Caspase-3 inhibits growth in *Saccharomyces cerevisiae* without causing cell death

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Abstract Caspase-3, a member of the caspase family of cell death proteases, cleaves cytoplasmic and nuclear substrates and promotes apoptotic cell death in mammalian cells. Although yeast homologs of apoptotic genes have not been identified, some components of apoptotic pathways retain function in yeast. Here we show that the expression of caspase-3 delays cell growth in *Saccharomyces cerevesiae* without causing cell death. Mutation of the caspase-3 QACRG active site abolished effects on yeast growth. Co-expression of caspase inhibitors alleviated growth inhibition in yeast as did the tripeptide caspase inhibitor ZVAD-fmk. These results suggest that substrates for caspase-3 are present in *S. cerevesiae* and may participate in the normal cell growth and division processes.

Key words: Caspase; Apoptosis; Yeast

1. Introduction

Caspases are a family of cytoplasmic cysteine endoproteases that function in cell death pathways in metazoans and higher organisms [1]. These enzymes are synthesized as inactive zymogens that are activated by proteolytic processing [2]. The active form of most caspases is composed of two polypeptides of approximately 20 (p20) and 10 kDa (p10) in a tetrameric structure of two p20-p10 heterodimers [3–10]. After an apoptotic stimulus, members of the caspase family are activated and cleave specific cytoplasmic and nuclear substrates such as fodrin and poly (ADP-ribose) polymerase (PARP) at preferred sites containing Asp in the P1 position [2,11,12]. Stringent regulation of caspase activity is likely to be vital for the cell survival.

A number of viral gene products function as caspase inhibitors and have provided important insights into caspase function and their role in proteolytic processing of caspase proenzymes. For example, the baculovirus p35 protein is a broad spectrum caspase inhibitor that blocks apoptotic cell death following any one of multiple stimuli in nematodes, *Drosophila* and mammalian cells [13,14]. In contrast, the cowpox virus serpin CrmA inhibits a limited number of apoptosis pathways as a selective antagonist of caspases −1, −8 and −10 [15–21]. A recently identified family of proteins called IAPs (inhibitors of apoptosis) has been characterized with broad activity for caspases [22–27]. Mammalian IAPs have recently been shown to inhibit the catalytic activity of three distal caspases (3, 7 and 9) in vitro [28–30]. The IAPs are the only mammalian proteins as yet shown to be specific inhibitors of caspases, suggesting that additional inhibitors remain to be discovered.

Single cell eukaryotes are not generally believed to possess homologous cell death machinery, yet several studies have described cell death or growth arrest in the yeasts *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe* following the expression of the pro-apoptotic Bax and Bak proteins [31–35]. As in mammalian cells, Bcl-2 and Bax are targeted to the mitochondria and Bax-induced cell death is associated with mitochondrial release of cytochrome *c* [36,37]. Bax lethality is prevented by the co-expression of anti-apoptotic Bcl-2 family members, suggesting that the molecular targets for certain cell death components are shared between yeast and metazoans [35,38].

Bax-induced killing in *S. cerevisiae* has been incorporated in a genetic screen for suppressors of Bax function [39,40]. Two isolates in the Bax screen have been described, the mammalian BI-1/TEGT gene encoding a resident ER protein and the yeast F0/F1 subunit ATPase 4 gene. These genes have comparable effects on mammalian apoptosis, demonstrating the utility of yeast-based screens for investigating cell death pathways in higher eukaryotes. However, since caspase inhibitors do not prevent Bax lethality in *S. cerevisiae* or *S. pombe*, Bax/Bcl-2-based screens will not identify apoptosis components that act on the caspase proteolysis pathway [35]. As for the Bcl-2 gene family, searches of the yeast genome database have not identified homologs of caspases. Despite this apparent lack of endogenous caspases in yeast, potential caspase cleavage sites are found in *S. cerevisiae* proteins (unpublished, MW and DH) and may function as substrates if exogenous active caspases are expressed. A recent report of the expression of procaspase-3 in the methylotrophic yeast *Pichia pastoris* described an inhibitory effect on cell growth.

In this report, we describe the effects of caspase-3 expression in *S. cerevisiae*. Caspase-3 expression in yeast is not le-

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Abbreviations: ZVAD-fmk, Z-Val-Ala-Asp-fluoro-methyl ketone; PARP, poly(ADP-ribose) polymerase; IAP, inhibitor of apoptosis; CrmA, cowpox-virus modifier A protein; CrmA-mut, cowpox-virus modifier A mutant protein; Gal, galactose; Glu, glucose; TRP, tryptophan; HIS, histidine; URA, uracil; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction

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thal, but it impairs cell growth. This growth inhibition is dependent on intact protease function, since either mutation of the active site cysteine, co-expression of an optimized CrmA caspase inhibitor or treatment with the broad spectrum caspase inhibitor ZVAD-fluoro-methyl ketone reverse caspase-3 growth inhibition in *S. cerevisiae*. Since caspase-3 functions, like those of Bax, may be recapitulated in yeast should allow rapid genetic or small compound-based screens for inhibitors of apoptotic pathways in higher eukaryotes.

2. Materials and methods

2.1. Yeast cell culture and transformations

All yeast strains were maintained on an appropriate dropout medium to select for plasmid marker expression. The yeast strains used in these studies were W303-1A (*MATa ade-2-1 his3-11 his3-15 leu2-3 leu2-112 trp1-1 ura3-1*) (kindly provided by Dr. Ted Young) and EGY48 (*MATα trp1 ura3 his3 leu2::(lexA* operators)$_6$*-LEU2*) (kindly provided by Dr. Roger Brent). Transformations were performed using the lithium acetate method using a total of 4–6 μg of yeast plasmid with 100 μg of sheared salmon sperm DNA. One tenth of each transformation reaction was plated onto selective media. ZVAD-fmk was resuspended in DMSO at 10 mM. It was applied to the yeast plates at a final concentration of 20 μm prior to plating of transformed yeast cells. Also colony growth was assessed at 96 h. For measurements of cell viability, cells were collected at 24 h intervals following plating and tested for their ability to exclude trypan blue. At least 300 cells were scored as live versus dead for each measurement.

2.2. Cloning of caspase-3 into yeast and bacterial expression vectors

The large 17 kDa subunit (amino acids 28–175) and the small 10 kDa subunit (amino acids 176–277) of caspase-3 were subcloned by polymerase chain reaction (PCR) from a human caspase-3 cDNA (pcDNA3) into the pYX233 (*TRP1*) and pYX213 (*URA3*) yeast expression vectors (Novagen), respectively, using standard recombinant DNA technologies (Maniatis). The mutant large 17 kDa subunit was generated using a two step PCR mutagenesis method that changed the amino acid cysteine-163 to glycine. The CrmA and CrmA-mut cDNAs were subcloned by PCR from pcDNA3 CrmA and pBluescript KS(+) CrmA-mut vectors into the pYX122 (*HIS3*) yeast expression vector (Novagen). For expression and quantification of recombinant protein, caspase-3 (amino acids 28–277) and CrmA were subcloned by PCR into pET28a(+) bacterial expression vector with the addition of a C-terminal His-6 tag (Novagen). CrmA-mut was excized from pBluescript KS(+) CrmA-mut as a *Sac*I-*Hin*dIII ×bp fragment and ligated to pRSET A histidine-tagged bacterial expression vector (Invitrogen) and linearized. All constructs were sequenced for authenticity and shown to be in frame. The details of the primer sequences used for PCR cloning will be provided upon request.

2.3. Immunoblotting assays

Overnight 100 ml cultures of caspase-3-transformed cells grown in 2% raffinose media were diluted to an OD_{600} of 0.8 into 2% galactose-containing media to induce caspase-3 expression. 10 ml aliquots were centrifuged at 5000 rpm and the cell pellet was washed twice with sterile de-ionized water. The yeast cells were resuspended in lysis buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA) with a protease inhibitor cocktail tablet (Boehringer, Mannheim) added immediately before using equal volumes of acid-washed 4 mm glass beads were added to each sample followed by vortexing for 2 min at room temperature. The protein content was quantitated using the Bio-Rad protein assay. The samples, containing 50 μg of protein, were resolved on a 12% SDS-polyacrylamide gel. The gel was transferred to nitrocellulose membranes and probed with an anti-caspase-3 polyclonal antibody (Santa Cruz) according to the manufacturer's instructions. 2 μg of purified recombinant caspase-3 was included as a positive control.

2.4. Expression and purification of His-tagged caspase-3, CrmA and CrmA-mut protein

The pET28a(+) caspase-3, pET28a(+) CrmA and pRSET A CrmA-mut bacterial expression plasmids were transformed into BL21(DE3) (Novagen) cells for protein expression and purification. 5 ml cultures containing the corresponding plasmids were grown overnight at 30°C. The following morning the overnight culture was diluted into a 1 l culture of Luria broth and grown at 37°C to an OD_{660} of 0.8. Isopropyl β-D-thiogalactopyranoside was added to each culture to a final concentration of 1 mM. Cultures were grown for an additional 3 h at 25°C. The cells were harvested and histidine-tagged fusion proteins were purified by a nickel chromatograph using a Qiagen Ni-NTA protein purification kit. Coomassie blue staining of SDS-polyacrylamide gels was used to determine the purity of the recovered proteins.

2.5. In vitro caspase-3 cleavage assays

[^{35}S]-labelled CrmA-mut protein was prepared from the pRSET A CrmA-mut plasmid by in vitro transcription and translation using a Promega TNT T7 transcription/translation kit. All caspase-3 cleavage reactions were performed in a buffer containing 20 mM Pipes, 100 mM NaCl, 10% sucrose and 10 mM dithiothreitol. 200 ng of recombinant caspase-3 was incubated with 3 μl of [^{35}S]methionine-labelled CrmA-mut protein in a total volume of 20 μl. 200 ng aliquots of recombinant caspase-3 were incubated with the indicated amounts of purified CrmA or CrmA-mut protein at 30°C for 15 min followed by the addition of 3 μl of in vitro translated [^{35}S]methionine-labelled CrmA-mut protein. After an additional h at 37°C the samples were subjected to 15% SDS-PAGE. The gel was dried and exposed to Kodak X-Omat AR X-ray film for 24 h at room temperature.

3. Results

We focused on studying the effects of caspase-3 in *S. cerevisiae* since previous studies have shown it to be an essential caspase required for mammalian apoptosis [41–43]. Caspase-3 preferentially cleaves DEVD-containing substrates and is activated as a central component in a proteolytic cascade in most, if not all, models of apoptosis [44]. Transient expression of pro-caspase-3 in Sf9 insect cells results in apoptosis, presumably as a consequence of autocatalytic proteolytic processing [45].

We initially inserted the cDNA encoding the 32 kDa caspase-3 pro-enzyme into the pYX243(*LEU2*) 2-micron yeast expression plasmid under the control of the GALx promoter. Galactose-induced expression of full length caspase-3 zymogen in yeast did not result in a detectable autoactivation of caspase-3 as judged by the appearance of processed large (17 kDa) and small (10 kDa) subunits (unpublished, MW), in contrast to a previous report in *P. pastoris*, and did not impair

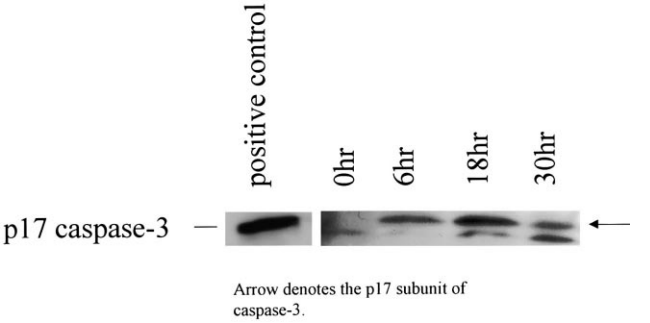

Fig. 1. Time course of caspase-3 expression in galactose media. W303-1A cells containing both the pYX233-p17 (*TRP1*) and pYX213-p10 (*URA3*) subunits of caspase-3 were grown overnight in YNB tryptophan- and uracil-deficient liquid media containing 2% raffinose. Cells were diluted to an OD_{660} of 0.8 into 2% galactose YNB tryptophan- and uracil-deficient liquid media to induce expression of the caspase-3 subunits. 10 ml aliquots of yeast cells were processed at the indicated time as described in Section 2. 50 μg of yeast lysate was loaded per lane and resolved on a 12% SDS-polyacrylamide gel. 2 μg of purified recombinant caspase-3 was loaded as a positive control.

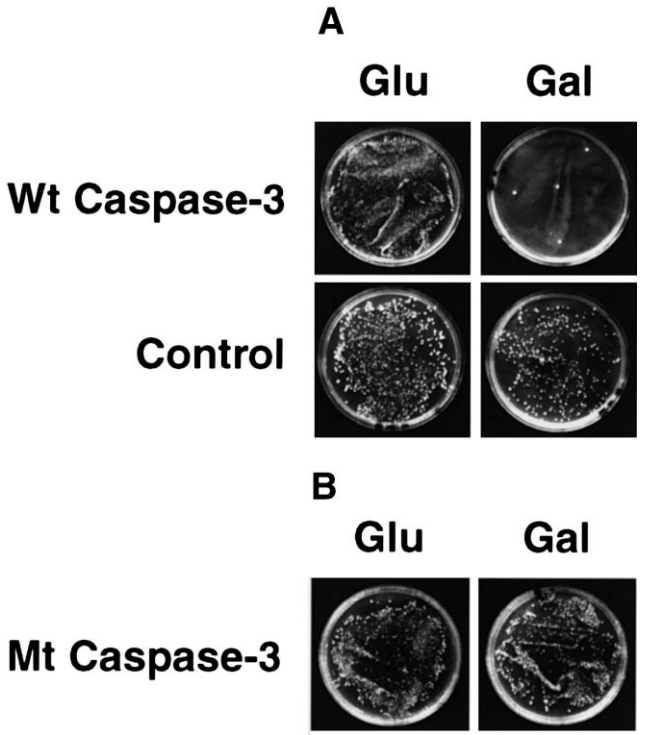

Fig. 2. Co-expression of the 17 and 10 kDa subunits of the caspase-3 enzyme inhibits the yeast growth. (A) W303-1A cells were transformed with the pYX233-p17 (*TRP1*) and pYX213-p10 (*URA3*) plasmids or control pYX233(*TRP1*) and pYX213 (*URA3*) plasmids. Cells were plated onto tryptophan- and uracil-deficient plates containing 2% glucose or galactose and incubated at 30°C for 96 h. (B) W303-1A cells were co-transformed with the mutant pYX233-p17(*TRP1*) and pYX213-p10(*URA3*) caspase-3 plasmids and incubated as described in Fig. 2A.

yeast growth or survival [46]. To circumvent the lack of caspase-3 autoactivation, we co-expressed the large and small subunits from separate plasmids to allow the intracellular assembly of active protease. cDNA fragments encoding the 17 and 10 kDa caspase-3 subunits were cloned into the 2-micron yeast episomal expression vectors pYX233 (*TRP1*) and pYX213(*URA3*), respectively, with each subunit under the control of the galactose-inducible GAL1 promoter. Using an antibody that recognizes the x, expression of the 17 kDa subunit was observed in galactose-containing media by 6 h (Fig. 1). Co-transformation with plasmids encoding each subunit of active caspase-3 resulted in no colonies on galactose plates (Fig. 2A). Colony formation in *S. cerevisiae* was similar to the vector controls when either the p17 or p10 subunit of caspase-3 was expressed alone (data not shown). To determine whether this effect was dependent on protease activity, we mutated the active site cysteine-163, which lies within the highly conserved QACRG pentapeptide sequence of the large subunit, to a glycine [41,45,47]. The expression of this mutant pYX233-p17 caspase-3 subunit with the normal pYX213 p10 subunit resulted in colony formation on galactose, comparable to vector controls (Fig. 2B).

To determine if the caspase-3 phenotype in yeast was due to cell killing, we compared the viability of transformants on galactose and glucose media by trypan blue dye exclusion. Viability of the two cultures over a 72 h period was greater than 90% under both conditions (Fig. 3A). However, a significant growth delay was evident for caspase-3-expressing cells in galactose media relative to glucose media when viable cell counts were compared (Fig. 3B). Thus, caspase-3 fails to induce cell death in *S. cerevisiae* but hinders cell growth. Mutant caspase-3 expression resulted in normal growth kinetics in liquid culture (Fig. 3A).

As an additional test for the requirement of specific caspase-3 protease activity in the yeast growth delay, we co-expressed caspase-3 with the cowpox virus serpin CrmA, or a modified version referred to as CrmA-mut [48]. CrmA is a potent inhibitor for caspases 1, 4 and 8, but relatively ineffective for caspases 2,3,6,7 and 10 and the *C.elegans* caspase homolog CED-3. While CrmA is a poor inhibitor of casp-

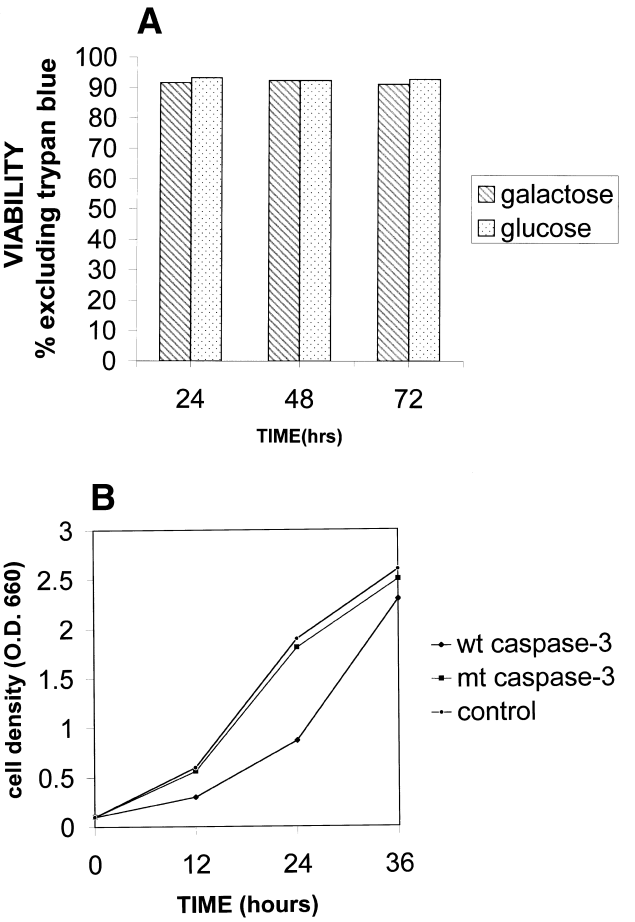

Fig. 3. Caspase-3 expression in yeast inhibits growth. (A) W303-1A colonies containing pYX233-p17(*TRP1*) and pYX213-p10(*URA3*) caspase-3 subunits were picked from tryptophan- and uracil-deficient glucose plates and re-streaked onto tryptophan- and uracil-deficient plates containing 2% glucose or galactose. Approximately 200 yeast cells were scored using trypan blue in triplicate at the specified time points. The results represent the percentage of yeast cells excluding trypan blue and are given as the mean ± S.D. These data are representative of at least three independent experiments. (B) Fresh yeast colonies containing wild-type or mutant caspase-3 plasmids were picked from a tryptophan- and uracil-deficient glucose plate and inoculated into 50 ml of YNB tryptophan- and uracil-deficient media containing 2% raffinose. After overnight growth at 30°C the culture was centrifuged and the cell pellet washed twice with sterile water. The cells were diluted to an OD_{660} of 0.10 into 50 ml of fresh YNB tryptophan- and uracil-deficient media containing 2% galactose. The OD_{660} of each culture was measured every 12 h over a 36 h time period. The data are representative of at least three independent experiments.

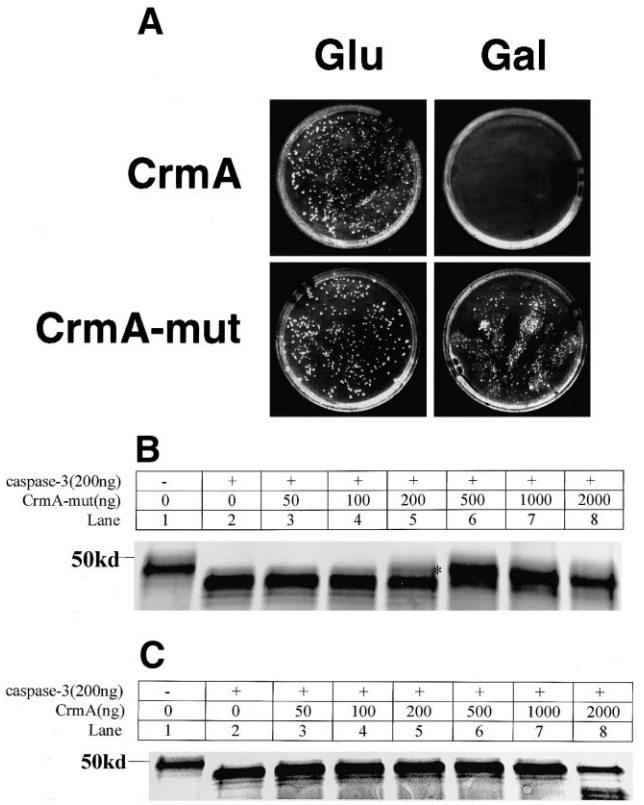

Fig. 4. Co-expression of CrmA-mut but not CrmA rescues the yeast growth defects caused by caspase-3 expression. (A) W303-1A cells were co-transformed with the pYX233-17 (*TRP1*) and pYX213-p10 (*URA3*) caspase-3 plasmids along with the pYX122CrmA3 (*HIS3*) or pYX122CrmA-mut (*HIS3*) plasmids. Transformants were grown for 96 h at 30°C on tryptophan-, uracil-, and histidine-deficient media containing glucose or galactose as described in Fig. 2A. (B) 200 ng aliquots of recombinant caspase-3 were incubated with the indicated amounts of purified CrmA-mut protein at 30°C for 15 min followed by the addition of 3 μl of in vitro translated [^{35}S]methionine-labelled CrmA-mut protein (*CrmA-mut). After an additional h at 37°C the samples were subjected to 15% SDS-PAGE. The gel was dried and exposed to Kodak X-Omat AR X-ray film for 24 h at room temperature. (C) 200 ng aliquots of recombinant caspase-3 were incubated with the indicated amounts of purified CrmA protein at 30°C for 15 min followed by the addition of 3 μl of in vitro translated [^{35}S]methionine-labelled CrmA-mut protein (*CrmA-mut). After an additional h at 37°C the samples were subjected to 15% SDS-PAGE. The gel was dried and exposed to Kodak X-Omat AR X-ray film for 24 h at room temperature.

ase-3, CrmA-mut contains a baculovirus p35 cleavage site inserted into the reactive loop of CrmA. p35 is cleaved by multiple caspases at this site and the resulting caspase-p35 complex is inactive. The CrmA-mut inhibitor was previously shown to be cleaved by CED-3 and capable of inhibiting CED-3 mediated apoptosis in *C. elegans*, while CrmA could not [48]. We cloned cDNAs for both inhibitors into the centromeric pYX122(*HIS3*) yeast episomal vector which placed their expression under the constitutive control of the yeast triose phosphate isomerase promoter. Yeast co-transformed with the caspase-3 and CrmA plasmids failed to form colonies on galactose, but growth suppression was relieved when the CrmA-mut plasmids were introduced with the caspase-3 plasmids (Fig. 4A). This result suggests that caspase inhibitors function similarly in yeast as in animal cells, and that CrmA-mut is an effective inhibitor of caspase-3. We confirmed the specificity of each inhibitor with an in vitro cleavage assay. With [^{35}S]methionine labelled CrmA-mut protein as a substrate, recombinant caspase-3 produced essentially complete cleavage (Fig. 4B, lane 2). The addition of purified recombinant CrmA-mut protein to the reaction caused a partial inhibition of this cleavage at a 1:1 molar ratio (Fig. 4B, lane 5) and a complete inhibition at a 2.5:1 molar ratio (Fig. 4B, lane 6). This is similar to the inhibitory properties of p35, which also has maximal effects at equimolar ratios with purified recombinant caspase. In contrast, CrmA failed to block caspase-3 activity even when present at a 10 fold molar excess (Fig. 4C, lane 8).

We also determined whether ZVAD-fluoro-methyl ketone, a broad spectrum irreversible caspase inhibitor, could suppress the growth defects induced by caspase-3. As shown in Fig. 5, plating of transformed yeast cells with caspase-3 onto plates containing 20 μM ZVAD-fmk prevented caspase-3-mediated growth inhibition.

4. Discussion

While most of the identified components of cell death pathways have regulatory functions, some proteins appear to have direct cytotoxic activities. Bax, a pro-apoptotic homolog of Bcl-2, produces apoptotic death after cell transfection with mitochondrial and microsomal derangements perhaps related to its function as a membrane channel [49]. The apoptotic caspase proteases can also have direct cytotoxic functions and are generally regarded as central executioners for programmed cell deaths. Transfection of single caspases can cause apoptotic cell death, following autoprocessing of the pro-enzyme [45,47,50–55]. We investigated whether, like Bax, the caspase-3 apoptotic protease is amenable to study in a heterologous yeast system that appears to lack a comparable cell death pathway.

Caspase-3 expression resulted in growth inhibition in *S. cerevisiae*, manifested as a delayed colony formation on agar and slow growth in liquid culture. Growth inhibition depended on the protease activity, as shown by its absence in yeast containing an active site mutation within the p17 catalytic subunit of caspase-3. Caspase-3 induced growth delay appeared to be dose-dependent, since co-expression of subunits from a low copy number centromeric yeast vector failed to produce a noticeable effect on *S. cerevisiae* cell growth (unpublished, MW). Previous studies have demonstrated that expression of two other mammalian proteins that pro-

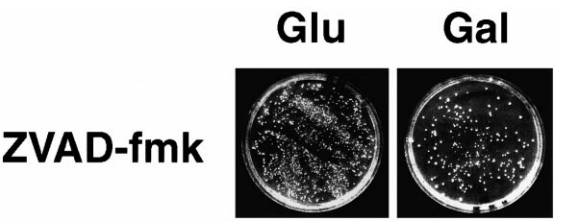

Fig. 5. The broad spectrum caspase inhibitor ZVAD-fmk alleviates caspase-3-mediated growth inhibition in yeast. W303-1A cells co-transformed with the pYX233-p17 (*TRP1*) and pYX213-p10 (*URA3*) caspase-3 plasmids were plated onto tryptophan- and uracil-deficient galactose or glucose plates containing 20 μM ZVAD-fmk. The transformed cells were incubated for 96 h at 30°C. The data are representative of at least three independent experiments.

mote apoptosis, Bax and Bak, is lethal in yeast. The lack of cell killing with caspase-3 expression in *S. cerevisiae* is perhaps not surprising, since additional caspases cannot be recruited by a proteolytic cascade in yeast, as they can in mammalian cells. Bax expression in respiratory-deficient yeast strains has also been reported to cause growth inhibition rather than cell death, suggesting that expression of other apoptotic factors may compromise but not curtail cell growth [33]. Interestingly, not all caspases had growth inhibitory effects in yeast. This is likely to reflect the functional differences between caspases [56–60]. Specifically, among caspases 2, 3, 6–9 expressed as active subunits in yeast, only caspase-3 resulted in a severe growth inhibition in yeast (unpublished, MW and DH). It can be inferred from these results that substrates for caspase-3 exist in *S. cerevisiae* and may include endogenous yeast proteins required for cell growth and division. These substrates may represent homologs of proteins that are cleaved by caspase-3 during mammalian apoptosis, as cleavage sites may be conserved (unpublished, MW and DH).

We also demonstrated that caspase inhibitors identified in higher eukaryotes are functional in *S. cerevisiae*. The yeast expression system discriminated between CrmA and CrmA-mut proteins as caspase inhibitors. These proteins are identical, except for the substitution of the amino acid sequence DQMDGFHD within the reactive cleavage loop of CrmA (aa 300–307: LVADCAST) [48]. The eight amino acid sequence in CrmA-mut is derived from the reactive cleavage site of the p35 baculovirus protein, which is preferentially cleaved by caspase-3-like proteases [61]. Previous studies demonstrated that CrmA-mut, but not CrmA, inhibited programmed cell death in *C. elegans*, consistent with its enhanced ability to be cleaved by the CED-3 protease in vitro [48]. CrmA and p35 are suicide inhibitors that form a stable protease-inhibitor complex after their cleavage. CED-3 and caspase-3 have similar substrate specificities and our results demonstrate that CrmA-mut is a better in vivo inhibitor of caspase-3 in yeast and in vitro inhibitor in the cleavage assay [60].

The broad spectrum caspase inhibitor ZVAD-fmk inhibits apoptosis in mammalian cells initiated by different stimuli, including the Fas family of death receptors and DNA damaging agents. The diversity of cell deaths inhibited by ZVAD-fmk underscores the significance of caspase activity during apoptosis. The yeast growth defect induced by caspase-3 expression was reversible with 20 μM ZVAD-fmk, a concentration routinely used to block mammalian cell culture models of apoptosis [62]. This result strongly suggests that the ZVAD-fmk moiety crosses the yeast cell membrane and maintains its inhibitory activity intracellularly. Therefore, this caspase-3 expression system in *S. cerevisiae* should prove to be useful for testing small compounds or screening mammalian cDNA expression libraries for novel caspase-3 inhibitors.

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