An IAP-IAP Complex Inhibits Apoptosis*

Regulators of apoptosis are thought to work in concert, but the molecular interactions of this process are not understood. Here, we show that in response to cell death stimulation, survivin, a member of the inhibitor of apoptosis (IAP) gene family, associates with another IAP protein, XIAP, via conserved baculovirus IAP repeats. Formation of a survivin-XIAP complex promotes increased XIAP stability against ubiquitination/proteasomal destruction and synergistic inhibition of apoptosis, which is abolished in XIAP−/− cells. Therefore, orchestration of an IAP-IAP complex regulates apoptosis.

Among the regulators of programmed cell death, or apoptosis (1), Bcl-2 proteins (2) control the release of apoptogenic proteins from mitochondria, notably cytochrome c (3), whereas members of the inhibitor of apoptosis (IAP) gene family act as endogenous inhibitors of caspases (4), the enzymatic effectors of apoptosis (1). The structural requirements of IAP-caspase(s) complexes have been defined in considerable detail (5).

Survivin is a structurally unique IAP protein that has been implicated in protection from apoptosis and regulation of mitosis (6). A role of survivin in cell division has been linked to spindle checkpoint function (8). In contrast, despite its implication in protection from apoptosis and regulation of mitosis (6), the mechanism(s) by which survivin inhibits apoptosis has remained elusive. This is important because IAPs, especially XIAP (9) and survivin (6), have emerged as critical regulators of cell survival in tumors and promising targets for rational anti-cancer therapy (10, 11).

In this study, we investigated the mechanism(s) of survivin cytoprotection. We found that in response to cell death stimulation, survivin physically associates with XIAP, and this complex promotes enhanced XIAP stability and synergistic inhibition of caspase-9 activation.

MATERIALS AND METHODS

Cell Culture—Breast carcinoma MCF-7, lymphoblastoid Raji, and kidney embryonic HEK293T cells were from the American Type Culture Collection (ATCC, Manassas, VA). Wild type (WT) or XIAP−/− mouse embryonic fibroblasts (MEF) (12) were the gift of Dr. C. Dukett (University of Michigan).

Protein-Protein Interactions—Affinity fractionation and immunoprecipitation experiments were carried out as described (10, 13). Full-length survivin, truncated survivin-BIR1(1–87), full-length XIAP, clpAIP, clpAIP, or the three isolated XIAP, BIR1(1–123), BIR2(123–259) and BIR3(260–336) were expressed as GST fusion proteins (14). Proteins were extracted from mammalian cells and used in pull-down experiments (15). Pull-down experiments with recombinant survivin (0.1–0.4 μg) and GST-XIAP, GST-clpAIP, GST-clpAIP (8 μg), or the individual GST-BIR1, -BIR2, or BIR3 of XIAP (10 μg) bound to glutathione beads (100 μl) were as described (13). Alternatively, XIAP was translated in vitro in the presence of [35S]methionine (Amersham Sciences), mixed with 5 μg of GST or GST-survivin, and used in pull-down experiments.

Modulation of XIAP Ubiquitination—HEK293T cells were transfected with FLAG-XIAP in the presence of survivin or control vector, pulse-labeled with 0.5 μCi of [35S]methionine and [35S]cysteine, chased with unlabeled methionine, and immunoprecipitated with an antibody to FLAG (Sigma). Bands were quantified by NIH Image analysis (1.62/3DV11.01/ppc).

In vitro ubiquitination reactions were performed for 1 h at 30 °C using 1 μg of survivin-His6 or control ΔN-TraF-His6 in 40 mM Tris, pH 7.5, 5 mM MgCl2, 1 mM DTT, 10% glycerol, 10 mM phosphocreatine, 100 μM creatine phosphokinase, 0.5 mM ATP, 1 mM MgCl2, 1 μM ubiquitin aldehyde, and 1 μl of in vitro translated [35S]labeled XIAP or [35S]labeled XIAP Lys258 → Arg mutant, with or without HEK293T extracts (30 μg). HEK293T cells were transfected with His6-ubiquitin, pcDNA3-Myc-survivin, and FLAG-XIAP, lysed after 24 h, and mixed with 60 μl of covalent-chelation resin, and bound proteins were analyzed by immunoblotting. In some experiments, XIAP expression levels and formation of a survivin-XIAP complex were analyzed in MCF-7 cells treated with staurosporine (1–1.5 μM for 12 h) with or without a caspase inhibitor, Z-VAD-fmk (20 μM), or proteasome inhibitor lactacystin (5 μM).

Analysis of Caspase Activity and Cell Death—Caspase assays were performed using recombinant caspase-9, XIAP, and survivin by continuously monitoring AFC release from the fluorogenic substrate Ac-LEHD-AFC. Alternatively, recombinant procaspase-9 (4 μg) was incubated with Apaf-1 (4 μM), cytochrome c (600 nM), dATP (200 μM), procaspase-3 (4 μM), and GST-XIAP in the presence of recombinant survivin for 30 min at 30 °C, and reaction mixtures were analyzed for cleavage of Ac-DEVAD-AFC. HEK293T cells were transfected with pEGFP (Clontech) and various plasmids (Superfect, Qiagen) and harvested after 24 h, and GFP cells scored for nuclear morphology of apoptosis by 4′,6-diamidino-2-phenylindole (DAPI) staining and fluorescence microscopy. Alternatively, WT or XIAP−/− MEF were transfected with pAd-GFP or pAd-survivin, exposed to staurosporine (0.75 μM) or UVB (1500 J/m2) for 18 h, and analyzed by DAPI staining and fluorescence microscopy.

RESULTS

Identification of a Survivin-XIAP Complex during Apoptosis—We fractionated Raji cell extracts by affinity chromatography over an antibody to survivin (14) and analyzed bound proteins by immunoblotting. Survivin co-eluted with the mo-

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† The abbreviations used are: IAP, inhibitor of apoptosis; BIR, baculovirus IAP repeat; WT, wild type; MEF, mouse embryonic fibroblast; GST, glutathione S-transferase; Z, benzoyloxycarbonyl; fmk, fluoromethyl ketone; AFC, amino-4-trifluoromethylcoumarin; GFP, green fluorescent protein; DAPI, 4′,6-diamidino-2-phenylindole.

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Fig. 1. Survivin-XIAP interaction. A, affinity chromatography. Raji cell extracts were fractionated on an anti-survivin column, and eluted fractions were analyzed by immunoblotting. B, co-immunoprecipitation. MCF-7 cells were immunoprecipitated (IP) with IgG or an antibody to survivin, followed by immunoblotting. C, co-immunoprecipitation from overexpressing cells. MCF-7 cells were transduced with pAd-survivin without or with staurosporine (STS), and immune complexes were analyzed by immunoblotting. D, survivin-XIAP interaction. Upper panel, GST-XIAP or GST was incubated with survivin, and bound proteins were analyzed by immunoblotting. Input, survivin (0.4 μg). Lower panel, 35S-labeled in vitro translated XIAP (Input) was incubated with GST-survivin or GST, followed by autoradiography. E, survivin-IAP interaction. The indicated GST-IAPs were incubated with survivin, and bound proteins were detected by immunoblotting. Input, survivin (0.4 μg). F, BIR interaction. The indicated GST-BIRs were mixed with survivin, followed by immunoblotting. The membrane was stained with Coomassie Blue for equal loading.

The molecular chaperone complex comprising both Hsp90 and Hsp70 (Fig. 1A), in agreement with previous observations (13). In addition, the survivin complex contained the IAP family protein XIAP (4) in the same fractions (Fig. 1A). We next immunoprecipitated endogenous survivin from MCF-7 cells with or without exposure to the apoptotic stimulus staurosporine and probed the immune complexes by immunoblotting. Immunoprecipitated survivin co-associated with endogenous XIAP, and this interaction was increased by staurosporine treatment (Fig. 1B). Immune complexes precipitated with a control IgG did not contain XIAP (Fig. 1B). Interactions of survivin with homologous IAP proteins, cIAP1 and cIAP2, were also detected but not with Bcl-2, Bcl-XL, or Bax (not shown). To investigate a role of apoptotic stimulation in the formation of a survivin-XIAP complex, we overexpressed survivin in MCF-7 cells using a replication-deficient adenovirus (pAd-survivin) and performed co-immunoprecipitation experiments with or without staurosporine. Exposure of MCF-7 cells to staurosporine increased the association of survivin with XIAP, whereas immunoprecipitates of non-immune IgG did not contain XIAP (Fig. 1C).

We next determined whether survivin and XIAP interacted directly. Recombinant survivin bound GST-XIAP in a concentration-dependent manner, whereas no interaction with GST was observed (Fig. 1D). Reciprocally, 35S-labeled in vitro translated XIAP associated with GST-survivin but not GST (Fig. 1D). We next expressed IAP family proteins cIAP1 and cIAP2 (4) and tested their association with survivin. Both cIAP1 and cIAP2 bound survivin directly, whereas no interaction with GST was detected (Fig. 1E). A role of homologous BIR sequences in IAP-IAP complex formation was investigated. Recombinant survivin associated with XIAP-BIR1 and XIAP-BIR3 and more weakly with XIAP-BIR2 (Fig. 1F). Reciprocally, a truncated survivin 1–87 mutant containing the single survivin BIR bound full-length XIAP (not shown).

**Regulation of XIAP Stability by Survivin**—MCF-7 cells transduced with pAd-GFP and exposed to staurosporine exhibited a time-dependent loss of XIAP expression (Fig. 2A). In contrast, transduction of MCF-7 cells with pAd-survivin preserved XIAP expression over time in the presence of staurosporine (Fig. 2A). We then performed [35S]l-methionine pulse-chase experiments in HEK293T cells expressing XIAP together with survivin or a control vector. Co-transfection of HEK293T cells with survivin resulted in persistence of XIAP levels over a 6-h interval after chase (Fig. 2B) and prolongation of XIAP half-life from 2 h in vector-transfected cells to 5.5 h (Fig. 2C). Addition of the proteasome inhibitor lactacystin prevented loss of XIAP expression in staurosporine-treated MCF-7 cells, whereas a caspase inhibitor, Z-VAD-fmk, was ineffective (Fig. 2D).

**Regulation of XIAP Ubiquitination by Survivin**—When mixed with ubiquitin, in vitro translated, [35S]labeled XIAP exhibited a ladder of bands typical of polyubiquitinated conjugates (Fig. 3A). In contrast, a XIAP (Lys228 → Arg) mutant lacking a critical lysine residue involved in ubiquitination of this protein exhibited minimal ubiquitin conjugation in vitro (Fig. 3A). Addition of recombinant survivin suppressed XIAP ubiquitin conjugate formation, whereas a control Traf3 protein was ineffective (Fig. 3B). Increasing concentrations of recombinant survivin inhibited the binding of the ubiquitin-conjugat-
ing enzyme, UbcH5, to GST-XIAP (Fig. 3C), suggesting that a survivin-XIAP complex may prevent substrate accessibility to the ubiquitination machinery. We then explored whether survivin interfered with XIAP ubiquitination in vivo. Polyubiquitinated XIAP conjugates were readily detectable in HEK293T cells transfected with control pcDNA3 in the presence of a proteasome inhibitor (Fig. 3D). In contrast, expression of survivin attenuated XIAP polyubiquitination in vivo (Fig. 3D), and produced a 4-fold reduction in the relative amounts of XIAP-ubiquitin conjugates, as compared with control cells (Fig. 3E).

Cytoprotective Mechanism of the Survivin-XIAP Complex—We next used purified components in vitro to test whether a survivin-XIAP complex synergistically suppressed caspase activity. Whereas survivin alone had essentially no effect on caspase-9 generation, the combination with a suboptimal concentration of XIAP (Fig. 4A, left panel) dose-dependently inhibited caspase-9 generation (Fig. 4A, right panel). Similarly, when combined with a suboptimal concentration of XIAP (Fig. 4B, left panel), survivin inhibited caspase activity in the context of a functional apoptosome in vitro (Fig. 4B, right panel). To determine whether XIAP and survivin synergistically inhibited apoptosis in vivo, we transfected HEK293T cells with survivin and XIAP alone or in combination, and stimulated apoptosis by expressing Bax or Fas, which activate mitochondrial or death receptor apoptosis, respectively. Expression of suboptimal amounts of survivin or XIAP in HEK293T cells individually did not significantly reduce apoptosis (Fig. 4C). In contrast, the combination of XIAP and survivin synergistically suppressed cell death induced by Bax (Fig. 4C, left panel), or Fas (Fig. 4C, right panel). Next, we used XIAP−/− MEF to determine whether a survivin-XIAP complex was required for cytoprotection in vivo. Transduction of WT MEF with pAd-survivin inhibited apoptosis stimulated by staurosporine or UVB (Fig. 4D). In contrast, survivin cytoprotection was completely lost in XIAP−/− MEF (Fig. 4D).
Survivin-XIAP Complex in Cytoprotection

Fig. 4. Survivin-XIAP complex inhibits apoptosis. A, inhibition of caspase-9 activity. Left panel, ΔCARD caspase-9 was mixed with GST-XIAP and analyzed for caspase-9 activity by Ac-LEHD-AFC hydrolysis. Right panel, a suboptimal concentration of XIAP (30 nM, left panel, arrow) was mixed with survivin and analyzed for caspase-9 activity by Ac-LEHD-AFC hydrolysis. B, inhibition of apoptosome-associated caspase activity. Left panel, GST-XIAP was mixed with procaspase-9, Apaf-1, dATP, cytochrome c, and procaspase-3 and analyzed for caspase activity by Ac-DEVAD-AFC hydrolysis. Right panel, a suboptimal concentration of GST-XIAP (0.5 nM, left panel, arrow) was mixed with survivin, and caspase activity (%Vmax) was monitored by AFC release. C, inhibition of apoptosis in vivo. HEK293T cells were transfected as indicated with pEGFP and survivin or XIAP, alone or with pcDNA3-Bax (left panel) or pcMV-Fas (right panel). GFP+ cells were scored for nuclear apoptosis. Data are the mean ± S.D. of at least three independent determinations. D, requirement of XIAP for cytoprotection. WT (left panel) or XIAP−/− (right panel) MEF were transduced with pAd-GFP (black bars) or pAd-survivin (gray bars), exposed to staurosporine or UVB, and scored by DAPI staining. Data are the mean ± S.E. of a representative experiment averaging 500 cells/condition. ***, p < 0.0001; ns, not significant.

DISCUSSION

In this study, we have shown that IAP family members XIAP and survivin form a heterocomplex in response to cell death stimulation in vitro. This interaction promotes cell survival by enhancing the stability of XIAP against proteasomal destruction and by synergistically antagonizing apoptosome-mediated cell death, in a pathway abolished in XIAP−/− cells.

Although the formation of heterocomplexes is a hallmark of Bcl-2 proteins (2), IAP-IAP interactions have not been described previously. This recognition involves survivin binding sites on all three BIRs of XIAP, albeit with different affinities, and may affect the interaction with other BIR-interacting partners, including XIAP antagonists Smac (3), Omi/HtrA2 (16), XAP (17), survivin co-factors (HBXIP) (18), and XIAP targets (caspase-3, -7, and -9) (4).

Formation of a survivin-XIAP complex resulted in increased stability of XIAP against polyubiquitination and proteasomal degradation in vitro and in vivo, potentially by excluding the ubiquitin-conjugating enzyme, UbcH5. Sudden changes in IAP stability influence cell viability (19), and ubiquitin-dependent proteasomal destruction of IAPs enhances cell death (20). Here, a stabilized survivin-XIAP complex synergistically suppressed caspase-9 processing/activity, alone or in the context of the apoptosome, and blocked apoptosis in vivo. Although the mechanism(s) of survivin cytoprotection have long remained elusive, these data suggest a model of intermolecular cooperation in which survivin enhances the anti-apoptotic activity of XIAP to suppress the upstream initiation of mitochondrial cell death (15, 18). Apoptotic stimulation induces the formation of a survivin-XIAP complex in vivo, and future studies will investigate whether this reflects post-translational modifications in survivin (10) and/or XIAP (21) enabling protein interaction or, alternatively, redistribution of survivin from a specialized subcellular pool during cell death (14).

Targeted antagonists of the IAP-IAP complex may be suitable to disable apoptosis resistance in tumors, where survivin (6) and XIAP (9) are commonly overexpressed.

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