The Structure and Interactions of the Proline-rich Domain of ASPP2*Ts

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ASPP2 is a pro-apoptotic protein that stimulates the p53-mediated apoptotic response. The C terminus of ASPP2 contains ankyrin (Ank) repeats and a SH3 domain, which mediate its interactions with numerous partner proteins such as p53, NFκB, and Bcl-2. It also contains a proline-rich domain (ASPP2 Pro), whose structure and function are unclear. Here we used biophysical and biochemical methods to study the structure and the interactions of ASPP2 Pro, to gain insight into its biological role. We show, using biophysical and computational methods, that the ASPP2 Pro domain is natively unfolded. We found that the ASPP2 Pro domain interacts with the ASPP2 Ank-SH3 domains, and mapped the interaction sites in both domains. Using a combination of peptide array screening, biophysical and biochemical techniques, we found that ASPP2 Ank-SH3, but not ASPP2 Pro, mediates interactions of ASPP2 with peptides derived from its partner proteins. ASPP2 Pro-Ank-SH3 bound a peptide derived from its partner protein NFκB weaker than ASPP2 Ank-SH3 bound this peptide. This suggested that the presence of the proline-rich domain inhibited the interactions mediated by the Ank-SH3 domains. Furthermore, a peptide from ASPP2 Pro competed with a peptide derived from NFκB on binding to ASPP2 Ank-SH3. Based on our results, we propose a model in which the interaction between the ASPP2 domains regulates the intermolecular interactions of ASPP2 with its partner proteins.

ASPP2 (see Fig. 1A) is one of three members of the ASPP (apoptosis-stimulating proteins of p53) family, which specifically stimulates the apoptotic response mediated by the tumor suppressor protein p53 (1). Human ASPP2 is a 1128-amino acid protein, whose C-terminal part (amino acids aa 600–1128) was originally identified as a protein that binds the p53 core domain and was named p53-binding protein 2 (53BP2) (2). Another truncated form of ASPP2 (aa 123–1128) was discovered as Bbp (Bcl-2-binding protein) (3) (Fig. 1A). Full-length ASPP2 was found to specifically stimulate the apoptotic function of p53 by enhancing the transactivation function of p53 on the promoters of pro-apoptotic genes, but it has no effect on genes involved in cell cycle arrest (1). ASPP2 contains several structural and functional domains (Fig. 1A): Its N terminus (residues 1–83) has the structure of a β-Grasp ubiquitin-like fold (4). It is followed by a predicted α-helical domain located between aa 123 and 323 (3), and a proline-rich (ASPP2 Pro) domain between aa 674 and 902 (3). The C-terminal part of ASPP2 contains four ankyrin repeats and an SH3 domain (ASPP2 Ank-SH3), as seen from its crystal structure in complex with p53 core domain (5). The crystal structure of the C-terminal domain of the homologous ASPP family member iASPP was solved recently, showing a similar structure of four ankyrin repeats and an SH3 domain. The C-terminal domain of iASPP formed a homodimer mediated by contacts between the N-terminal part of one molecule and the SH3 domain and the third ankyrin repeat of a second molecule (6). The ASPP2 Ank-SH3 domains mediate the interactions of ASPP2 with numerous partner proteins, most of which are involved in apoptosis or its regulation. Among these partner proteins are p53 (2), the anti-apoptotic protein Bcl-2 (3), the p65 subunit of the transcription factor NFκB (7), protein phosphatase 1 (8), the pro-apoptotic Yes-associated protein 1 (YAP1) (9), the N-terminal 46 residues of the hepatitis C virus (HCV) core protein (10), and the p53 tumor suppressor protein p63 and p73 (11). The role of the ASPP2 Pro domain is unclear. This domain was not shown to bind other proteins, with the only exception being the interaction with YAP1. This interaction is mediated predominantly via the ASPP2 SH3 domain, but there is an additional binding site in the C terminus of the ASPP2 Pro domain, which is adjacent to the ASPP2 Ank-SH3 domains (9).

Peptides are valuable experimental tools to study proteins in many cases. This is particularly useful in the case of natively unfolded protein domains: Peptides, which are in most cases also unstructured, are excellent tools to study such domains. Peptides are also good models for binding studies, because they may gain their native structure upon ligand binding. This was...
shown for example for peptides derived from the BH4 domain of Bcl-2 (12) and from the pro-apoptotic Bak (13). Peptides are also useful to map interaction sites between proteins: Peptide arrays provide a highly efficient way for identifying the location of the binding sites in both interaction partners (14). There have been numerous applications of peptide arrays for the study of epitope mapping, enzyme binding, and protein–protein interactions (15–19). Here we used peptides to gain information about the structure and interactions of ASPP2 domains using both methodologies: peptides as models for the ASPP2 Pro domain, which we found to be natively unfolded (see below), and peptide arrays to identify the precise binding sites involved in intramolecular and intermolecular interactions of ASPP2.

Our aim in the current study was to elucidate the structure and the interactions of the ASPP2 Pro domain to gain insight into its function. Our results show that the ASPP2 Pro domain is natively unfolded. We found that ASPP2 Pro makes an interaction with the ASPP2 Ank-SH3 domains and mapped the interaction sites in both domains. However, according to our results ASPP2 Pro does not mediate the interactions of ASPP2 with peptides derived from its partner proteins. ASPP2 Pro-Ank-SH3 bound a peptide derived from its partner protein NFkB weaker than ASPP2 Ank-SH3 bound this peptide. This suggested that the presence of the proline-rich domain inhibited the interactions mediated by the Ank-SH3 domains. This was confirmed by our observation that a peptide from ASPP2 Pro competed with a peptide derived from NFkB on binding to ASPP2 Ank-SH3. Based on our results, we propose a model in which the interaction between the ASPP2 domains regulates the intramolecular interactions of ASPP2 with its partner proteins.

**EXPERIMENTAL PROCEDURES**

**Expression Plasmids**—The constructs pRHislipoTEV (pHLT) ASPP2 Ank-SH3, containing ASPP2 893–1128 was a kind gift of Dr. Mark Allen, Cambridge, UK. The construct pGEX2TK ASPP2 Pro-Ank-SH3, containing ASPP2 693–1128 amino acids was a kind gift from Prof. Xin Lu, Ludwig Institute for Cancer Research, London, UK. Point mutations were introduced into the gene encoding ASPP2 using the Site-Direct Mutagenesis kit (iNtRON). The construct pHLT ASPP2 Pro-Ank-SH3 was obtained by cloning of the ASPP2 693–1128 fragment from pGEX2TK ASPP2 Pro-Ank-SH3 into the pHLT pERM2 ANK-SH3 construct. This was done in the following manner: pGEX2TK ASPP2 Pro-Ank-SH3 vector was restricted with BamHI and PstI enzymes. The fragment from this reaction was then used to replace the original BamHI–PstI fragment in the pHLT ASPP2 Ank-SH3, creating pHLT ASPP2 Pro-Ank-SH3. The truncated form pHLT ASPP2 Pro containing ASPP2 amino acids 693–918 was obtained by introducing two stop codons in positions 919 and 920 on the pHLT ASPP2 Pro-Ank-SH3 template, using site-directed mutagenesis with the following oligonucleotides: (i) 5′-GGCTCAGAGGCTATCCTCATATAATAGAGGTTGAAT-TCAAACC-3′; (ii) 5′-GGGGGTTGAATTTCACCCTCTAA- TAATGAGGCTACGCCTGAGCC-3′. All constructs were verified by DNA sequence analysis (Center for Genomic Technologies, the Hebrew University).

**Protein Expression and Purification**—We expressed and purified three truncated ASPP2 variants with and without the HLT tag containing His tag, Lipo domain, and TEV protease cleavage site (20) (Fig. 1B). All pHLT constructs were expressed in *Escherichia coli* BL21 pLYsS cells (Novagen). Cells were grown in 2× YT media containing 1% glucose. Induction was done at 30°C using 0.6 with 0.1 mM isopropyl-β-D-thiogalactopyranoside for the pHLT ASPP2 Pro and for pHLT ASPP2 Pro-Ank-SH3, and with 1 mM isopropyl-β-D-thiogalactopyranoside for pHLT ASPP2 Ank-SH3. All cells were harvested after 14-h incubation at 22°C for constructs ASPP2 Ank-SH3 and ASPP2 Pro and 16°C for construct ASPP2 Pro-Ank-SH3.

**Purification of ASPP2 Pro-Ank-SH3**—Cells were lysed by French Pressure cell press (SIM-AMINCO) and purified on a nickel-Sepharose HP column (Amersham Biosciences) using an AKTA explorer system (Amersham Biosciences). The protein was eluted using imidazole. The eluted protein was digested overnight at 24°C with His-tagged TEV protease (21) and further purified on a Superdex 200 column (Amersham Biosciences). Eluted protein was further purified on nickel-NTA beads (Qiagen) to trap the His-tagged TEV protease and residual uncleaved protein.

**Purification of ASPP2 Pro**—Cells were lysed by French Pressure cell press and purified on a nickel-Sepharose HP column using an AKTA explorer system (Amersham Biosciences). The protein was eluted using imidazole. The protein was further purified on Superose 12 (Amersham Biosciences). Part of the protein was aliquoted and stored at −80°C; the rest of the sample was digested overnight at 24°C with His-tagged TEV protease (21) and further purified on 10-ml nickel-NTA column (Qiagen) to trap the His-tagged TEV protease, the HLT tag, and residual uncleaved protein.

**Purification of ASPP2 Ank-SH3**—Cells were lysed by microfluidizer processor M-110EHI and purified a nickel-Sepharose HP column using an AKTA explorer system (Amersham Biosciences).
and eluted by NaCl gradient. The eluted protein was digested overnight at 30 °C with His-TEV protease (21) and subsequently reapplied onto the nickel-NTA column (Qiagen) to separate the His-tagged TEV protease and residual uncleaved protein. The unbound fraction was further purified on a Sephacryl S-100 column (Amersham Biosciences).

Analytical Size Exclusion Chromatography—HislipoTEV (HLT) 350 μl of ASP2 Pro at 4.1 μM was injected to an analytical gel filtration Superose 12 (300 × 10 mm) column, on an AKTA design Explorer instrument equipped with a Monitor UV-900 detector and the Unicorn software package. The running buffer was 20 mM phosphate buffer, pH 7.0, 150 mM NaCl, 10% glycerol at 4 °C. 150 μl of 86 μM ASP2 Ank-SH3 was separated in 1-ml fractions from an analytical Superose 12 (300 × 10 mm) column equilibrated with 25 mM phosphate buffer with 150 mM NaCl at 4 °C. Molecular mass standards were run under the same conditions, and their elution volumes were used to create a calibration curve. The markers were: thyroglobin (669 kDa), ferritin (440 kDa), catalase (232 kDa), β-amylase (200 kDa), aldolase (163 kDa), bovine serum albumin (67 kDa), ovalbumin (44 kDa), and RNase A (13.7 kDa).

Peptide Array Screening—The peptide arrays were synthesized by Jerini Bio Tools GmbH (Berlin). The peptides were acetylated at their N terminus and attached to cellulose via their C terminus by an amide bond. Binding of the human ASP2 893–1128 (ASPP2 Ank-SH3) to the cellulose-bound peptides was screened as described previously (17) with the following modifications: The peptide array was prewashed in binding buffer (30 mM Tris-HCl, pH 7.6, 0.075 mM NaCl, 5 mM MgCl₂, 5% sucrose, 0.05% Tween 20). ASP2 Ank-SH3 in a final concentration of 6.8 μM or ASP2 Pro (final concentration, 1.4 μM) was incubated with the array in binding buffer overnight at 4 °C with gentle shaking. The electrotransfer was performed in four steps: 10-min transfer followed by replacement of the polyvinylidene difluoride membrane and continuation of the transfer for additional 30 min; afterward all the “sandwiched” (blotting paper and polyvinylidene difluoride membrane) was replaced, and the transfer was continued for two more 30-min intervals. Transferred ASP2 was detected using enhanced chemiluminescence blotting substrate SuperSignal reagent (Pierce) was used according to the manufacturer’s instructions for detection using ECL.

Nickel-affinity Pulldown Assay—59 μl of 45 μM ASP2 Ank-SH3 was incubated for 1 h with gentle agitation at 4 °C with 500 μl of 5.3 μM HLT ASP2 Pro in 25 mM NaH₂PO₄, 150 mM NaCl, 10% glycerol, and with 500-μl wash buffer (20 mM Tris-HCl, pH 7.0, with 150 mM NaCl, 10% glycerol, and 10 mM imidazole) alone. 20 μl of nickel-NTA beads (Qiagen) equilibrated with wash buffer was added, and the incubation continued for another 1 h. The samples were centrifuged for 4 min at 3500 rpm at 4 °C, and the suspension was collected and defined as unbound. Samples were centrifuged, unbound material was collected, and beads were washed five times with buffer before elution with buffer containing 250 mM imidazole. Finally, samples were analyzed on a 12% SDS-PAGE gel.

CD—CD spectra were recorded using a J-810 spectropolarimeter (Jasco) in 5 mM phosphate buffer, pH 7.5, 30 mM NaCl, and 2% glycerol in a 0.1-cm quartz cuvette for far-UV CD spectroscopy. Far-UV CD spectra were collected in a spectral range of 185 to 200 nm. The changes in CD spectra with temperature were measured in a temperature range of 20–90 °C in 10 °C steps. Prior to each experiment ASP2 Pro was centrifuged in 13,200 rpm for 10 min at 4 °C, and the UV spectrum between 200 and 400 nm was measured. The amount of secondary structure was analyzed using DichoWeb (22) with K2D software.

Fluorescein Labeling—The peptides were labeled using 5’ (and 6’)-carboxyfluorescein succinimidyl ester (Molecular Probes) at the N terminus as described before (23).

Fluorescence Anisotropy—Measurements were performed at 10 °C by using a PerkinElmer Life Sciences LS-50b spectrofluorometer equipped with a Hamilton microLab M dispenser (24, 25). Titration of ASP2 Ank-SH3 into the fluorescein-labeled peptides was performed in 20 mM Hepes at pH 7.3 with ionic strength (IS) of 50 mM, 100 mM, and 150 mM. Titration of ASP2 Ank-SH3 and of ASP2 Pro-Ank-SH3 into the fluorescein-labeled NFκB 303–332 was performed in 20 mM NaH₂PO₄ at pH 7.0 with an IS of 50 mM. Fluorescence was measured with excitation at 480 nm and emission at 530 nm. The bandwidths were changed depending on the amount of the labeled molecule used. The labeled peptide was placed in the cuvette in a volume of 1 ml, at a concentration of 100 nM, and 150–200 μM of ASP2 Ank-SH3 was placed in the dispenser. Additions of 5 μl were titrated at 90-s intervals, the solution was stirred for 10 s, and the fluorescence and anisotropy were measured. Dissociation constants (Kₐ) were calculated by fitting the anisotropy titration curves (corrected for the dilution effect) by using Kaleidagraph (Synergy Software, Reading, PA). The following equation was used for the single-site model,

\[
    r = r_0 + \frac{r_{[\text{ASPP2 Ank-SH3}]} - K_d}{K_d + [\text{ASPP2 Ank-SH3}]} \quad (\text{Eq. 1})
\]

Where, \( r_0 \) is the initial anisotropy, \( r_i \) is the difference in anisotropy between unbound and bound anisotropy, \( K_d \) is the dissociation constant, \([\text{ASPP2 Ank-SH3}]\) is the molar concentration of ASP2 Ank-SH3. Each experiment was performed at least three times, and \( K_d \) values were always reproducible. The experimental errors for \( K_d \) values are the result of the data fitting.

In the different competition binding assays, the conditions were as following: 150 μl of 750 μM ASP2 723–737 was added to a 1150-μl mixture of 87 nm fluorescein-labeled NFκB 303–332 and 2 μM ASP2 Ank-SH3 at 50 mM IS; 150 μl of 195 μM NFκB 303–332 was added to a 1150-μl mixture of 87 nm fluorescein-labeled ASP2 723–737 and 10.5 μM ASP2 Ank-SH3 at 50 mM IS; 100 μl of 1500 μM ASP2 723–737 was added to a 1200-μl mixture of 83 nm fluorescein-labeled ASP2 723–737 and 26.5 μM ASP2 Ank-SH3 at 150 mM IS.
Disorder Predictions—We have used the following servers for disorder prediction for ASPP2: NORS (26), Disopred (27), PONDR (28), GlobPlot (29), RONN (30), PreLink (31), IUPred (32), SEG (33), DisPro (34), Disprot (35), and FoldIndex (36). In all cases, the whole sequence of ASPP2 (SP: ASPP2_HUMAN) was subjected to disorder prediction using default server parameters.

Cross-linking—ASPP2 Pro-Ank-SH3 and ASPP2 Ank-SH3 (3.75 μM final concentration) were incubated at 37 °C for 30 min in a pH 7.0 buffer containing 20 mM NaH2PO4, 110 mM NaCl, 5 mM β-mercaptoethanol, and 10% glycerol. The reaction mixtures were treated with the cross-linking agent BS3 (25, 50, or 100 eq, Pierce) for 30 min at 37 °C. The reaction mixtures were denatured and analyzed by 10% SDS-PAGE (37, 38).

Analytical Ultracentrifugation—AUC experiments were performed at 10 °C using a Beckman Optima XLI analytical ultracentrifuge with an An60Ti rotor. A 2.9 μM sample of ASPP2 Pro-Ank-SH3 (693–1128) was subject to sedimentation equilibrium experiments. The buffer was 25 mM NaH2PO4, pH 7.0, 150 mM NaCl, 10% glycerol, 0.02% NaN3, and 10 mM β-mercaptoethanol. The reaction mixtures were treated with the cross-linking agent BS3 (25, 50, or 100 eq, Pierce) for 30 min at 37 °C. The reaction mixtures were denatured and analyzed by 10% SDS-PAGE (37, 38).

RESULTS

ASPP2 Pro Domain Is Natively Unfolded—We used analytical size-exclusion chromatography to study the oligomerization state and compactness of the ASPP2 variants. ASPP2 Pro (aa 693–918) and ASPP2 Ank-SH3 (aa 893–1128) both eluted from the gel-filtration column as single peaks, indicating the existence of a single oligomeric form in both cases. The ASPP2 Pro domain eluted earlier than predicted from its molecular mass, and its apparent molecular mass estimated from the calibration curve was ~130,000 Da (Fig. 2A), a value five times higher than the theoretical value of 24,500 Da. This early elution in the gel filtration may be attributed to an extended or unfolded native structure of the protein (39) or to putative oligomerization. The structured ASPP2 Ank-SH3 eluted as expected from its molecular weight. For this protein, the calculated and the estimated molecular mass were the same, with a value of 26,600 Da (Fig. 2B).

To determine the basis for the early elution of ASPP2 Pro in the gel-filtration experiments, we analyzed its secondary structure using CD spectroscopy. The far-UV CD spectrum of the recombinant purified protein exhibited a minimum at only ~200 nm (Fig. 3A), suggesting a highly unstructured conformation. To further test this, we monitored changes in the far-UV CD signal as a function of temperature (Fig. 3B). No change in the signal was observed, indicating no structural transition upon temperature increase. This is typical for unstructured proteins (40). Taken together, the CD spectra indicate a high content of unstructured regions in ASPP2 Pro domain.

We used computational methods to verify whether ASPP2 Pro is unstructured. We have submitted the sequence of ASPP2 to twelve publicly available servers that implement algorithms for protein disorder prediction (26–36, 41). These algorithms are based on different criteria to predict disordered conformation from sequence, as reviewed in Ferron et al. (41). In all cases we submitted the full-length ASPP2 sequence (1128 aa) and used the default parameters. The C-terminal part of ASPP2 (926–1128) is known to be structured (5) and thus serves as a positive control for the disorder prediction. The results show that the sequence of the ASPP2 Pro domain is predicted to be disordered, whereas the sequence of residues 918–1128, which contains the Ank-SH3 domains, is predicted to be folded, as expected, by the different prediction methods (Fig. 3C). 57% of the amino acids in ASPP2 693–918 (ASPP2 Pro) are typical for disordered regions (Glu, Lys, Arg, Gly, Gln, Ser, and Pro) (42–44) (Fig. 3D), compared with only 34% in the ASPP2 Ank-SH3 domains. On the other hand, the amino acids Ile, Leu, Val, Trp, Phe, Tyr, Cys, and Asn, which are not favored in disordered domains. On the other hand, the amino acids Ile, Leu, Val, Trp, Phe, Tyr, Cys, and Asn, which are not favored in disordered regions, make only up 27% of the ASPP2 Pro domain sequence. Together, our results imply that the ASPP2 Pro domain is highly unstructured with an extended conformation.

Domain-Domain Interaction between ASPP2 Pro and ASPP2 Ank-SH3—Proline-rich domains are known to serve as binding regions for SH3 domains (45, 46). This raised the possibility of a putative domain-domain interaction between the proline-rich and Ank-SH3 domains of ASPP2. We performed a nickel-affin-
Domain-Domain Interaction in ASPP2

The Sites in ASPP2 That Mediate the Domain-Domain Interaction—To identify the precise residues in ASPP2 Pro and ASPP2 Ank-SH3, which mediate the domain-domain interaction, we designed membrane-bound peptide arrays containing partly overlapping peptides derived from the ASPP2 sequence (for peptide sequences see supplemental data). The peptide arrays were screened for binding the ASPP2 Pro and the ASPP2 Ank-SH3 domains. ASPP2 Pro-bound peptides from the first Ank repeat (aa 931–961) and from the SH3 domain (aa 1083–1096) (Table 1 and Fig. 5, A, B, and D). Screening a second peptide array revealed that ASPP2 Ank-SH3 bound several peptides derived from ASPP2 Pro (Table 2 and Fig. 5, C and D). These peptides are located between residues 693 and 752 at the N-terminal region of the ASPP2 Pro domain, and between residues 893 and 912 at its C terminus.

To quantify the binding of the ASPP2 Pro peptides to ASPP2 Ank-SH3, we synthesized fluorescein-labeled peptides derived from the overlapping binding sequences discovered in the peptide array and tested their binding to ASPP2 Ank-SH3 using fluorescence anisotropy. ASPP2 Ank-SH3 bound ASPP2 Pro-Ank-SH3 with a $K_d$ of 16 μM (Fig. 5E) at physiological IS. Binding to ASPP2 Pro 693–712 was evident but did not reach saturation and was too weak to quantify (data not shown). ASPP2 Pro 723–737 to ASPP2 Ank-SH3 at different IS and found that the $K_d$ at IS = 100 mM was 5 μM and at IS = 50 mM was 0.6 μM (Fig. 5E). The IS dependence of $K_d$ indicates an electrostatic contribution to the interaction. We could not quantify the binding of the ASPP2 AnK-SH3 peptides to ASPP2 Pro, because of the instability of ASPP2 Pro that prevented us from reaching the high concentrations required for these studies.

ASPP2 Pro-Ank-SH3 but Not ASPP2 Pro Mediates Interactions with Peptides Derived from ASPP2-binding Proteins—To determine whether ASPP2 Pro is involved in the binding of ASPP2 to its partner proteins, we carried out another peptide array experiment. We designed an array of cellulose-bound partly overlapping peptides, derived from several proteins known to bind ASPP2 (Table 3, see supplemental data for peptide sequences). These proteins include the p65 subunit of NFκB (7), protein phosphatase 1 (8), HCV core protein (10), YAP1 (9) and the Bcl-2 family member Bcl-W.4 The peptides were designed based on the domains in these proteins that are known to bind ASPP2 (Table 3). The peptides from YAP1 and HCV core protein were derived from short sequences already known to bind experiment. Taken together, our data suggest that the proline-rich domain of ASPP2 is necessary for the dimer formation. To test the native oligomeric state of ASPP2 Pro-Ank-SH3 we used AUC. Sedimentation equilibrium experiments showed that the protein was a monomer, with a molecular mass of 43126 ± 2047 Da (Fig. 4D). This supports our idea that the residual dimer formation in the cross-linking experiments may be due to the experimental conditions, resulting in some intermolecular domain-domain interactions in addition to the intramolecular ones.

ASPP2 and served as positive controls (9, 10). We screened ASPP2 Pro and ASPP2 Ank-SH3 for binding the peptide array (Fig. 6). ASPP2 Pro did not bind the peptides in the array except for one positive spot, which is attributed to the YAP peptide that is known to bind this domain (Fig. 6A). ASPP2 Ank-SH3 bound to several peptides derived from each of the partner proteins (Fig. 6A and Table 3). The peptides from YAP1 and HCV core protein that served as positive controls indeed bound to ASPP2 Ank-SH3 in the array screening. The peptides from NFκB (p65), which we found to bind ASPP2, represent sites in NFκB that are known to mediate intermolecular interactions (Fig. 6B). The ASPP2 binding site between amino acids 303 and 355 of ASPP2 Ank-SH3 and fluorescently labeled NFκB 303–332 at IS = 50 mM, competition took place and the anisotropy decreased almost completely back to the initial values (Fig. 7A). The same trend was observed when the competition was performed whether NFκB 303–332 and the ASPP2 Pro-derived peptide ASP2 723–737 compete on the same binding site in ASP2 Ank-SH3. When unlabeled ASP2 723–737 was added to a pre-formed complex of ASP2 Ank-SH3 and fluorescently labeled NFκB 303–332 at IS = 50 mM, competition took place and the anisotropy decreased almost completely back to the initial values (Fig. 7B). The same trend was observed when the competition was performed in the opposite way: Unlabeled NFκB 303–332 displaced fluorescently labeled ASP2 723–737 even quicker (Fig. 7C), because its affinity to ASP2 Ank-SH3 was much tighter. As a control, we showed that there is competition between labeled and unlabeled ASP2 723–737 (Fig. 7D). In summary, our results show that the interaction between ASP2 Pro and ASP2 Ank-SH3 inhibits the intermolecular interaction of the latter with the NFκB peptide.

The ASPP2 Pro Domain Inhibits the Binding of ASPP2 Ank-SH3 to an NFκB Peptide—To gain insight into the biological role of the domain-domain interaction in ASPP2, we tested whether binding of ASPP2 Pro to ASP2 Ank-SH3 affects the intermolecular interactions mediated by ASP2 Ank-SH3. As a model, we used the NFκB-derived peptide comprising residues 303–332, which was found to bind ASP2 Ank-SH3 in the peptide array screening. We used fluorescence anisotropy to test the affinity of ASP2 Ank-SH3 and ASP2 Pro-Ank-SH3 to this peptide. ASP2 Ank-SH3 bound NFκB 303–332 with $K_d = 0.27 \text{ nM}$, whereas ASP2 Pro-Ank-SH3 bound this peptide with $K_d = 1.2 \text{ nM}$, an order of magnitude weaker (Fig. 7A). This shows that the presence of the proline-rich domain inhibits the interaction of ASP2 Ank-SH3 with the NFκB peptide. Next, we tested by competition fluorescence anisotropy whether NFκB 303–332 and the ASPP2 Pro-derived peptide ASP2 723–737 compete on the same binding site in ASP2 Ank-SH3. When unlabeled ASP2 723–737 was added to a pre-formed complex correspends to the known binding site for IκB, the natural inhibitor of NFκB, which contains ankyrin repeats like ASPP2 (47). The second ASPP2-binding site, between amino acids 21 and 50, is found in the DNA binding site of NFκB (48). We conclude that the proline-rich domain of ASP2 is not involved in the interactions of ASP2 with the partner proteins tested here. These interactions are mediated by ASP2 Ank-SH3 domains.

**FIGURE 4. Direct interaction between the ASPP2 Pro and ASPP2 Ank-SH3 domains.** A, nickel-affinity pull-down assay of ASPP2 Ank-SH3 by N-terminal His-tagged ASP2 Pro. ASPP2 Ank-SH3 was incubated with His-tagged ASP2 Pro (upper panel) or with wash buffer (lower panel) for 1 h at 4 °C and then incubated for another hour with nickel-NTA beads. The samples were centrifuged, and the suspension was kept (lane 1). The beads were washed with wash buffer containing 10 mM imidazole for five times (lanes 2–6), and then the proteins were eluted by the elution buffer, containing 250 mM imidazole, four times (lanes 8–11). ASPP2 Ank-SH3 was found in the elution fractions (8–11) only when incubated with ASP2 Pro, but not with the buffer, indicating binding between the proteins. LMW (Amersham Biosciences) marker was used in lane 7. B, incubation of ASPP2 Pro-Ank-SH3 with different concentrations of the cross-linker BS3 resulted in some formation of dimers. Shown are the results for 25 equivalents of BS3 (lane 2), 50 equivalents of BS3 (lane 3), and 100 equivalents of BS3 (lane 4). No dimer was observed in the absence of the cross-linker (lane 1); C, incubation of ASPP2 Pro-Ank-SH3 with ASP2 Pro-Ank-SH3 with or without BS3 (lanes 5 and 6), resulted also in some formation of a second type of dimer with a different molecular weight, corresponding to a dimer between ASPP2 Pro-Ank-SH3 and ASP2 Ank-SH3. Incubation of ASP2 Pro-Ank-SH3 with or without BS3 (lanes 1 and 2) and incubation of ASP2 Ank-SH3 with or without BS3 (lanes 3 and 4) are shown as controls. D, AUC analysis of ASPP2 Pro-Ank-SH3 (top panel). Sedimentation equilibrium experiments shows that the protein is monomeric with molecular mass = 43,126 ± 2,047 Da. The expected molecular mass of the protein is 48,300 Da. The **bottom panel** represents the residuals for the fit.
**DISCUSSION**

In the current study we performed detailed quantitative characterization of the structure and interactions of the ASPP2 Pro domain. We found that ASPP2 Pro is natively unfolded and that it binds the Ank-SH3 domains of ASPP2, but not peptides derived from ASPP2 binding proteins. The domain-domain interaction has a large inhibitory effect on the binding of ASPP2 Ank-SH3 to NF\(\text{H9260}\)B peptide. We suggest that the unstructured proline-rich domain of ASPP2 has a role in regulating the protein-protein interactions of ASPP2 by forming domain-domain interaction with the structured ASPP2 Ank-SH3.

**FIGURE 5.** The specific binding sites between the ASPP2 Pro and ASPP2 Ank-SH3 domains. A, the sites in ASPP2 Ank-SH3 that bind ASPP2 Pro: An array consisting of peptides derived from ASPP2 was screened for binding ASPP2 Pro. Every dark spot represents binding between the corresponding peptide and ASPP2 Pro. For sequences of binding peptides see Table 1. For the complete peptide list see the supplemental data.

**TABLE 1** Binding of ASPP2 Pro to ASPP2-derived peptides: screening of peptide array #1

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<th>Peptide number</th>
<th>Binding residues</th>
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<td>14–15</td>
<td>931–961</td>
<td>LDSSLGEPDLVQRITYVEVDPPKLPMTEIT</td>
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<tr>
<td>38</td>
<td>1083–1096</td>
<td>CNTTHREDEDEIE</td>
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**TABLE 2** Binding of ASPP2 Ank-SH3 to ASPP2-derived peptides: screening of peptide array #2

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<td>693–712</td>
<td>IPRPLSPTKLPPSNPYPNN</td>
</tr>
<tr>
<td>3</td>
<td>713–732</td>
<td>QSDMELALKLNNAPRPL</td>
</tr>
<tr>
<td>4</td>
<td>723–742</td>
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<tr>
<td>5</td>
<td>733–752</td>
<td>KKRSISITPEPQNPQPNQLK</td>
</tr>
<tr>
<td>21</td>
<td>893–912</td>
<td>EITQQVSLPPGKRTLKSTG</td>
</tr>
</tbody>
</table>

**FIGURE 6.** Binding of ASPP2 to peptides derived from its binding proteins is mediated by ASPP2 Ank-SH3 but not by ASPP2 Pro. A peptide array consisting of peptides derived from the ASPP2-binding proteins specified at Table 3 (Bcl-W in red, p65 in green, protein phosphatase 1 in pink, HCV core protein in orange, and YAP1 in blue) was screened for binding of ASPP2 Pro (A), which did not bind almost any of the peptides in the array and, ASPP2 Ank-SH3 (B). For sequences of binding peptides see Table 3. For the complete peptide list see the supplemental data.

**TABLE 3** Binding of ASPP2 Ank-SH3 to ASP2-binding proteins: screening of peptide array #3

<table>
<thead>
<tr>
<th>ASPP2-partner protein</th>
<th>Residues in the partner protein</th>
<th>Binding peptides number</th>
<th>Binding residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-W</td>
<td>1–188</td>
<td>5</td>
<td>10–35</td>
</tr>
<tr>
<td>p65 (NF(\text{H9260})B) (7)</td>
<td>176–405</td>
<td>9</td>
<td>57–86</td>
</tr>
<tr>
<td>PP1 (8)</td>
<td>1–317</td>
<td>58</td>
<td>19–48</td>
</tr>
<tr>
<td>HCV core protein (10)</td>
<td>1–46</td>
<td>86</td>
<td>1–20</td>
</tr>
<tr>
<td>YAP1 (9)</td>
<td>84–208</td>
<td>89</td>
<td>81–100</td>
</tr>
</tbody>
</table>

**DISCUSSION**

In the current study we performed detailed quantitative characterization of the structure and interactions of the ASPP2 Pro domain. We found that ASPP2 Pro is natively unfolded and that it binds the Ank-SH3 domains of ASPP2, but not peptides derived from ASPP2 binding proteins. The domain-domain interaction has a large inhibitory effect on the binding of ASPP2 Ank-SH3 to NF\(\text{H9260}\)B peptide. We suggest that the unstructured proline-rich domain of ASPP2 has a role in regulating the protein-protein interactions of ASPP2 by forming domain-domain interaction with the structured ASPP2 Ank-SH3.

**The Unstructured Nature of the ASPP2 Pro Domain—Our results, obtained using CD, analytical gel filtration, and theore-
NF-B 303–332 tighter than ASPP2 Pro-Ank-SH3: fluorescence anisotropy studies. In control experiment of competition between unlabeled ASPP2 723–737 and fluorescein labeled NF-B 303–332 competes for binding ASPP2 Ank-SH3: fluorescence anisotropy competition experiments. Shown are the following competition experiments: Left panel in B–D shows the binding curves for the titration of ASPP2 Ank-SH3 into a fluorescein-labeled peptide, and the right panel describes the competition, where unlabeled peptide was titrated into the fully formed complex. Shown are the following competition experiments: unlabeled ASPP2 723–737 and fluorescein labeled NF-B 303–332 (B), unlabeled NF-B 303–332 and fluorescein labeled ASPP2 723–737 (C), and a control experiment of competition between unlabeled ASPP2 723–737 and fluorescein labeled ASPP2 723–737 (D).

The Sites in ASPP2 That Mediate Domain-Domain Interaction—We have mapped the sites in ASPP2 Pro that are involved in binding the ASPP2 Ank-SH3 domains and found four binding sequences that map to three regions in the proline-rich domain (Fig. 5D). The tightest binding peptide, spanning residues 723–737, lacks the typical PXXP motif and is one of the only positively charged segments (charge = +6) in the highly acidic 53BP2 region of ASPP2 (pl = 4.75). The intrinsic strength dependence of the affinity of this peptide to ASPP2 Ank-SH3 indeed shows that there is a strong electrostatic contribution to the binding. Therefore, the overall binding between the domains could be initiated by an electrostatic attraction between the positive ASPP2 723–737 and the negative ASPP2 Ank-SH3, with subsequent fine-tuning of the interaction by the other ASPP2 Pro sequences involved. Additional binding peptides, which contain the PXXP motif, were identified in the peptide array screening but failed to show significant binding as free peptides in solution. It is possible that, although the binding of each of these peptides, which are not as positively charged as the 723–737 peptide, is weak, there is a cooperative effect within the full protein that promotes the binding.

The sites in ASPP2 Ank-SH3 that mediate the binding to ASPP2 Pro are located both in the SH3 domain and in the first proline-rich domain (Fig. 6) as well as data in the literature about interactions between the full-length proteins (7–10) show that the Ank-SH3 domains are those that predominantly mediate the protein-protein interactions of ASPP2. If so, then what is the role of the proline-rich domain? Our data show that it is involved in an interaction with other ASPP2 domains: the ankyrin repeats and SH3 domain. We conclude that the ASPP2 Pro domain represents a unique case of a natively unfolded domain, because it mediates binding to another domain in the same protein but does not mediate almost any intermolecular interaction with the peptides and proteins tested so far (9).

ASPP2 belongs to the group of proteins that contain natively unfolded domains. Such domains thermodynamically favor an unstructured rather than a folded state. Almost 30% of the eukaryotic genome encodes for proteins that are completely or partly unstructured (43, 50, 51). Natively unfolded proteins and protein domains are characterized by an almost complete lack of secondary and tertiary structure and an extended conformation with high flexibility (52). The lack of structure in natively unfolded proteins does not mean lack of function, and natively unfolded proteins present a case where the active state is unstructured (51, 53, 54). The structural flexibility of natively unfolded proteins and protein domains enables them to bind a large number of partner ligands by induction of folding (52). In our case, the natively unfolded ASPP2 Pro does not behave as a typical natively unfolded domain, because it does not interact with many proteins. On the contrary, our peptide array results (Fig. 6) as well as data in the literature about interactions between the full-length proteins (7–10) show that the Ank-SH3 domains are those that predominantly mediate the protein-protein interactions of ASPP2. If so, then what is the role of the proline-rich domain? Our data show that it is involved in an interaction with other ASPP2 domains: the ankyrin repeats and SH3 domain. We conclude that the ASPP2 Pro domain represents a unique case of a natively unfolded domain, because it mediates binding to another domain in the same protein but does not mediate almost any intermolecular interaction with the peptides and proteins tested so far (9).
Domain-Domain Interaction in ASPP2

![Diagram of domain-domain interaction in ASPP2](attachment://domain-domain_inter.png)

**FIGURE 8.** A proposed mechanism by which the ASPP2 Pro domain regulates the protein-protein interactions of ASPP2. The unstructured ASPP2 Pro domain binds the ASPP2 Ank-SH3 domains. This interaction could be either intramolecular within a given ASPP2 monomer, or intermolecular resulting in the formation of dimers. According to our results this equilibrium favors the intramolecular interaction and not dimerization. We propose that, by doing so, ASPP2 regulates its protein-protein interactions. The binding of the ASPP2 Pro domain to the ASPP2 Ank-SH3 domains blocks other protein-protein interactions that these domains mediate. Following a yet unknown control signal, the ASPP2 Pro domain is released from the ASPP2 Ank-SH3 domains, making them available to bind the partner proteins.

Implications for Regulation and Function of ASPP2—Based on our results, we propose a model according to which the interaction between the ASPP2 domains may serve as a control mechanism for regulating the interactions of ASPP2 with other proteins (Fig. 8). According to our model, the unfolded ASPP2 Pro domain, probably from the same ASPP2 monomer, wraps around the structured Ank and SH3 domains, preventing them from intermolecular binding to other proteins or to another ASPP2 molecule. When the ASPP2 Ank-SH3 domains are released from this interaction, following one or more as yet unknown control mechanisms, they are available for binding the specific target protein. Our results show that the presence of the proline-rich domain weakened the binding of the Ank-SH3 domains to a model target peptide derived from NFkB. These results support the model and confirm that the interactions of ASPP2 Ank-SH3 with ASPP2 Pro and with its binding proteins may take place via the same site.

The putative control mechanism that switches between the domain-domain and intermolecular interactions in ASPP2 could potentially involve post-translational modifications such as phosphorylations, because disorder-promoting residues are known to surround phosphorylation sites and it is assumed that protein phosphorylation occurs mainly within intrinsically disordered protein regions (56). Indeed, it was found that Bbp (ASPP2 123–1128) is phosphorylated and that constitutively active kinases weaken the interaction between YAP1 and Bbp in *vivo*, probably by phosphorylating Bbp (9). In *vitro*, various phosphopeptides derived from the YPPYPPPPYPS motif of ASPP2 (aa 866–876) also inhibit the interaction of Bbp with the YAP1 WW1 domain (9). ASPP2 423–848 was also found to be ubiquitinated, and its level is regulated by proteasomal degradation (57, 58). It is also possible that the interaction between ASPP2 Pro and ASPP2 Ank-SH3 also modulates the degradation of ASPP2.

Our model explains the reported observation that the interaction of Bbp and YAP1 in *vivo* is drastically increased when only the C-terminal part of Bbp (amino acids 852–1128, which is also the C-terminal part of ASPP2) was used (9). This C-terminal truncated form does not contain the regions in the proline-rich domain, which we found to bind the ASPP2 Ank-SH3 domains. Deletion of the proline-rich domain releases the Ank-SH3 domains from an intramolecular interaction, making it available to bind its target protein (YAP1 in this case).

Our study focused on the C-terminal part of ASPP2. We cannot exclude the possibility that the N-terminal domains of the protein also participate in intramolecular or intermolecular domain-domain interactions as a mechanism of regulating the activity of the protein. We are currently performing additional experiments to account for such possible interactions. In any case, the unfolded nature of the proline-rich domain makes it particularly suitable for interactions with other domains also in the context of the full-length protein.

Our results shed light on the structure and possible function of the ASPP2 Pro domain and extend the group of natively unfolded proteins. Future studies in cells will have to be performed to prove the proposed mechanism *in vivo* and to reveal the control mechanisms by which the domain-domain interaction in ASPP2 is regulated.
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REFERENCES