Regulation of Survivin Stability by the Aryl Hydrocarbon Receptor-interacting Protein

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Survivin is a multifunctional member of the IAP (inhibitor of apoptosis) family, but its molecular interactions in protection from cell death and regulation of cell division have not been completely elucidated. In a proteomics screening to identify novel survivin-binding partners, we found that the aryl hydrocarbon receptor-interacting protein (AIP) directly associates with survivin in vitro and in co-immunoprecipitation experiments in vivo. This interaction is mediated by the carboxyl-terminal end of AIP, which contains three tetratricopeptide motifs, and involves the carboxyl terminus coiled coil in survivin with critical roles of Asp142 in AIP recognition. A survivin mutant lacking only Asp142 fails to bind AIP and exhibits accelerated degradation in vivo in a reaction reversed by a proteasome inhibitor. Acute knock-down of AIP by short interference RNA or competition of the survivin-AIP complex by peptidyl mimicry destabilizes survivin levels in cells, with enhanced apoptosis but no changes in cell cycle progression. Therefore, AIP regulates survivin stability, thus elevating a cellular anti-apoptotic threshold. The survivin-AIP complex may influence the cellular xenobiotic response to environmental toxin(s) and contribute to subcellular chaperone trafficking during cell death regulation.

The IAP (inhibitor of apoptosis) family comprises evolutionarily conserved members that act to buffer cell death stimuli by associating with caspases, the effector enzymes of apoptosis, and prevent their catalytic activation and/or activity (1). Among IAP proteins, survivin is structurally and functionally unique, as has been implicated not only in preservation of cell viability but also in essential regulatory circuitries of cell division (2), potentially by regulating chromosomal segregation (3) and mitotic spindle formation (4). Recently, a third emerging function of survivin has been identified in the cellular stress response. This is centered on two potentially intersecting pathways: the association of survivin with the molecular chaperone Hsp90 (5), which is required to preserve survivin stability in vivo, and dynamic changes in survivin levels that occur rapidly in response to noxious environmental stimuli, including hypoxia, irradiation, exposure to chemotherapeutic drugs, and heat shock (5, 6). Although changes in survivin gene transcription have been invoked in the latter response (7), other results have highlighted the role of rapid adaptive mechanisms regulating survivin protein expression, either through post-translational modifications that affect survivin stability, such as phosphorylation (8), or dynamic redistribution of survivin pools among subcellular compartments (9, 10), including mitochondria (6).

To further define the molecular partners that affect survivin stability/subcellular shuttling and thus regulate its multiple functions (2), we have undertaken a proteomics screening to identify novel survivin-associated molecules. We found that the aryl hydrocarbon receptor-interacting protein (AIP)3 (11), a critical modulator of the cellular xenobiotic response to environmental toxin(s) and a component of the Hsp90 chaperone system (12), directly associates with survivin, thus preserving protein stability and a cytosolic anti-apoptotic threshold.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Cervical carcinoma HeLa, breast adenocarcinoma MCF-7, and lymphoblastoid Raji cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in culture as recommended by the supplier. An antibody to AIP was purchased from NOVUS Biologicals (Littleton, CO). A rabbit polyclonal antibody to survivin was from NOVUS. Antibodies against XIAP and phosphorylated eukaryotic initiation factor 2α were obtained from BD Biosciences (San Diego, CA), and Cell Signaling (Danvers, MA), respectively. Biotin-conjugated peptides duplicating a survivin-binding site for AIP or its scrambled sequence were synthesized, purified by high pressure liquid chromatography to >95% purity, and confirmed by mass spectrometry (Peptron, Daejeon, South Korea). Biotin-conjugated peptides were made cell-permeable by fusing the human immunodeficiency virus Tat protein basic domain cell-penetrating sequence YGRKKRRQRRR at the NH2 terminus of each peptide, as described (13). Amplification and cloning oligonucleotides were purchased from Invitrogen.

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3 The abbreviations used are: AIP, aryl hydrocarbon receptor-interacting protein; siRNA, short interference RNA; GST, glutathione S-transferase; BSA, bovine serum albumin; PBS, phosphate-buffered saline; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; dsRNA, double-stranded RNA; HA, hemagglutinin; TPR, tetratricopeptide repeat; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
Survivin-AIP Interaction

**Molecular Cloning of AIP, Survivin, and Deletion Mutants**—Total RNA was isolated from HeLa cells using an RNasy mini kit (Qiagen) according to the manufacturer’s specifications. After first strand cDNA synthesis using SuperScript (Invitrogen), an AIP transcript was amplified using primers 5′-AAAAGAATTCCGAAGGAGATGGCGGATACG-3′ and 5′-AAAACTCGAGTCATGGGAGATCCGATCTG-3′. A carboxyl-terminal fragment of AIP comprising residues 170–330 was amplified with 5′-H11032ATCCACGCGGAACCAGATC-3′ and 5′-AAAAAATTTCATGACAGACGAAGAGAAGGCAAAG-3′. An amino-terminal fragment of AIP containing residues 1–169 vectors to generate pcDNA-AIP and pGEX-AIP, respectively. pcDNA3.0 (Invitrogen) or pGEX-4T (Amersham Biosciences) products were validated by DNA sequencing and inserted in the SWISS-PROT and NCBI databases using the search program ProFound (prowl.rockefeller.edu).

**Protein Expression and Purification**—Recombinant proteins were expressed as GST fusion proteins in Escherichia coli BL21- CodonPlus-RIL (Stratagene) strain with induction in 0.2 mM isopropyl β-D-thiogalactopyranoside for 5 h at 30 °C. The cells were harvested by centrifugation at 6,000 × g for 20 min, suspended in 50 mM Tris, pH 7.4, 500 mM NaCl, 5 mM MgCl2, and 1 mM dithiothreitol, and lysed by sonication. After centrifugation at 15,000 × g for 30 min, the soluble fractions were mixed with glutathione-agarose beads (Sigma-Aldrich) and incubated for 1 h at 4 °C. After centrifugation at 1,000 × g for 1 min, beads containing proteins were washed three times in 50 mM Tris, pH 7.4, 500 mM NaCl, 5 mM MgCl2, and 1 mM dithiothreitol and further purified by chromatography over Econo-column (Bio-Rad) after overnight thrombin (Sigma) treatment to release the GST frame. The protein concentrations were measured using a protein assay reagent (Bio-Rad) with BSA as standard.

**Identification of Novel Survivin-binding Protein(s)**—HeLa cells were harvested by scraping and centrifugation followed by washing in ice-cold PBS. The cell pellet was suspended in lysis buffer containing 25 mM Hepes, pH 7.5, 100 mM KCl, 1% Triton X-100, plus protease inhibitors (Roche Applied Science) under continuous agitation for 30 min at 4 °C. After removal of the insoluble fraction, the cell extract was precleared with glutathione-agarose beads (Sigma-Aldrich) for 4 h at 4 °C. The lysate was mixed with control GST beads or GST beads coupled to recombinant survivin and washed with 20 bead volume of PBS, and bound proteins were eluted in 20 mM Tris, pH 7.4, 2 mM EDTA, 0.1% CHAPS, and 1 mM NaCl. The eluates were concentrated by precipitation with ProteoExtract protein precipitation kit (Calbiochem), and the pellets were analyzed by two-dimensional gel electrophoresis, followed by silver staining (Genomed, Pohang, Kyungbuk, South Korea). Image analysis of two-dimensional gel electrophoresis gels was performed using the PDQuest software according to the protocols provided by the manufacturer (Bio-Rad). The two-dimensional gel electrophoresis gel images obtained from GST or GST-survivin eluates were matched after normalization of total valid spot intensities. Spots detected exclusively in the eluate from GST-survivin were excised from the gel and enzymatically digested with sequence grade trypsin (Promega). After extraction from the gel, the peptides were identified using an Ettan matrix-assisted laser desorption ionization time-of-flight system (Amer sham Biosciences), and candidate sequences were matched to the SWISS-PROT and NCBI databases using the search program ProFound (prowl.rockefeller.edu).

**In Vitro Pulldown, Capture Assay, and Immunoprecipitation**—GST or GST-survivin equilibrated in 20 mM Hepes, pH 7.7, 75 mM KCl, 0.1 mM EDTA, 2.5 mM MgCl2, 1 mg/ml BSA, and 0.05% Nonidet P-40 was incubated with aliquots of Raji cell extracts for 16 h at 4 °C in the presence of protease inhibitors. Bound proteins were recovered by centrifugation at 14,000 × g for 30 min, separated by SDS-polyacrylamide gel electrophoresis, and detected by Western blotting, as described (5). Alternatively, 35S-labeled survivin or 35S-labeled AIP was generated using a TNT quick coupled transcription/translation system (Promega) in the presence of [35S]methionine (Amer sham Biosciences), mixed with GST, GST-AIP, or GST-survivin, and bound proteins were detected by autoradiography. In another series of experiments, Raji cell extracts were incubated with control IgG or an antibody to survivin, and the immune complexes were precipitated by the addition of protein A-Sepharose beads (Amer sham Biosciences) in buffer containing 50 mM Tris, pH 7.4, 120 mM NaCl, 0.5% Triton X-100. After washes, the pellets or supernatants were separated by SDS gel electrophoresis and analyzed by Western blotting.

**Transfection Experiments**—Two independent double-stranded (ds) RNA oligonucleotides targeting the AIP sequence CCAUGACAGAGAACAA (AIP 1) or UGGUGUGACGAGGAGAGUACUA (AIP 2) were synthesized (Dharmacon). A control dsRNA oligonucleotide (VIII) ACUCUAUCUGCCAGGCUGAC matched for length and GC content was also synthesized. For gene silencing by small interfering RNA (siRNA), dsRNA oligonucleotides were mixed with oligofectamine (Invitrogen) in OPTI-MEM I (Invitrogen) medium, and the mixture was incubated with HeLa cells for 4 h at 37 °C, as described (14). The cultures were supplemented with 10% serum and 1% antibiotics and further incubated for 48 or 72 h before analysis of AIP knock-down and modulation of survivin expres-
Identification of AIP as a Novel Survivin-interacting Protein—

We used a proteomics screening to identify novel proteins that bind to survivin and may regulate its function(s). A protein spot with apparent molecular weight of ~37,600, and a pI of 6.8 was specifically detected in eluates from GST-survivin beads, but not GST alone (Fig. 1A). This candidate survivin-interacting molecule was identified from peptide mass fingerprinting with 31% sequence coverage as the AIP (Fig. 1B), also designated ARA9 or XAP2 (16–18).

RESULTS

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A schematic diagram of AIP and (AIP1–169) and (AIP170–330) fragments. The FKBP homology region and three TPR domains are indicated. B, schematic diagram of survivin. The dimer interface, baculovirus IAP repeat, and carboxy-terminal coiled-coil region are indicated. C, AIP-binding site. The indicated constructs AIP1–169 (AIP N) or AIP170–330 (AIP C) were expressed as GST fusion proteins, incubated with recombinant survivin, and bound proteins were detected by Western blotting. D, survivin-binding site. The indicated constructs survivin1–87 (SVV N) or survivin88–142 (SVV C) were expressed as GST fusion proteins and mixed with 35S-labeled in vitro transcribed/translated AIP, and bound proteins were detected by autoradiography. E and F, survivin deletion mutants. The indicated constructs survivin88–140 (SVV 88–140), survivin88–141 (SVV 88–141), survivin88–142 (SVV 88–142), survivin136–142 (SVV 136–142), survivin137–138 (SVV 137–138), survivin138–139 (SVV 138–139), and survivin140–142 (SVV 140–142), survivin141–142 (SVV 141–142) or survivin142–143 (SVV 142–143) were expressed as GST fusion proteins and incubated with 35S-labeled AIP, and bound proteins were detected by autoradiography. Comparable input of the various survivin mutant proteins was confirmed by Coomassie Blue staining of the gel. G, requirement of Asp142 in survivin-AIP interaction and role of Hsp90. Wild type survivin or a single amino acid deletion mutant, survivin1–141 (SVV 141) was 35S-labeled in reticulocyte extracts and mixed with GST-AIP in the presence or absence of the Hsp90 ATPase pocket antagonist, 17-allylamino-17-demethoxygeldanamycin (17-AAG), and bound proteins were detected by autoradiography.

Characterization of a Survivin-AIP Complex—In pulldown experiments, incubation of Raji cell extracts with GST-survivin resulted in strong association with AIP, by Western blotting (Fig. 2A). In contrast, GST did not bind AIP (Fig. 2A). In addition, 35S-labeled in vitro transcribed and translated AIP strongly associated with GST-survivin, whereas no interaction with GST was observed (Fig. 2B). To determine whether the association between AIP and survivin was direct, we performed in vitro pulldown experiments with isolated recombinant proteins. In these experiments, survivin and AIP reciprocally interacted, whereas no binding of either protein to GST was demonstrated (Fig. 2C). Finally, survivin immune complexes precipitated from Raji cell extracts contained the molecular chaperone Hsp90, in agreement with previous observations (5), and AIP in vivo (Fig. 2D). In contrast, immune complexes precipitated with a control IgG did not contain Hsp90 or AIP (Fig. 2D).

Structure-Function Analysis of the Survivin-AIP Complex—Several recombinant deletion mutants of AIP or survivin were generated and characterized in pulldown experiments. A schematic diagram of AIP (Fig. 3A) and survivin (Fig. 3B) and their respective functional domains is shown. Full-length AIP or AIP170–330 (AIP-C) comprising its three tetra-tricopeptide (TPR) repeats (18) strongly associated with recombinant survivin in pulldown experiments (Fig. 3C). In contrast, AIP1–169 (AIP-N) containing the FKBP12 homology region or GST did not bind recombinant survivin (Fig. 3C). In reciprocal experiments, the carboxyl-terminal α-helical coiled-coil region of survivin (survivin188–142, SVV-C) strongly associated with 35S-labeled in vitro transcribed and translated AIP (Fig. 3D). In contrast, survivin1–87 (SVV-N) containing the baculovirus IAP repeat only very weakly associated with recombinant AIP, and GST showed no reactivity (Fig. 3D).

To map more precisely the binding site for AIP, serial deletion mutants of the survivin carboxyl-terminal region were generated and analyzed in pulldown experiments. Survivin deletion mutants with sequential removal of the carboxyl terminus (SVV 88–138 and SVV 88–140) lost the ability to bind AIP, and even a survivin mutant (SVV 88–141) lacking only the...
Regulation of Survivin Stability by AIP—AIP contains an immunophilin homology domain (17, 18) and may exhibit chaperone-like activity (19). To begin to investigate the functional relevance of a survivin-AIP complex, we studied a potential role for AIP in regulating survivin stability. For these experiments, we transfected cells with HA-tagged full-length survivin (HA-survivin) or HA-survivin<sup>1–141</sup>, which is defective in AIP binding (Fig. 3, F and G). In cycloheximide block experiments, SVV 1–141 exhibited accelerated degradation as compared with full-length, wild type survivin (Fig. 4A). Densitometric quantification of protein bands revealed that deletion of the last survivin amino acid Asp<sup>142</sup> resulted in a ~5-h half-life, as opposed to the 10-h half-life of wild type survivin (Fig. 4B). We next asked whether AIP directly contributed to survivin stability. For these experiments, we used two independent siRNA sequences to knock down AIP expression in MCF-7 cells and monitored survivin levels. Transfection of MCF-7 cells with control siRNA did not affect survivin or AIP levels, by Western blotting (Fig. 4C). In contrast, acute knock-down of AIP resulted in significant reduction of survivin expression, whereas levels of another IAP family protein, XIAP, were not affected (Fig. 4C). Under these experimental conditions, the addition of the proteasome inhibitor lactacystin restored survivin levels in AIP knock-down cells to those of control siRNA-transfected cultures (Fig. 4C).

Modulation of Cell Viability by the Survivin-AIP Complex—Consistent with the data reported above (Fig. 4C), acute knockdown of AIP resulted in reduction of survivin levels in MCF-7 cells, whereas expression of β-actin was not affected (Fig. 5A). In addition, transfection with control or AIP-directed dsRNA oligonucleotides did not result in eukaryotic initiation factor 2α phosphorylation in these cells, thus ruling out activation of an interferon response (Fig. 5A). Under these experimental conditions, the loss of survivin after AIP knock-down was not associated with significant alterations in cell cycle progression as revealed by DNA content analysis, and mitotic transition, a cell cycle phase typically modulated by survivin, was not affected (Fig. 5B). The addition of wild type survivin (HA-survivin) or SVV 1–141 to AIP (Fig. 3G), thus indicating that Hsp90 expression or function does not modulate the survivin-AIP complex.

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membrane integrity (Fig. 5C), and further reduction in long-term cell viability, by MTT analysis (Fig. 5D). siRNA knock-down of AIP was also associated with further reduction in cell viability induced by the DNA-damaging agent etoposide as compared with control siRNA-treated cultures (Fig. 5D). To further test a role of AIP in regulating survivin stability and cytoprotection, we used the survivin carboxyl-terminal peptide or the survivin sequence SVV136–142 comparably to AIP, intracellular penetration. MCF-7 cells were incubated with control scrambled peptide or survivin peptide (closed circles) to AIP. B, intracellular penetration. MCF-7 cells were incubated with control scrambled peptide or survivin peptide were analyzed by Western blotting. D, cell proliferation/cell viability. MCF-7 cells were treated with the cell death stimuli staurosporine (STS) or etoposide in the presence or absence of control scrambled peptide or survivin peptide (μM) and analyzed for cell viability by an MTT assay. ***, p = 0.0005; **, p = 0.0023–0.0028; *, p = 0.01.

DISCUSSION

In this study, we have used a proteomics screening to identify the AIP (16–18) as a novel survivin-binding partner. Survivin associates with AIP directly in vitro and in vivo through a recognition largely mediated by the last carboxyl terminus residue in survivin, Asp142. Experiments with acute AIP knock-down by siRNA, competition of the survivin-AIP complex using synthetic peptidyl mimicry, and expression of a survivin mutant defective in AIP binding (SVV1–141) all pointed to a role for this interaction in promoting survivin stability in vivo and preserving an anti-apoptotic threshold in the cytosol.

Contributing to its multifunctional role in cell division and cell viability (2), survivin exhibits adaptive changes in protein stability and dynamic trafficking among multiple subcellular compartments (9), which include various aspects of the mitotic apparatus (10), the nucleus (20), and a specialized mitochondrial pool specifically involved in apoptosis inhibition (6). In this context, AIP recognition provides a novel mechanism for regulation of survivin stability, potentially linked to the Hsp90 chaperone system. AIP, also known as ARA-9 or XAP-2, binds the aryl hydrocarbon receptor, a ligand-activated member of the basic-loop-helix/Per-Arnt-Sim family of transcription factors (12). This molecule is the main effector of the cellular response to environmental toxins, especially 2,3,7,8-tetrachlorodibenzo-p-dioxin, commonly referred to as TCDD or dioxin, and acts via induced transcription of phase I detoxification cytochrome P-450 genes (12). Despite its structural similarities with immunophilins, AIP is thought to stabilize the unliganded aryl hydrocarbon receptor in the cytosol via its carboxyl-terminal TPR motifs (11), thus preventing ubiquitin-dependent proteasomal destruction. In turn, this causes increase in overall cytosolic receptor levels (21) and enhanced detoxification signaling potentially via inhibition of receptor nuclear-cytoplasmic shuttling (22). A third component of the aryl hydrocarbon receptor-AIP complex is Hsp90, which associates with AIP through the TPR motifs (23) and binds the aryl hydrocarbon receptor via its middle domain (23). A similar paradigm may apply to survivin, which binds independently to AIP (this study) and Hsp90 (5), with both interactions required to enhance its stability in the cytosol by preventing ubiquitin-dependent proteasomal destruction. Structurally, the last carboxyl terminus residue in survivin, Asp142, has been shown to play a critical role in AIP recognition. This is reminiscent of the binding requirements between the TPR motifs in the co-chaperone p60hsp90 and the carboxyl-terminal domain of Hsp90, as determined by biochemical analysis and x-ray crystallography (24, 25). In addition to deletion experiments, blocking the carboxylate group at the survivin carboxyl terminus using an HA tag also suppressed the interaction with AIP (not shown), suggesting that an association between two carboxylate groups, one from Asp142 and the other from the peptide backbone, may provide most of the binding interface for the TPR-containing domain of AIP.

The more functional consequences of the survivin-AIP complex were probed using two independent approaches: acute...
knock-down of AIP levels by siRNA and peptidyl mimicry of the interaction using a cell-permeable peptide modeled on the carboxyl-terminal survivin-AIP binding interface. Both approaches resulted in only a partial suppressive effect on either AIP expression or the integrity of the survivin–AIP complex. However, both strategies resulted in destabilization of survivin levels via proteasome-dependent destruction, reduced cell viability and enhanced apoptosis by suboptimal doses of cell death agonists, including UVB, etoposide, and staurosporine. This phenotype is consistent with several previous studies demonstrating that acute loss of survivin levels in tumor cells results in spontaneous or synergistic induction of apoptosis by cell death stimuli (2), potentially by critically lowering an anti-apoptotic threshold in the cytosol. In addition, survivin cytoprotection has been consistently associated with its cytosolic pool (9), in dynamic equilibrium with a fraction of survivin compartmentalized in mitochondria and discharged in the cytosol in response to cell death stimuli (6). In this context, it is intriguing that AIP has been implicated not only in affecting nucleo-cytoplasmic shuttling of the aryl hydrocarbon receptor (22), but also in more global chaperone mechanisms of mitochondrial import of preproteins via an interaction between its TPR motifs and the carboxyl-terminal region of the translocase of the outer membrane molecule, Tom20 (19). Whether AIP promotes survivin stability in the cytosol and enhances its mitochondrial import requires further investigation. However, the data presented here suggest that the survivin–AIP complex may be selectively involved in cytoprotection, which is typically mediated by mitochondrial survivin discharged in the cytosol (6), rather than mitotic regulation that involves a distinct subcellular pool of the molecule (9).

In sum, we have identified survivin as a novel component of a dynamic chaperone complex that controls cellular responses to environmental toxins via a direct recognition of AIP. This is consistent with an emerging role for survivin in preserving cell viability and potentially cell proliferation during the cellular stress response and fits well with the already documented interaction between survivin and Hsp90 (5) as an additional component of the AIP complex (11). It has been speculated that signaling through the aryl hydrocarbon receptor and its complex extends beyond detoxification mechanisms against environmental threats and may also involve modulation of endogenous intracellular signaling pathways linked to cell proliferation (26, 27), cell metabolism (28), and differentiation/cell viability (29). Survivin, with its multifunctional roles (2), appears ideally positioned to participate not only in the exogenous stress response mediated by this chaperone complex but also in potential endogenous pathways of cellular adaptation, which may require elevation of survivin levels via rapid changes in protein stability and dynamic shuttling among specialized subcellular compartments (9).

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