Caspase-3 and inhibitor of apoptosis protein(s) interactions in *Saccharomyces cerevisiae* and mammalian cells

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Abstract Using a heterologous yeast expression assay, we show that inhibitor of apoptosis proteins (IAPs) suppress caspase-3-mediated cytotoxicity in the order of XIAP > c-IAP2 > c-IAP1 > survivin. The same ordering of IAP activities was demonstrated in mammalian cells expressing an auto-activating caspase-3. The relative anti-apoptotic activities of each IAP depended on the particular death stimulus. For IAP-expressing cells treated with camptothecin, survival correlated with their intrinsic anti-caspase-3 activity. However, c-IAP1-transfected cells were disproportionately resistant to tumor necrosis factor-α, suggesting that its anti-apoptotic activities extend beyond caspase-3 or -7 inhibition. Yeast-based caspase assays provide rapid, reliable information on specificity and activity of the IAPs and aid in identifying critical targets in mammalian apoptotic pathways.

Key words: Caspase; Inhibitor of apoptosis protein; Apoptosis; Yeast

1. Introduction

The family of caspase cysteine proteases is involved in carrying out programmed cell death in metazoans in a process referred to as apoptosis [1]. There are 14 identified mammalian caspases, each synthesized as a zymogen which becomes activated through proteolytic cleavage at sites with aspartic acid in the P1 position by a subset of activated caspases [1]. When cells receive signals to die, specific caspases become activated in a hierarchical fashion that leads to the processing and activation of several critical effector caspases [2]. One of these effector caspases, caspase-3, promotes cell disassembly through the cleavage of several structural proteins and repair enzymes necessary for cellular homeostasis. The proteolytic activity of caspase-3 is a critical determinant of whether a cell commits to death, and as a result, multicellular organisms have evolved strategies to tightly regulate caspase-3 activity within the cell [1].

One mechanism used to control caspase activity is the expression of endogenous protease inhibitors. The foremost example is a family of caspase inhibitors called inhibitor of apoptosis proteins (IAPs) [3,4]. The IAPs were originally described by an activity that prevents apoptotic cell deaths caused by multiple triggers in *Drosophila*, *Caenorhabditis elegans* and mammalian cells [5,6]. Subsequently, several labs demonstrated by in vitro assays that IAPs function as direct inhibitors for specific caspases [7–9]. Specifically, three mammalian IAPs, c-IAP1, c-IAP2, and XIAP, are direct inhibitors of recombinant caspases-3, -7, and -9 [7–9]. This is generally accepted to be the principal mechanism for the anti-apoptotic activity of the IAPs in various experimental settings [4].

In this report, we examined the activities of the four known mammalian IAPs, XIAP, c-IAP1, c-IAP2, and survivin, in cellular assays dependent on caspase-3 function. Using a novel functional assay based on heterologous expression in yeast, we tested the ability of individual IAPs to correct a growth defect in *Saccharomyces cerevisiae* expressing active human caspase-3. Since yeasts lack endogenous caspases, this assay provides a selective measure of IAP activity against the exogenously introduced caspase. These results were compared with the anti-apoptotic activities of these IAPs in mammalian cells expressing an activated caspase-3 or treated with tumor necrosis factor-α (TNFα) or camptothecin, a topoisomerase I inhibitor.

The different results with these assays of cellular IAP function indicate the importance of inhibiting multiple caspases in mammalian apoptotic pathways and suggest that the in vivo activity profile is distinct for each IAP.

2. Materials and methods

2.1. Yeast cell culture and transformations

The *S. cerevisiae* W303-1A (*MATa ade2-1 his3-11 his3-15 leu2-112 trp1-1 ura3-1*) strain was used for the yeast expression studies. Transformations were performed using lithium acetate. One tenth volume of each transformation reaction was plated onto selective media including 0.5 mM $ZnCl_2$. This concentration of $ZnCl_2$ had no effect on normal yeast growth or caspase-3-mediated cytotoxicity in yeast (data not shown). Yeast colony counts were determined 48 or 96 h after plating at 30°C. Colony counts were determined in triplicate.

2.2. Construction of the pRS424GAL1-10 yeast expression vector and derivatives

The pRS424*GAL1-10* yeast expression vector was constructed by ligating a *Eco*RI–*Bam*HI fragment containing the complete *GAL1-10* promoter from the pBM150 plasmid into the 2μ pRS424 episomal vector [10]. The pRS424*GAL1-10*-caspase-3 vector was constructed by inserting a *Eco*RI–*Xho*I cDNA fragment encoding the 17 kDa subunit of caspase-3 (amino acids 1–175) downstream of the *GAL1* promoter and inserting a *Bam*HI–*Sac*I cDNA fragment encoding the 10 kDa subunit of caspase-3 (amino acids 179–277) downstream of the *GAL10* promoter. The 20 kDa and 10 kDa subunit cDNA fragments of caspase-3 were amplified by polymerase chain reaction (PCR).

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Abbreviations: GAL, galactose; HEK, human embryonic kidney; IAP, inhibitor of apoptosis protein; RCF, relative colony frequency; Rev-caspase-3, reversed caspase-3; TNF, tumor necrosis factor

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2.3. Cloning of inhibitor of apoptosis genes into mammalian and yeast expression vectors

XIAP, c-IAP1, c-IAP2, and survivin cDNAs were PCR-amplified from poly(A)$^+$ RNA extracted from the human Jurkat T-cell line. PCR primers added a c-*myc* epitope (EQKLISEEDL) in frame following the C-terminal residue. The c-*myc*-tagged IAP PCR products were cloned into pcDNA3 mammalian and pYES2 yeast expression vectors.

2.4. Construction of Bcl-X_L, baculovirus p35, caspase-3 (C163S), and dnFADD(81–209) mammalian and yeast expression vectors

Human Bcl-X_L and baculovirus p35 cDNAs were cloned into the pYES2 vector as *Eco*RI–*Xba*I fragments [11]. The dncaspase-3 construct with an active site mutation (C163S) was derived by overlap extension PCR mutagenesis of pcDNA3-caspase-3, converting cysteine 163 of caspase-3 to a serine [12,13]. The dnFADD (amino acids 81–209) construct was created by PCR amplification of pcDNA3-FADD [14] and cloned into the pcDNA3 vector.

2.5. Immunoblotting assays

293HEK cells (1×10^6) were seeded into 100 mm dishes and transfected the next day with 5 μg of pcDNA3-IAP plasmids using calcium phosphate. At 24 h post-transfection, cells were washed in ice-cold phosphate-buffered saline and resuspended in 500 μl lysis buffer (20 μM Tris–HCl pH 8.0, 100 mM NaCl, 0.1% Triton X-100, 1 mM EDTA) supplemented with a protease inhibitor cocktail tablet (Roche). The lysates were microcentrifuged at 10 000 rpm for 30 min. The protein concentration was quantitated by Bradford assay (Bio-Rad). 50 μg of protein was fractionated on 12% SDS–polyacrylamide gels followed by Western blot analysis using a monoclonal antibody against the c-*myc* epitope (BabCo).

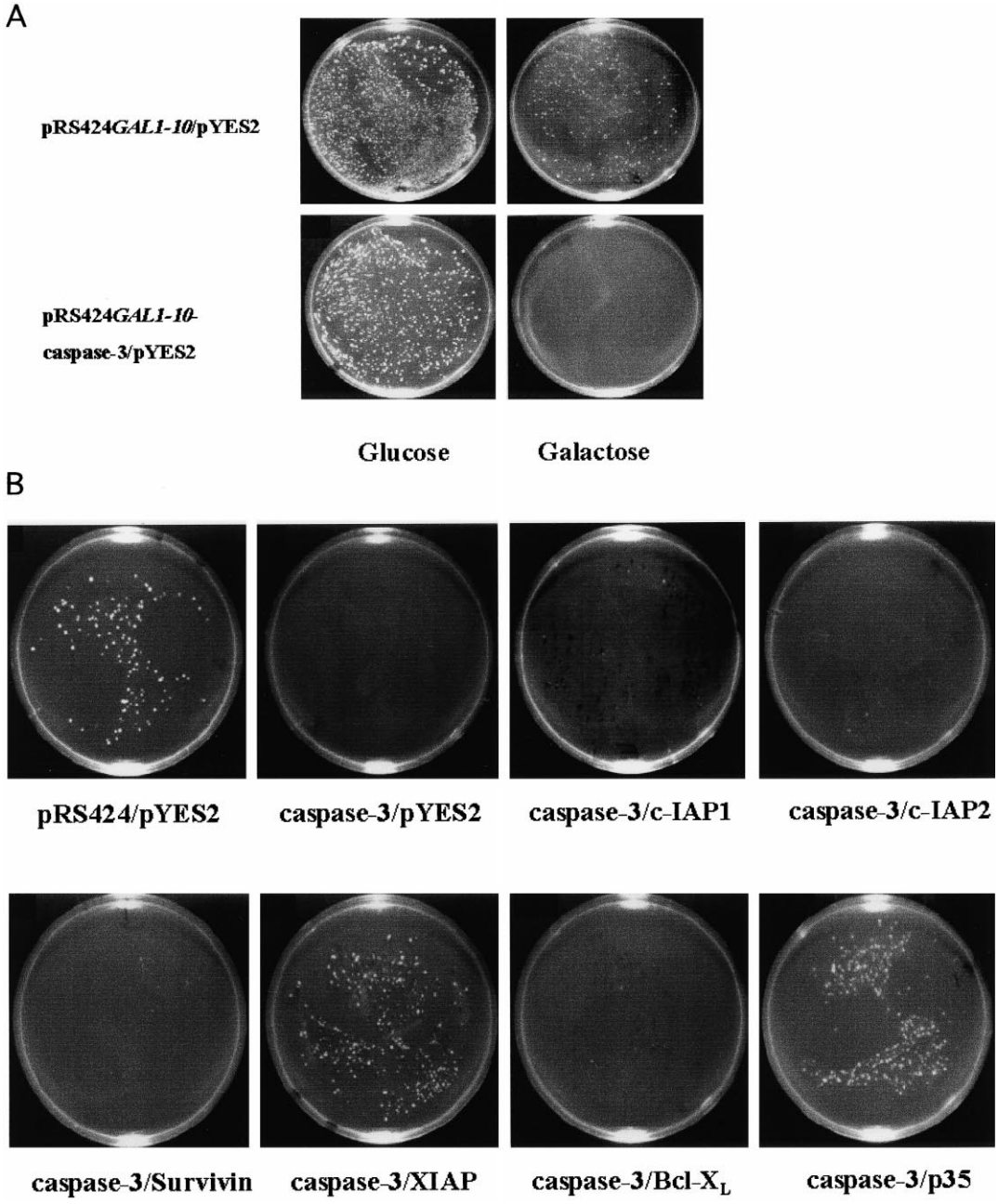

Fig. 1. Expression of caspase-3 inhibits yeast growth. A: W303-1A cells were transformed with pRS424*GAL1-10*-caspase-3 or control vector along with the pYES2 vector and plated onto galactose- or glucose-selective media. Colony growth is shown at 48 h. B: W303-1A cells were co-transformed with pRS424*GAL1-10*-caspase-3 and test cDNAs. Colony growth is shown at 48 h. The results are representative of at least three independent experiments.

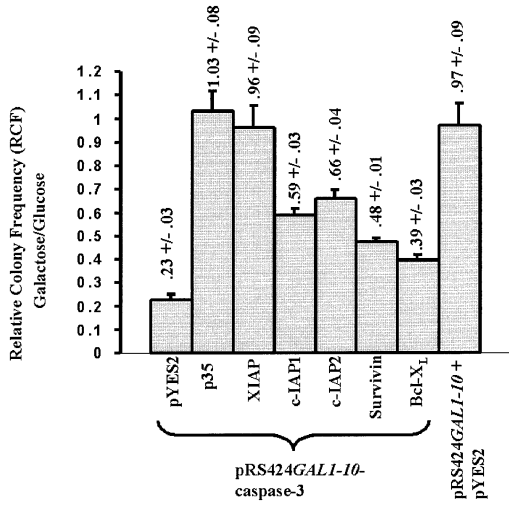

Fig. 2. Inhibition of caspase-3-mediated cytotoxicity in yeast by IAPs. Relative colony frequency was assessed at 96 h following co-transformation of pRS424*GAL1-10*-caspase-3 and pYES2 vectors expressing baculovirus p35, XIAP, c-IAP1, c-IAP2, survivin, or Bcl-X_L cDNAs.

2.6. Cell death assays

2.6.1. Reversed (rev) caspase-3-mediated cell death. 293HEK cells were seeded at a density of 9.0×10^4 cells per well in 12 well tissue culture plates. On day 2, cells were re-fed and the following day the cells were co-transfected with 50 ng Of the pRSC-rev-caspase-3 vector and 1450 ng of pcDNA3 vector or test pcDNA3 plasmids using calcium phosphate. Cells were fixed in 0.5% glutaraldehyde at 24 h post-transfection. Cells were incubated in X-gal staining solution overnight at 37°C. Dead cells were identified by a rounded, condensed morphology, while viable cells were flattened and extended. Apoptosis was determined as the ratio of apoptotic blue cells over the total number of blue cells per well. A minimum of 300 blue cells was scored in triplicate assays.

2.6.2. TNFα-mediated cell death. 293HEK cells were seeded at a density of 5.0×10^4 cells per well in a 12 well tissue culture plate. On day 2, the cells were re-fed and the following day the cells were co-transfected with 2 ng of pcDNA3-β-galactosidase plasmid and 1500 ng of the pcDNA3 vector or test pcDNA3 plasmids. The medium was replaced at 24 h with medium (2% fetal calf serum) containing 80 ng/ml of recombinant TNFα (R&D Systems). Apoptotic cells were counted after 24 h as described above.

2.6.3. Camptothecin-mediated cell death. 293HEK cells were seeded at a density of 5.0×10^4 cells per well in a 12 well tissue culture plate. The cells were re-fed the next day. On day 3, the cells were co-transfected with 2 ng pcDNA3-β-galactosidase plasmid and 1500 ng of pcDNA3 vector or test plasmids. The medium was replaced at 24 h with fresh medium containing 2 μM camptothecin (Sigma). The cells were scored for apoptosis at 24 h.

3. Results

We previously reported that expression of active human caspase-3 in *S. cerevisiae* results in severe growth delay and inhibition of colony formation [15]. For co-transformation studies, we generated a single yeast expression vector containing both 20 kDa and 10 kDa subunits of caspase-3 downstream of the *GAL1* and *GAL10* promoters, respectively. Transformation of yeast with the pRS424*GAL1-10(TRP1)*-caspase-3 vector inhibited colony formation on galactose-containing (GAL) media (Fig. 1A). Colony formation in the pRS424*GAL1-10*-caspase-3 transformants could be rescued by co-expression of a strong caspase inhibitor, baculovirus p35 protein (Fig. 1B) [15].

We employed this caspase-3-dependent phenotype in yeast to test individual IAPs. Yeast were co-transformed with the

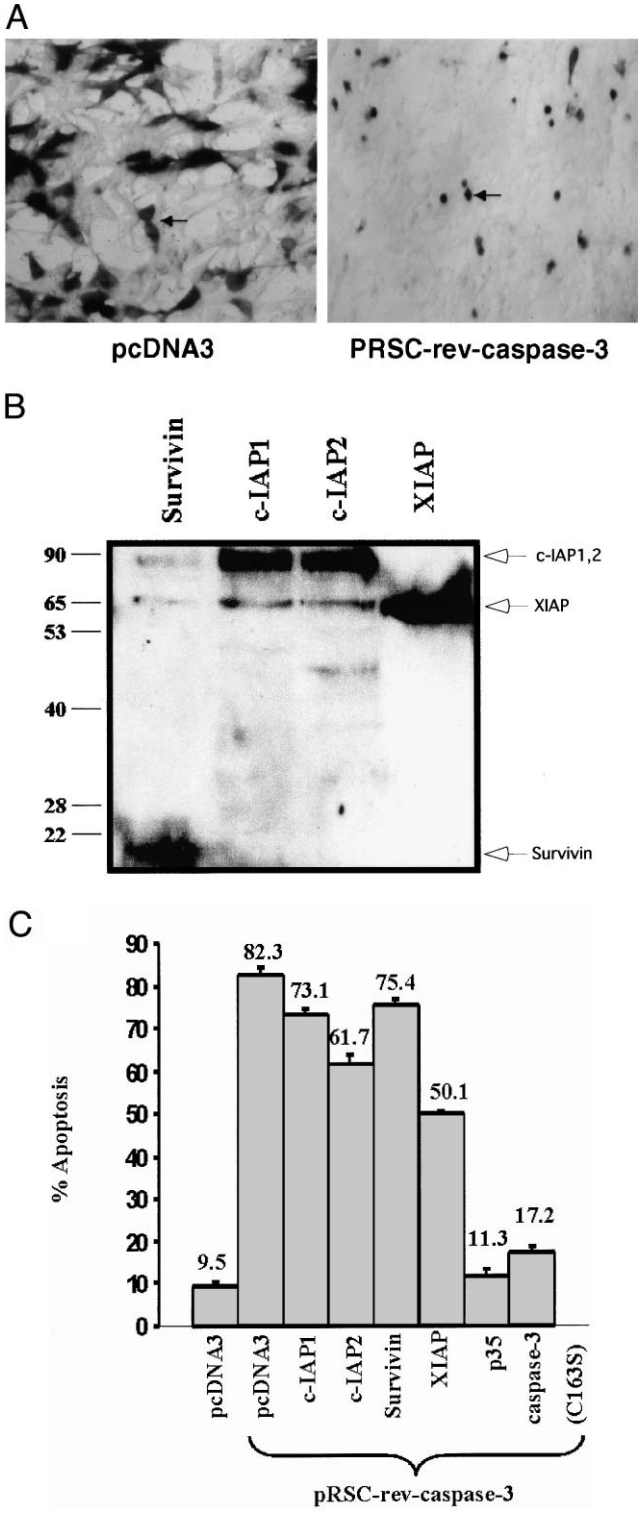

Fig. 3. Inhibition of caspase-3-induced apoptosis by IAPs. A: 293HEK cells stained for, β-galactosidase expression at 24 h post-transfection with control plasmid (left) or pRSC-rev-caspase-3 (right). B: Western blot analysis of IAP protein expression in 293HEK cells. C: The pRSC-rev-caspase-3 plasmid was co-transfected into 293HEK cells with test cDNAs. Apoptosis was assessed at 24 h.

pRS424*GAL1-10*-caspase-3 vector and a galactose-inducible pYES2 vector with either c-IAP1, c-IAP2, XIAP, survivin, baculovirus p35 or Bcl-X_L cDNAs. XIAP expression restored normal colony growth of caspase-3 transformants on GAL medium as effectively as baculovirus p35 (Fig. 1B). However, no colonies formed on GAL plates by 48 h for c-IAP1, c-IAP2, survivin and Bcl-X_L co-transformants.

c-IAP1 and c-IAP2 are direct inhibitors of caspase-3 in vitro, although their activities are 2–3 logs lower than XIAP [8]. To quantitate lower levels of anti-caspase-3 activity, we delayed counting colonies in the caspase-3 transformants on GAL medium until 96 h to allow slower-growing colonies to be scored. At 96 h, a small number of colonies are detected in caspase-3 transformants. We expressed the growth delay in caspase-3 transformants as relative colony frequency (RCF), the ratio of colonies formed on GAL medium divided by colonies on glucose-containing medium at 96 h. Co-transformation of pRS424*GAL1-10*-caspase-3 and the control pYES2 plasmid produced a RCF value of 0.23, while baculovirus p35 and XIAP produced RCF values close to 1 (Fig. 2). c-IAP1, c-IAP2, and survivin gave intermediate results, with RCF values of 0.59, 0.66, and 0.48 respectively, while Bcl-X_L, which acts upstream of caspase activation in apoptosis, resulted in an RCF value of 0.39 (Fig. 2).

We next examined the anti-apoptotic activity of individual IAPs in mammalian apoptosis induced by transient overexpression of caspase-3. We introduced activated caspase-3 in 293HEK cells by transient transfection, using a pRSC expression vector harboring rev-caspase-3 [16]. The rearranged protein produced by this cDNA is more efficiently processed in transfected cells than native procaspase-3. Transfected cells expressed β-galactosidase and appeared blue with X-gal staining (Fig. 3A). Transfection of rev-caspase-3 induced cell shrinkage and nuclear condensation typical of apoptosis (Fig. 3A).

Individual IAPs tagged with a *myc* epitope were expressed at comparable levels in transfected 293HEK cells (Fig. 3B). We co-transfected single IAPs with rev-caspase-3 and counted β-galactosidase-positive cells at 24 h. As shown in Fig. 3C, co-expression of XIAP with rev-caspase-3 decreased the percentage of blue cells with condensed nuclei (50.1%), while c-IAP2, c-IAP1, and survivin provided less protection (61.7%, 73.1%, and 75.4% apoptotic cells, respectively). Baculovirus p35 and a dominant-negative mutant caspase-3 (C163S) were strongly protective in this assay and decreased apoptosis to 11.3% and 17.2% respectively.

We compared these results to the ability of each IAP to block apoptosis following death receptor signaling or genotoxic damage. At 24 h after transient transfection of pcDNA3-IAP plasmids, we treated 293HEK cells with TNFα or camptothecin. TNFα induced apoptosis in 77.0% of mock-transfected 293HEK cells by 24 h, while co-transfection of either baculovirus p35, dncaspase-3 (C163S), or a dominant-negative FADD(89–209) reduced levels of apoptosis to 34.7%, 20.8%, and 16.9%, respectively (Fig. 4A). XIAP and Bcl-X_L provided an intermediate level of protection against TNFα, decreasing apoptosis to 48.4% and 48.7%. c-IAP1, which had relatively low activity against caspase-3 in both yeast and 293HEK cell assays, was as effective as Bcl-X_L and XIAP in reducing TNFα-induced death (44.3%) (Fig. 4A). In contrast, c-IAP2, which ranked second to XIAP in caspase-3 inhibition, provided minimal resistance in TNFα-induced apoptosis, along with survivin (64.8% and 69.1% respectively).

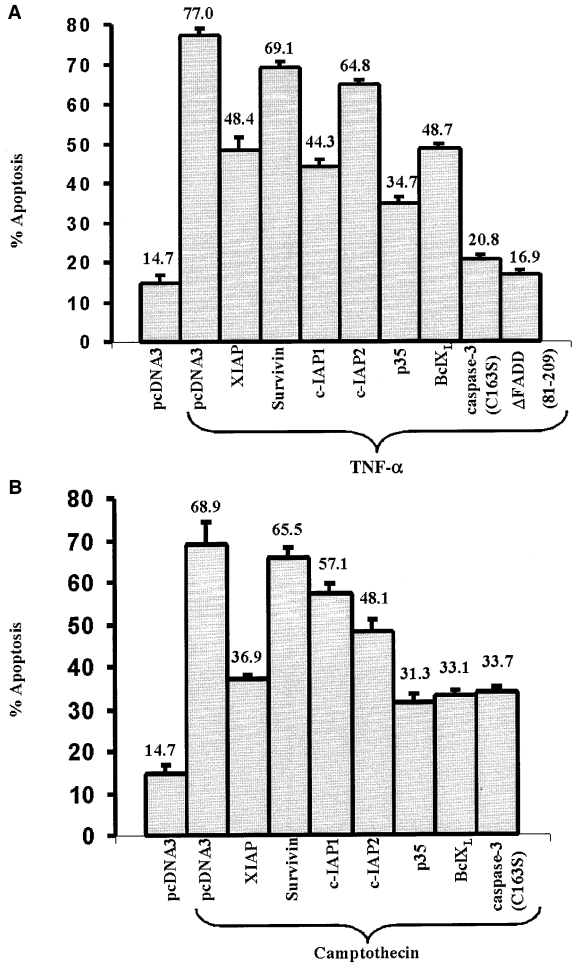

Fig. 4. TNFα- and camptothecin-induced apoptotic cell deaths are differentially regulated by IAP proteins. 293HEK cells were transiently transfected with test cDNAs. A: Apoptosis was assessed 24 h after addition of TNFα. B: Apoptosis was assessed 24 h after treatment with 2 μM camptothecin.

Treatment of 293HEK cells with the DNA topoisomerase I inhibitor, camptothecin, resulted in moderate levels of apoptosis at 24 h (68.9%), which was inhibited by prior transfection of baculovirus p35, Bcl-X_L or dncaspase-3 (31.3%, 33.1%, and 33.7% apoptosis, respectively). XIAP was again found to be the strongest inhibitor of the four IAPs for camptothecin-induced apoptosis (36.9%), while c-IAP2, c-IAP1, and survivin had lesser activities (48.1%, 57.1%, and 65.5% apoptosis, respectively) (Fig. 4B).

4. Discussion

4.1. Suppression of a caspase-3-dependent phenotype in S. cerevisiae

Functional studies on individual components of apoptotic pathways in higher eukaryotes are complicated by problems of redundancy and specificity, related to the existence of families of apoptotic effectors/regulators and extensive crosstalk between signaling pathways. The absence of endogenous homologs provides an advantage for functional analysis of selected mammalian genes in yeast expression models [17,18].

Heterologous expression of the mammalian cell death regulators Bcl-2 and Bax in *S. cerevisiae* has been utilized successfully to identify and test functional suppressors of these genes [19]. We exploited the lack of endogenous caspases in yeast to analyze the IAP class of caspase inhibitors in an in vivo setting with a single introduced caspase. We previously showed that co-transformation and expression of separate plasmids encoding the 20 kDa and 10 kDa subunits of active caspase-3 caused growth inhibition of *S. cerevisiae* [15].

Among individual IAPs tested, XIAP completely suppressed caspase-3-mediated growth suppression in yeast, while c-IAP1, c-IAP2, and survivin had lower, but detectable activities. These results are consistent with the in vitro activities of each IAP against recombinant active caspase-3, with the exception of survivin [7–9]. It was recently reported that bacterially expressed recombinant survivin, which binds to activated caspases-3 and -7, is inactive against caspase-3 in vitro, although it provided some protection from apoptosis induced by overexpression of procaspase-3 [20]. The yeast experiments indicate that survivin is a bona fide, although weak, in vivo inhibitor of caspase-3 [20]. The K_is of recombinant XIAP, c-IAP2, and c-IAP1 for caspase-3 are 0.7 nM, 35 nM, and 108 nM, respectively [7,8]. These IAPs ranked in the order XIAP > c-IAP2≥c-IAP1 as in vivo inhibitors of caspase-3 in yeast. Based on the RCF values, we can predict that the K_i of survivin for caspase-3 is greater than that of c-IAP1 (108 nM). Currently, difficulties with production of bioactive survivin protein in bacteria preclude these studies. The yeast heterologous expression system provides an alternative method for studying the relative inhibitory activities of IAPs against individual caspases.

4.2. IAP and caspase-3 interactions in mammalian cells

We next tested the ability of each IAP to inhibit cell death induced by overexpression of active caspase-3 in mammalian cells. Each of the IAPs bind to processed caspase-3, and XIAP and survivin have been demonstrated to inhibit apoptosis caused by overexpression of procaspase-3 [20]. All of the IAPs identified to date are inhibitors of multiple caspases and their ability to inhibit apoptosis may depend on the specific cellular apoptotic pathways activated as well as the spectrum of anti-caspase activity for each IAP. Co-transfection of the IAPs with rev-caspase-3 suppressed apoptosis in 293HEK cells in the order XIAP > c-IAP2 > c-IAP1 > survivin. These results are consistent with the yeast expression studies, and confirm that caspase-3 is the critical target in these cell death assays.

4.3. IAPs in TNFα- and camptothecin-induced apoptosis

Stimuli for mammalian apoptosis are transmitted through specific signal transduction pathways that converge on the proteolytic activation of one or more caspases [21,22]. We extended our studies of IAPs in mammalian cells to investigate their ability to suppress apoptosis after TNFR-I receptor engagement or exposure to camptothecin, a DNA topoisomerase I inhibitor. There were distinct differences observed in the anti-apoptotic activities of the IAPs for these cell death triggers. The dominant-negative mutant of caspase-3 (C163S) efficiently blocked TNFα-induced cell death, confirming the importance of caspase-3 in apoptotic signaling downstream of TNFα. As expected, XIAP, a potent inhibitor of caspases-3, -7 and -9, efficiently blocked TNFα-induced cell death [7,8]. Somewhat surprisingly, c-IAP1 also strongly inhibited TNFα-induced cell death, and c-IAP2 and survivin had minimal activity. Published studies have shown that c-IAP1 and c-IAP2 are direct inhibitors of caspases-3, -7, and -9, suggesting that these caspases are the principal targets of c-IAP1 and c-IAP2 anti-apoptotic activity [8,9]. For caspase-3 and caspase-7, cIAP2 has greater or equivalent inhibitory activity as c-IAP1 [8]. In light of these findings, our results suggest that c-IAP1 has additional activities that are important in regulating TNFα-induced apoptosis. C-IAP1 and c-IAP2 were initially identified in association with the TNFR-II receptor complex and bind to the receptor-associated cytoplasmic proteins, TRAF1 and TRAF2 [23]. c-IAP1 is also recruited to the TNFR-I signaling complex upon receptor engagement and blocks TNFα-induced cell death if co-expressed with TRAF1/TRAF2 [24]. Differences between c-IAP1 and c-IAP2 in their effects on TNFα signal transduction in 293HEK cells may contribute to their relative anti-apoptotic effects in this model.

Camptothecin, a DNA topoisomerase I inhibitor, induces DNA double-stranded breaks and, like other genotoxic agents, results in apoptosis secondary to mitochondrial release of apoptogenic factors [23]. The IAPs block chemotherapy-induced apoptosis in different experimental protocols [4]. For camptothecin, the IAPs ranked in potency as XIAP > c-IAP2 > c-IAP1 > survivin. In this case, the anti-apoptotic activity of the IAPs in the camptothecin model tracks with their ability to inhibit caspase-3 [7,8]. Like TNFα-induced death, the dominant-negative caspase-3 mutant efficiently blocked camptothecin-induced apoptosis. Previous reports indicate that chemotherapeutic drugs result in activation of caspase-3 through the assembly of the APAF-1/cytochrome *c*/caspase-9 apoptosome complex [25–27]. As XIAP, c-IAP1 and c-IAP2 block cytochrome *c*-induced activation of caspase-9 as well as caspase-3 activity, the relative potency of IAPs in camptothecin-induced apoptosis can be explained by their known activities as caspase inhibitors [7–9].

In summary, we describe the use of a yeast heterologous expression assay to evaluate the relative activity of IAP family members as inhibitors of a specific human caspase, caspase-3. This novel in vivo system can be used in parallel with in vitro biochemical studies of recombinant caspase inhibitor proteins and provides an alternative method of analysis in the event that bioactivity cannot be preserved during bacterial expression and purification. By correlating profiles of caspase inhibitory activity with studies of IAPs in apoptosis, additional insights into how IAPs function in apoptosis can be gained. In addition to inhibition of caspase activity and/or processing, roles for IAPs in death receptor signaling and centrosome regulation during the cell cycle have been proposed [4,28,29]. Elucidating the relationships between IAPs and their cellular targets should increase our understanding of their functions during homeostasis and disease.

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