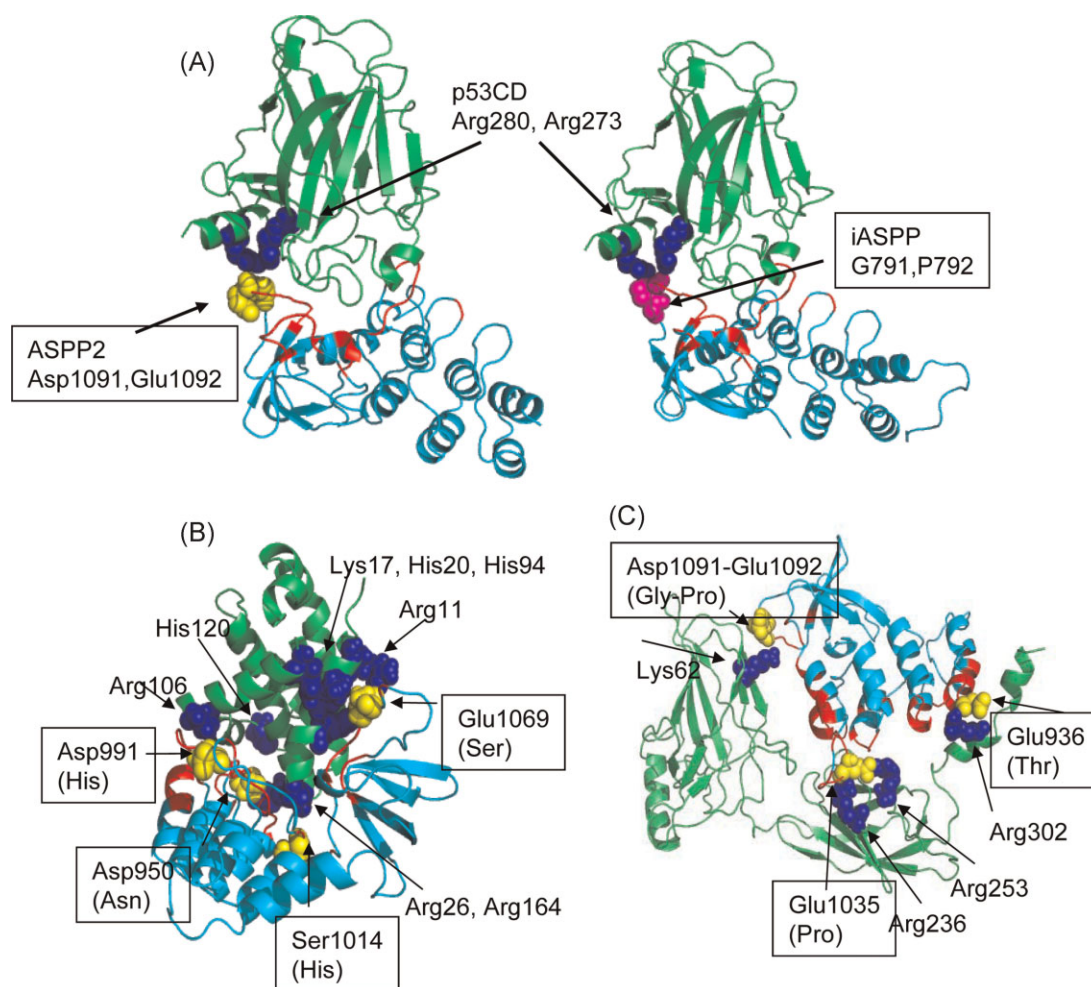


Figure 5. Gain of negative charge at the binding sites of ASPP2_{ANK-SH3} with p53, Bcl-2, and NFκB(p65). Contacts between ASPP2 residues with gain of negative charge and the partner protein are depicted in spheres. In green, the binding partner, in cyan: ASPP2_{ANK-SH3}; red: partner protein binding loops in ASPP2; blue: positively charged residues in the partner proteins, which bind ASPP2 residues that have gained negative charge (colored yellow). Framed residue labels mark residues with gain of negative charge. The residue with numbering is according to ASPP2, with the residue id in iASPP in parentheses. (A) Gain of negative charge at the binding site to p53CD. Left: the crystal structure of the complex between ASPP2_{ANK-SH3} and the core domain of p53 (PDB: 1ycs; Gorina and Pavletich, 1996). Right: iASPP_{ANK-SH3} (PDB: 2vge; Robinson *et al.*, 2008) superimposed on PDB: 1ycs (Gorina and Pavletich, 1996). In green, p53 core domain; cyan: iASPP_{ANK-SH3}; red: p53 binding loops; blue: p53 R273, R280; purple: G791, P792. (B) Gain of negative charge at the binding site to Bcl-2. The modeled complex of ASPP2_{ANK-SH3} and Bcl-2 is depicted in cartoons (Katz *et al.*, 2008). (C) Gain of negative charge at the binding site to NFκB(p65). The modeled complex of ASPP2_{ANK-SH3} and NFκB(p65) is depicted in cartoons (Benyamini *et al.*, 2009).

determined (Gorina and Pavletich, 1996), and we have recently published experimentally supported models for the interactions with Bcl-2 (Katz *et al.*, 2008) and NFκB(p65) (Benyamini *et al.*, 2009). Peptide binding data also exists for the intramolecular interaction between ASPP2_{ANK-SH3} and ASPP2_{PRO} (Rotem *et al.*, 2008). A combined view on the four binding sites revealed two new observations: (i) the binding sites of ASPP2_{ANK-SH3} with p53CD, Bcl-2 and NFκB(p65) are different, while the binding site to ASPP2_{PRO} partially overlaps the three binding sites, supporting a role in regulating the intermolecular interactions of the binding region (Figure 2); (ii) the binding sites are all found on the same face of the ANK-SH3 binding region, represented by a view where the ankyrin repeats are on the left side and the SH3 domain is on the right side. The main residue alterations between ASPP1/2 and iASPP can also be mapped on these two molecule faces: gain of negative charge is observed on the binding face while gain of positive charge is observed on the non-protein binding face of the molecule. Such a division may play a role in electrostatic

guidance of protein partners toward the binding face of the molecule.

Gain of negative charge at binding sites to the major apoptosis regulators

Our results reveal that gain of negative charge occurred in ASPP1 and ASPP2 in residues that bind positively charged residues of p53CD, Bcl-2, and NFκB(p65), suggesting tighter affinity of ASPP1/2 relative to the affinity of iASPP to these proteins. The binding affinity between the ANK-SH3 domains of ASPP family members and the p53CD was reported in several studies. The reported binding affinity of ASPP1 and ASPP2 to p53CD is at the low micromolar range, and was measured using different methods such as fluorescence anisotropy (measured for a peptide from ASPP2, 1089–1097; Friedler *et al.*, 2002), fluorescence spectroscopy (Ahn *et al.*, 2009), isothermal titration calorimetry (Tidow *et al.*, 2006; Patel *et al.*, 2008; Ahn *et al.*, 2009), and NMR (Ahn *et al.*, 2009).

Table 2. Energy assessment for ASPP family complexes^a

		p53CD			Bcl-2		
Algorithm ^b /ASPP		1	2	i	1	2	i
FastContact	Electrostatic ^c	-38.7	-38.3	-29.5	-12.7	-12.3	n/a
	Desolvation ^c	11.25	9.44	7.59	12.63	13.17	n/a
CoilCheck	Total ^d	-489	-514	-233	-212	-270	708
	Energy/res. ^d	-1.27	-1.34	-0.59	-0.70	-0.55	1.76
FireDock	Global ^e	-14.9	-13.0	10.7	-9.8	-13.7	13.4

^a The table details the energy assessment for known or suggested complexes of the ASPP family with p53CD or Bcl-2.

^b The algorithms FastContact (Camacho and Zhang, 2005), CoilCheck (Alva *et al.*, 2008), and FireDock (Andrusier *et al.*, 2007) were used for energy assessment of the complexes of the three ASPP family members with p53CD and Bcl-2.

^c Electrostatic and desolvation free energy (kcal/mol).

^d Total stabilizing energy, energy per residue (kJ/mol).

^e Global energy score.

For p53CD, ASPP1 and ASPP2 are suggested to bind tighter than iASPP. For Bcl-2, ASPP1 and ASPP2 are suggested to bind with similar affinity, while iASPP is suggested to not form a complex with Bcl-2: CoilCheck and FireDock suggest positive binding energy; FastContact was not able to complete the calculation probably due to high calculated energy.

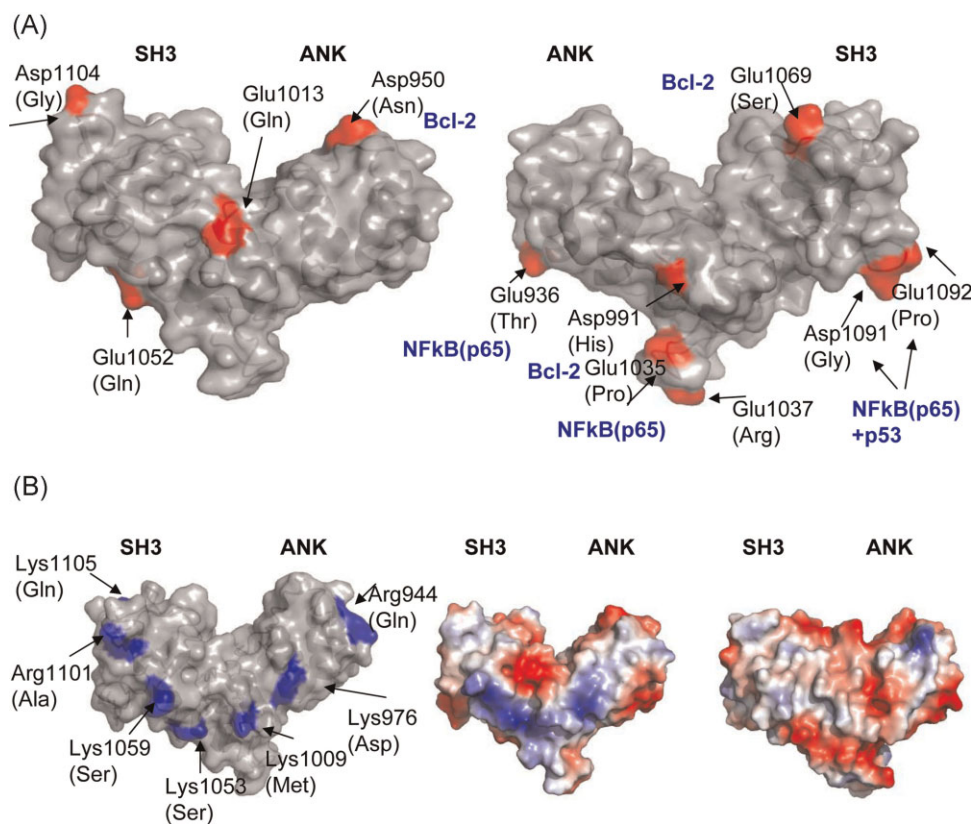


Figure 6. Electrostatic differences between ASPP1/2 and iASPP binding regions. (A) Gain of negative charge in ASPP1/2 relative to iASPP at the protein binding face of ANK-SH3. Residues where a gain of negative charge in ASPP1/2 is observed are colored red. Seven out of 11 are at the protein binding face of the molecule (right). The names of protein partners that bind a certain residue are bolded and colored blue next to the residue id. The residue id without parentheses is of ASPP2, in parentheses of iASPP. (B) Gain of positive charge at the non-protein binding face of ANK-SH3. Right: iASPP (PDB: 2vge; Robinson *et al.*, 2008). Middle and left: ASPP2 (Gorina and Pavletich, 1996). On the left, residues with gain of positive charge are colored blue on the surface of ASPP2_{ANK-SH3}. In the middle and right, electrostatic potentials of ANK-SH3 of ASPP2 and iASPP generated using Pymol (Delano, 2002). The figure demonstrates the gain of positive charge at the opposite of the protein binding face of ASPP2.

The results of Ahn *et al.* with low micromolar binding constant for the ASPP2_{ANK-SH3}-p53CD interaction show that the affinity of ASPP2_{ANK-SH3} is ~60 fold tighter (1.3–2.3 μ M vs. 76–100 μ M for iASPP_{ANK-SH3}), consistent with our analysis comparing the different composition of amino acids at the binding site, which suggests tighter binding of the ASPP1/2_{ANK-SH3} to p53CD compared to that of iASPP_{ANK-SH3}. Only one study (Robinson *et al.*, 2008) reported similar K_d values for ASPP2_{ANK-SH3} and iASPP_{ANK-SH3} at the nanomolar range (26 and 23 nM for ASPP2_{ANK-SH3} and iASPP_{ANK-SH3}, respectively). The difference is attributed to the different techniques used (Ahn *et al.*, 2009): NMR, ITC, and fluorescence spectroscopy (Ahn *et al.*, 2009) vs. enzyme linked immunosorbent assay (Robinson *et al.*, 2008). As detailed above, all the studies in solution, using various experimental methods, have measured K_d values within the low micromolar range (Friedler *et al.*, 2002; Tidow *et al.*, 2006; Patel *et al.*, 2008; Ahn *et al.*, 2009), which are also consistent with the binding constants of p53CD with other proteins such as 53BP1-BRCT, Rad51, and HIF-1 α (Tidow *et al.*, 2006). To the best of our knowledge, no binding constants were measured yet for the interactions between the ANK-SH3 domains of the ASPP family and Bcl-2 or NF κ B(p65). However, in support of our suggestion, in the Bcl-2 case, the role of electrostatic interactions between the Bcl-2 BH4 motif and ASPP2_{ANK-SH3} was demonstrated using ELISA for mutated peptide binding (Katz *et al.*, 2008). Replacement of the positively charged residues at the BH4 motif of Bcl-2 significantly reduced their binding to ASPP2_{ANK-SH3}, supporting the role of electrostatics in binding of Bcl-2 by ASPP2. For NF κ B(p65), the electrostatic binding of the positively charged nuclear localization signal of NF κ B(p65) is considered the essence of NF κ B(p65) inhibition by I κ B and hence highly likely to be important in ASPP2 binding of NF κ B(p65), which is suggested to occur via the same binding mechanism (Benyamini *et al.*, 2009). An assessment of the binding energies of the ASPP proteins complexes with p53 and Bcl-2 in the current study using different algorithms also suggested tighter binding of ASPP1/2 over iASPP to these proteins. For the Bcl-2 case, the energy assessment suggested that a complex of Bcl-2 with iASPP is even unlikely to occur.

Four cases of gain of negative charge were also observed on the binding site of ASPP2_{ANK-SH3} to ASPP2_{PRO}. Since we do not have a solved or modeled structure for this interaction, the meaning of these alterations is yet unknown. However, as ASPP2 723–737, the peptide from ASPP2_{PRO} that binds tightest to ASPP2_{ANK-SH3}, is rich in positively charged residues (peptide sequence: KKLSNAPRPLKRRSS), it is likely that gain of negative charge may contribute to tighter binding of ASPP1/2_{ANK-SH3} in this case as well.

The ANK-SH3 protein-binding region of the human ASPP family provides an example of an evolutionary process where a change of surface electrostatic features is associated with novel protein function, in this case promotion of cellular apoptosis. It will be interesting to examine how the functional diversity within the ASPP family relates to the protein sequences evolution in both the binding domains and the full-length proteins.

METHODS

Sequence alignment

The sequences of the ANK-SH3 parts of ASPP1, ASPP2, and iASPP were aligned using ClustalW.

Surface accessibility

The surface exposure of ASPP2 residues was measured using the G-X-G method (Chothia, 1976).

Residue properties assignment

Five properties were assigned to all the amino acids: Hydrophobic: A,C,G,I,L,M,V,P; Aromatic: F,Y,W,H; Positively charged: H,K,R; Negatively charged: D,E; Polar (non-charged): Q,S,T,N.

Graphic representation

All Figures were prepared using Pymol (Delano, 2002).

Protein structures

For a structural analysis of the possible effect of residue alterations on ASPP protein interactions, we used the determined structure of the complex of ASPP2_{ANK-SH3} with p53CD (Gorina and Pavletich, 1996), the structural model of the complex ASPP2_{ANK-SH3} with Bcl-2 (Katz *et al.*, 2008), and the structural model of the complex ASPP2_{ANK-SH3} with NF κ B(p65) (Benyamini *et al.*, 2009).

Energy assessment of the interactions of p53 or Bcl-2 with ASPP family members

Energy assessment algorithms: we used three different algorithms namely FastContact (Camacho and Zhang, 2005), CoilCheck (Alva *et al.*, 2008), and FireDock (Andrusier *et al.*, 2007). FastContact and CoilCheck output an estimated binding energy in kcal/mol or kJ/mol, while FireDock output an energy-based score.

Protein structures for energy assessment: for interactions of ASPP proteins with the core domain of p53 (p53CD), the reference was the crystal structure of the complex between ASPP_{ANK-SH3} and p53CD (Gorina and Pavletich, 1996). The original structure of the complex was used for the complex p53CD-ASPP2. For iASPP, we replaced the structure of ASPP_{ANK-SH3} with the crystal structure of iASPP_{ANK-SH3} (PDB: 2vge; Robinson *et al.*, 2008). For ASPP1, a structural model for the ANK-SH3 region was built using SWISSMODEL (Schwede *et al.*, 2003), based on the solved structure of ASPP2_{ANK-SH3} as a template, given the high degree of sequence homology (81% identical and 89% similar residues). Similar procedure was performed for the interaction with Bcl-2, with the experimentally based structural model of Bcl-2 with ASPP2_{ANK-SH3} serving as the reference structure. The structural model for the interaction between NF κ B(p65) and ASPP2 could not be used in a similar manner as it is a molecular dynamics model in which the structure of ASPP2 itself underwent structural changes. Thus, in this case ASPP2 could not simply be replaced by the crystal structure of iASPP or the model for ASPP1.

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