Cascades of Mammalian Caspase Activation in the Yeast Saccharomyces cerevisiae*

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Caspases (aspartate-specific cysteine proteases) play a critical role in the execution of the mammalian apoptotic program. To address the regulation of human caspase activation, we used the yeast Saccharomyces cerevisiae, which is devoid of endogenous caspases. The apical procaspases, -8β and -10, were efficiently processed and activated in yeast. Although protease activity, per se, was insufficient to drive cell death, caspase-10 activity had little effect on cell viability, whereas expression of caspase-8β was cytotoxic. This lethal phenotype was abrogated by co-expression of the pan-caspase inhibitor, baculovirus p35, and by mutation of the active site cysteine of procaspase-8β. In contrast, autoactivation of the executioner caspase-3 and -6 zymogens was not detected. Procaspase-3 activation required co-expression of procaspase-8 or -10. Surprisingly, activation of procaspase-6 required proteolytic activities other than caspase-8, -10, or -3. Caspase-8/β or -10 activity was insufficient to catalyze the maturation of procaspase-6. Moreover, a constitutively active caspase-3, although cytototoxic in its own right, was unable to induce the processing of wild-type procaspase-6 and vice versa. These results distinguish sequential modes of activation for different caspases in vivo and establish a yeast model system to examine the regulation of caspase cascades. Moreover, the distinct terminal phenotypes induced by various caspases attest to differences in the cellular targets of these apoptotic proteases, which may be defined using this system.

Apoptosis is a highly regulated program of cellular suicide critical for the development and homeostasis of multicellular organisms (1, 2). Many human diseases, such as neurodegenerative conditions, AIDS, and several forms of cancer, have been attributed to deregulation of the apoptotic program (3). The controlled disassembly of cellular structures in response to a wide variety of apoptotic signals is characterized by distinct biochemical and morphological changes (4, 5). The coordinated activation of a family of cysteine proteases that cleave at specific aspartic acid residues, termed caspases, appears crucial for the execution of the apoptotic program (6–9). Strict sequence specificity limits the endoproteolytic activity of the mature caspases to a few sites within a fairly small number of defined substrates. Nevertheless, these cleavages result in the activation of pro-apoptotic functions such as the caspases themselves and DNA fragmentation factor, as well as the inactivation of proteins involved in DNA repair, mRNA splicing, or the maintenance of nuclear architecture (6–11).

Over 11 human caspases have been identified that share similarities in sequence and activity. Phylogenetic analyses distinguish two subfamilies comprising the interleukin 1β-converting enzyme-like proteases (caspase-1, -4, -5, and -13) and the CED-3/CPP32 proteases (caspase-2, -3, -6, -7, -8, -9, and -10) (6, 9, 11). These proteins are expressed as precursor zymogens or procaspases, whose activation involves sequential proteolytic cleavages at aspartic acid residues to liberate the large and small subunits of the mature caspase as well as the prodomain (6, 9, 11). The crystal structures of mature caspase-1 and caspase-3 indicate a heterotetramer where two small subunits are flanked by two large subunits, with one of each subunit contributing to the active sites of the heterodimeric enzyme (11, 12). The ability of certain caspases to autoactivate or catalyze the processing of other caspase zymogens suggests the sequential activation of caspases in protease cascades (7, 9, 13, 14). This is supported by an analysis of cleavage sites within the caspase zymogens and the use of specific tetrapeptide inhibitors to abolish specific caspase activities (9, 15, 16).

Among the CED-3 subfamily, the proteases can be further subdivided on the basis of prodomain structure (6, 9, 11). Caspases-3, -6, and -7, for example, contain short prodomains for which no function has been ascribed. In contrast, caspase-2, -8, -9, and -10 contain large prodomains of varying structure, and these caspases are thought to act upstream of caspase-3, -6, and -7. The large prodomains of caspase-10 and -8 each contain two domains homologous to the death effector domain (DED)† of FADD (17, 18). Homophilic interactions between the DED domains of the caspase-8 zymogen and FADD result in the recruitment of caspase-8 to the cell death receptor FAS/APO-1/CD95 following ligand binding (17–19). The formation of this death-inducing signal complex initiates the proteolytic processing of caspase-8, perhaps as a result of receptor oligomerization, to liberate the mature heterodimeric p18/p11 enzyme (20, 21). This, in turn, suggests that caspase-8 functions at the apex of a cascade of sequential proteolytic cleavages culminating in the activation of the executioner caspases-3, -7, and -6. Presumably a similar mechanism would apply to caspase-10. However, caspase-10 also appears to be involved in the activation of caspase cascades in response to

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different apoptotic stimuli, such as DNA-damaging agents and staurosporine (14, 22).

The definition of caspase cascades in mammalian cells has largely relied on biochemical analyses of caspase activities in cell-free systems, the use of specific inhibitors, or a temporal ordering of sequential caspase processing in apoptotic cells (9–11, 23). However, the identification of at least 11 human caspase genes, coupled with complex patterns of caspase gene expression in most cells, precludes a direct analysis of individual caspase function in the absence of other caspases. To circumvent these issues, we expressed human caspase-8β, -10, -3, and -6 in the yeast Saccharomyces cerevisiae to investigate the autocatalytic and sequential proteolytic processing of these zymogens, in the absence of other caspase-like activities. Searches of the yeast genome failed to identify caspase homologs, and caspase-like activities have not been detected in yeast cell extracts. The utility of this genetically tractable organism in studying apoptotic mechanisms was recently demonstrated by the identification of yeast and human genes that regulate the proapoptotic activity of Bax in yeast (24–27). Here, we demonstrate that in yeast it was possible to establish different cascades of caspase activation and directly assess the regulation of proapoptotic activity of Bax in yeast (24–27).

Materials, Yeast Strains, and Plasmids—100 μM stocks of Ac-DEVD-CHO (Biomol) and Ac-IETD-AFC (Enzyme Systems, Inc.) and a 20 mM stock of Ac-DEVD-AMC (Bachem) were prepared in dimethyl sulfoxide (Me2SO) and stored at -20 °C.

Yeast strain FY250 (Mata, ura3-52, his3220, leu2Δ1, trp1Δ63) was kindly provided by Fred Winston (Harvard Medical School, Boston). Petri dishes lacking mitochondrial DNA (FY250 ρ−) were selected by incubating exponentially growing cells in synthetic complete media (SC) supplemented with 2% dextrose or galactose. Alternatively, the cultures were diluted 10-fold and plated onto selective media supplemented with dextrose or galactose. Accumulation of yeast was confluent, and shaking cells cultured on selective medium plates including dextrose or galactose at 30 °C was determined with a final 2% dextrose (SC – uracil + dextrose or SC – leucine + dextrose media, respectively) (33). Cells co-transformed with UR3 and LEU2 marker plasmids were plated on SC – uracil – leucine + dextrose media. To assess the effects of caspase expression on cell viability, exponentially growing cultures of individual transformants were serially diluted 10-fold in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 5-μl aliquots were spotted onto selective media supplemented with dextrose or galactose. Alternatively, the cultures were diluted 1:100 in selective medium containing raffinose and induced at an A595 = 0.3 with a final 2% galactose (31). At the times indicated, aliquots were serially diluted 10-fold and plated onto selective medium supplemented with dextrose. The number of viable cells forming colonies at 30 °C was determined. Additional samples were processed for microscopy and caspase activity assays (see below).

Fluorescence Microscopy—Galactose-induced cells were collected by centrifugation, washed with 0.25 volume 0.2 M Tris-HCl (pH 7.5), 1 M sorbitol and fixed in the same buffer plus 70% ethanol (34). After 1 h at room temperature, the fixed cells were stored at -20 °C or processed for microscopy. For the latter, the cells were pelleted and resuspended in 0.2 M Tris-HCl (pH 7.5), 1 M sorbitol containing 3–5 μl of DAPI (1 mg/ml) to stain the DNA. Following three consecutive washes with 0.2 M Tris-HCl (pH 7.5), 1 M sorbitol, the cells were resuspended in the same buffer, mounted on glass slides, and viewed on a Nikon Optiphot photomicroscope with an epi-fluorescence attachment EF-D mercury set with UV filter block.

EXPERIMENTAL PROCEDURES

Caspase Processing and Activity—Crude cell extracts of galactose-induced cultures (15 μl) were prepared essentially as described (35), except the lysate buffer was supplemented with 0.1% CHAPS. A battery of protease inhibitors (100 μM phosphomethylsulfonyl fluoride, 800 μM sodium bisulfite, 100 μM sodium fluoride, 20 μM benzamidine, 3 μM pepstatin A, 3 μM leupeptin, 1 μM chymostatin, 2 μM aprotinin, 1 μM phosphoramid, 7 μM E-64, 2.5 μM PMSF) was added in all buffer sets. Extracts were prepared to cell extracts and in caspase activity assays to prevent non-specific proteolytic degradation. Following cell lysis by vortexing with glass beads (35), the protein concentration of the clarified extracts was determined with the Bio-Rad assay.

To assess caspase proenzyme processing, cell extracts (100 μg of total protein) were electrophoretically resolved in 12% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes. The blot was blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.4% Tween 20 (TTBS), washed with TTBS, and incubated with a horseradish peroxidase-conjugated mAb specific for the T7 epitope (Novagen). After extensive washing with TTBS, the T7 epitope-tagged polypeptides were visualized by chemiluminescence using chemiluminescent-horseradish peroxidase reagents supplied by Novagen or ECL reagents from Amersham Pharmacia Biotech. Caspase proenzyme expression and processing were also assessed using rabbit polyclonal antibodies raised against the large subunits, followed by chemiluminescent detection using an alkaline phosphatase-coupled goat anti-rabbit antibody and CL-AP reagents from Novagen.

Caspase activity was assessed by fluorometric detection of Ac-DEVD-AMC and Ac-IETD-AFC cleavage in crude cell extracts (36) per the manufacturer’s recommendations. In 10-μl reaction volumes, 4 μg of protein were incubated with a final 50 μM Ac-DEVD-AMC or Ac-IETD-AFC in lysis buffer at 37 °C. After 30 min, the reactions were quenched by the addition of lysis buffer (90 μl or 1 ml, as indicated). AMC and AFC cleavage (emission at 460 and 505 nm, respectively) was measured in a Perkin-Elmer Luminescence Spectrometer LS50B following excitation at 380 and 400 nm, respectively. Where indicated, the caspase inhibitor Ac-DEVD-CHO was included in the reaction at a final 100 μM.

Isolation of Yeast Genomic DNA—Yeast genomic DNA was isolated as described (30). Briefly, 10-ml cultures of FY250 ρ− cells were harvested by centrifugation following galactose induction for 20 h. The cells were washed with 1 ml of distilled H2O, resuspended in 150 μl of ZSB...
buffer (100 mM sodium citrate, 1 M sorbitol, 60 mM EDTA, 0.5 mg/ml Zymolyase 20T (Seikagaku, Inc.), and 100 mM β-mercaptoethanol) and incubated for 40 min at 37 °C. This was followed by the addition of 150 μl of 0.1 M Tris (pH 8.0), 10 mM EDTA, 2% SDS for 10 min at 60 °C and 150 μl of 5 mM potassium acetate (pH 4.3) on ice for 30 min. Following centrifugation at 18,000 × g at 4 °C, the genomic DNA in the supernatant was precipitated, resuspended in 200 μl of distilled H₂O, and successively treated with 100 μg/ml RNase and 20 μg/ml proteinase K for 10 min at 37 °C. The DNA was phenol-extracted, ethanol-precipitated, and resuspended in 50 μl of 20 mM Tris (pH 8.0), 10 mM EDTA. The integrity of 5 μg of DNA was assessed by agarose gel electrophoresis.

RESULTS

Inducible Expression of Human Caspases in Yeast—To begin assessing the autocatalytic and sequential proteolytic processing of human caspases in yeast cells lacking endogenous caspase activities, caspase-3, -6, -8β, and -10 cDNAs were cloned into single copy yeast expression vectors, under the galactose-inducible GAL1 promoter (Fig. 1A). These enzymes include those thought to initiate apoptotic caspase cascade(s) in response to ligation of receptors like FAS/APO-1/CD95 (caspase-8β and -10) or other stimuli such as DNA-damaging...
agents (caspase-10), as well as more downstream caspases implicated in the execution of the apoptotic program (caspase-3 and -6) (6, 9, 11, 17, 37). The constructs included an N-terminal T7 epitope tag to allow for an unambiguous assessment of zymogen expression in immunoblots of whole cell extracts, as well as the sequential proteolytic processing of the procaspases. The assignment of processed bands was confirmed with polyclonal antibodies.

To assess the effects of galactose-induced caspase expression on yeast cell viability, FY250 cells were transformed with the indicated combinations of caspase expression vectors and selected on media supplemented with dextrose to suppress pGAL1 expression. To induce caspase expression, exponentially growing cultures of individual transformants were serially diluted 10-fold and 5-μl volumes were spotted onto selective agar containing dextrose (represses pGAL1 expression) or galactose (induces pGAL1 expression).

In mammalian cells, overexpression of some caspasezymogens is lethal (9, 11, 37). In yeast, however, galactose-induced expression of caspase-3 or -10 alone had no detectable effect on cell viability (Fig. 1B), suggesting a lack of zymogen processing and/or caspase activity. In contrast, yeast cells co-transformed with caspase-3 and -10 expression vectors experienced a 50–100-fold drop in viability on galactose plates (Fig. 1B), presumably in response to caspase activation.

Galactose-induced expression of caspase-8β, on the other hand, was extremely toxic. In comparison to the uninduced controls, a 3-log drop in cell viability accompanied caspase-8β expression (Fig. 1B). This lethal phenotype appeared to be a direct consequence of procaspase-8β autoactivation, as expression of procaspase-8βC345S, in which the active site cysteine was mutated to serine, had little effect on cell viability (Fig. 1B). Moreover, the cytotoxic activity of caspase-8β was suppressed by co-expression of the baculovirus p35 gene product, a specific caspase inhibitor (6, 10, 38), yet exacerbated in the presence of procaspase-3 (Fig. 1B). These data suggest caspase-8β is autoprocessed and that this p35 inhabitable activity suffices to promote yeast cell death and initiate caspase-3 processing.

**Caspase-10 and -8β Autoactivation in Yeast**—To address this, caspase zymogen processing and activity were assayed. Cultures of exponentially growing cells were induced with galactose at an A595 = 0.3. To assess cell viability, aliquots were taken at the times indicated (Fig. 2), serially diluted, and the number of viable cells forming colonies determined. Caspase expression and processing were monitored in immunoblots of crude cell extracts probed with a monoclonal antibody specific for the N-terminal T7 epitope tag (Figs. 3 and 4). Caspase activity was also assayed with synthetic fluorescent tetrapeptide substrates (Table I).

Consistent with the results in Fig. 1B, galactose-induced expression of caspase-10 or -8β alone had demonstrably different effects on cell viability. In Fig. 2A, the induction of caspase-10 expression had no adverse effects; the growth curve of these cells was indistinguishable from that of the vector control. Surprisingly, not only was the caspase-10 zymogen efficiently expressed, as evidenced by the presence of the intact p57 band in the immunoblot shown in Fig. 3A, but it was also processed. The p45 band corresponds to the N-terminal product of the proteolytic cleavage between the large and small subunit domains, whereas a second proteolytic cleavage would liberate the p28 prodomain. A band corresponding to the large p17 subunit was also detected with specific polyclonal antibodies (data not shown). A time course of caspase-10 zymogen processing indicates the p45 band accumulates prior to the p28 prodomain (data not shown), supporting a model of sequential cleavages between the large and small subunits followed by the liberation of the prodomain in the maturation of caspase-10 (16). Regardless of the order of these events, however, the proteolytic processing of procaspase-10 correlates with detectable caspase activity in vivo. When incubated with the fluorometric substrate DEVD-AMC (Table I, 100-μl reaction), a 5-fold increase in substrate cleavage was detected in extracts of caspase-10 expressing cells in comparison with substrate alone.

**Fig. 2. Time course of galactose-induced caspase expression.** Caspase expression in exponentially growing FY250 cells, transformed with the indicated YCpGAL1-caspase constructs (caspase-3, -10, or -10/-3 in A) (caspase-3, -8β, or -8β/-3 in B), was induced by the addition of a final 2% galactose (t = 0). At the times indicated, aliquots were serially diluted and plated onto S.C.-uracil-leucine plates containing dextrose. The number of viable cells forming colonies, relative to t = 0, was determined. The vector controls were transformed with URA3/LEU2, pGAL1 plasmids lacking caspase cDNAs. The values are an average of three experiments.
or with extracts derived from the vector controls. Moreover, this activity was inhibited by the caspase suicide substrate DEVD-CHO (Table I).

In contrast, the induction of caspase-8β produced a dramatic reduction in cell viability (Fig. 2B). The time course of cell lethality correlated with the accumulation of processed caspase-8β; the p55zymogen was rapidly cleaved to yield the p43 prodomain/large subunit, followed by the liberation of the p25 prodomain (Fig. 4, A and B). The identity of these bands, as well as the appearance of the caspase-8β p18 subunit, was confirmed with specific polyclonal antibodies (data not shown). Caspase-8β activity was also detected in crude cell extracts with DEVD-AMC, which was DEVD-CHO inhibitable (Table I). The autocatalytic processing of procaspase-8β was confirmed in similar studies with the caspase-8βC345S mutant. Substitution of Ser for the active site Cys inhibited the efficient processing of the caspase-8β zymogen (Fig. 5, A and B) and completely abolished DEVD-AMC cleavage in crude cell extracts (Fig. 5C). The appearance of an N-terminal fragment slightly smaller than the p25 prodomain band detected following activation of wild-type caspase-8β (small and large arrows, respectively, in Fig. 5B) was consistently observed in yeast cells expressing either form of caspase-8β. However, the initial cleavage event between the large and small subunits, shown for wild-type caspase-8β in Figs. 4A and 5B, was not detected in these blots of caspase-8βC345S (Fig. 5, A and B). Although the responsible proteolytic activity is presumably yeast-encoded, it was insufficient to catalyze the maturation of procaspase-8β.

Consensus cleavage sites for many caspases, including caspases-3, -6, and -8, have been defined using combinatorial libraries of tetrapeptide substrates (15, 39). Structural similarities between caspase-8β and -10 might predict common substrates, consistent with their presumed role in initiating the proteolytic processing of downstream caspases (18, 21). However, several observations suggest that significant differences exist. For example, immunostaining of proteins derived from caspase-10 and -8β expressing cells with the T7-specific mAb or caspase-specific polyclonal antibodies indicates thezymogens were efficiently expressed and processed (Figs. 3 and 4, data not shown). Yet, as only caspase-8β activity was cytotoxic, these mature caspases appear to have different cellular targets. In addition, the relative levels of caspase-10 or caspase-8β activity detected in crude cell extracts depended upon the tetrapeptide substrate used. In assays with DEVD-AMC, caspase-10, and -8β activities were roughly equivalent (Table I). However, in assays with IETD-AFC, which contains the cleavage site in procaspase-3 found between the large and small subunits, caspase-8β activity was almost 4-fold over that detected in extracts of cells expressing caspase-10 (Table I). Whereas these data demonstrate the efficient expression and activation of caspase-10 and -8β in yeast, apparent differences in the substrate specificities of these distinct caspases distinguish the cytotoxic activities of the mature enzymes.

Caspase-3 Zymogen Processing Is Promoted by Caspase-10 or -8β—The induction of caspase-3 expression, on the other hand, had no discernible effect on cell viability (Figs. 1A and 2, A and B), and processing of the procaspase p34 zymogen was not detected (Figs. 3, A and B, and 4B). Moreover, despite high levels of procaspase-3 expression, no significant cleavage of the substrates DEVD-AMC or IETD-AFC was detected in crude extracts of caspase-3 expressing cells (Table I). Although the initiator caspases, -10 and -8β, were capable of autoactivation in yeast, the executioner caspase-3 was not.

To establish the ability of mature caspase-10 or caspase-8β to initiate the proteolytic processing of the caspase-3 zymogen, yeast cells co-expressing caspase-10 or -8β with caspase-3 were examined. In the case of caspase-10/3, an immediate decrease in cell viability was observed following galactose induction (Fig. 2B), with a 5-fold reduction in the number of viable cells at 6 h. In cell extracts, the relative levels of processed caspase-10 products were slightly elevated in the presence of caspase-3 (Fig. 3A). However, processing of the caspase-3 zymogen, as evidenced by the accumulation of the p20 prodomain/large subunit polypeptide, absolutely required co-expression of caspase-10 (Fig. 3A). The identity of the p20 band and liberation of the large subunit were confirmed with polyclonal antibodies (Fig. 3B). A 3-fold increase in DEVD-AMC cleavage by caspases-10/3 was also evident in these extracts, in comparison to that detected in extracts of cells expressing caspase-10 alone (Table I).

More pronounced effects were observed with cells co-expressing caspases-8β/3. At 6 h, there was a 20-fold decrease in cell viability of cells expressing both caspases in comparison to those expressing caspase-8β alone (Fig. 2B). This coincided with the maturation of the caspase-3 zymogen (Fig. 4B) and a greater than 4-fold increase in DEVD-AMC cleavage (Table I). As with caspase-8β alone and caspases-10/3, cleavage of DEVD-AMC in the caspases-8β/3 extracts was completely blocked by the inhibitor DEVD-CHO (Table I). Lower levels of intact p55 caspase-8β zymogen and intermediate p43 bands, as well as low levels of processed caspase 3, were consistently observed in extracts of cells co-expressing caspase-8β/3 (compare caspase-8β and caspases-8β/3 lanes in Fig. 4B). Despite the inclusion of osmotic support, this reflects the preferential lysis of cells that have accumulated the mature caspases as the total protein in each lane was the same. Nevertheless, in all cases, caspase-8β, -8β/3, and 10/3-induced lethality was sup-

**Fig. 3.** The caspase-10 zymogen is efficiently processed and can initiate the maturation of caspase-3. Galactose-induced cultures were prepared as described in the legend to Fig. 2. At 8 h following galactose induction, cells were collected by centrifugation, and extracts were prepared as described under "Experimental Procedures." 100 μg of total protein were resolved by PAGE. A, caspase-10 and -3 bands were visualized by immunostaining with a T7-specific mAb and chemiluminescence. In a parallel blot B, caspase-3 bands were immunostained with a caspase-3-specific polyclonal antibody, followed by a second antibody and chemiluminescent staining (see "Experimental Procedures"). The identity and size of the procaspases and processed bands are indicated on the right. Vector extracts were prepared from cells transformed with vector controls.
Human Caspase Zymogen Processing in Yeast

**TABLE I**

| Substrate | Tetrapeptide cleavage (fold increase relative to vector control) |
|-----------|---------------------------------------------------------------|
| DEVD (100-μl reaction) | 1.1 ± 0.1, 4.5 ± 0.4, ND*, 5.3 ± 0.4, ND |
| DEVD | 1.1 ± 0.1, 2.2 ± 0.2, 9.7 ± 0.6, 2.6 ± 0.5, 7.9 ± 1.7 |
| DEVD + inhibitor | ND, 1.1 ± 0.1, 1.2 ± 0.3, 1.1 ± 0.1, 1.4 ± 0.2 |
| IETD | 1.3 ± 0.1, 8.7 ± 0.1, 5.6 ± 0.1, 2.4 ± 0.2, 1.8 ± 0.1 |

* Extracts of yeast cells expressing the indicated caspases were prepared as described in Fig. 3. After correcting for total protein, caspase activity was detected by the cleavage of fluorometric tetrapeptide substrates Ac-DEVD-AMC or Ac-IETD-AFC as detailed under "Experimental Procedures." For 100-μl reaction volumes, the 10-μl reactions were quenched with 90 μl of lysis buffer. All other reactions were quenched with 1 ml. Where indicated, the caspase inhibitor Ac-DEVD-CHO was included in the reaction. Average fluorescence units from four different experiments were divided by that obtained with extracts of cells transformed with the vector controls. Standard deviations are as indicated.

Not determined.

**Fig. 4.** Procaspase-8β is efficiently processed and activates the processing of procaspase-3. Following galactose induction, extracts were prepared from cells expressing procaspase-8β, -3, or -8β plus -3 (as described in Fig. 2 and "Experimental Procedures") at the times indicated (A) or at 8 h (B). 100 μg of protein were resolved by SDS-PAGE, and the indicated caspase bands were visualized by immunostaining (T7 mAb) and chemiluminescence. Vector extracts were prepared from cells transformed with the vector controls.

pressed by treatment with the cell-permeable caspase inhibitor, Z-VAD-fluoromethyl ketone (data not shown).

Caspase-3 exhibits a higher affinity for DEVD substrates than caspases-8 and -10 (9, 10, 15). This is also evident in the DEVD-AMC cleavage assays summarized in Table I. Synthetic fluorometric substrates containing the sequence IETD, the presumptive cleavage site between the large and small subunit domains in the caspase-3 zymogen (9, 11), were a poor substrate for caspase-10. The extent of IETD-AFC cleavage was also diminished in caspases-10/-3 and caspases-8β/-3 extracts, relative to that observed in extracts of cells expressing caspase-10 or -8β alone (Table I). Although the autoprocessing of caspase-3 presumably cleavage at IETD, the same sequence of amino acids in the context of a fluorescent substrate appears to inhibit caspase-3 activity. Whether or not mature caspase-3 is capable of proteolytically processing its own zymogen in yeast remains to be determined.

Caspase-6 Zymogen Activation Is Not Induced by Caspase-8β, -10, or -3—When the activation of procaspase 6 was investigated, the results were quite surprising. As in the case of caspase-3, pGAL1-promoted expression of caspase-6 alone had no adverse effect on yeast cell viability (Figs. 6A and 7B). In contrast, yeast cell viability was also unaffected by co-expression of caspase-6 and -10, and the cytotoxic activity of caspase-8β was not augmented by co-expression of caspase-6 (Fig. 6A). This was reflected in the absence of detectable processing of procaspase-6 in cells expressing caspase-6 alone or co-expressing caspases-6/-8β or caspases-6/-10 (Fig. 6B). Moreover, no increment in DEVD-AMC cleavage was detected in extracts of cells co-expressing caspase-6 (data not shown). Thus, whereas mature caspase-8β and -10 clearly initiate the activation of procaspase-3, they were insufficient to induce caspase-6 zymogen processing.

To address the possibility that additional caspase activities, such as caspase-3, are required for the sequential activation of procaspase-6, constitutively active forms of caspase-3 and -6 (caspase-3-rev and caspase-6-rev, respectively) were used. As diagrammed in Fig. 7A, the reversal of the large and small subunit sequences in the caspase-3-rev or -6-rev zymogens results in a constitutively active form of each caspase (32). In contrast to the wild-type zymogens, galactose-induced expression of caspase-3-rev or caspase-6-rev produced over a 4-log decrease in yeast cell viability (Fig. 7B) and a dramatic increase in DEVD-AMC cleavage in crude cell extracts (data not shown). However, neither the cytotoxicity nor catalytic activity of caspase-3-rev was enhanced by co-expression of wild-type caspase-6. Similar results were obtained with caspase-6-rev/wild-type caspase-3 (Fig. 7B and data not shown). Moreover, there was no evidence of caspase-3-rev-induced processing of procaspase-6-rev or caspase-6-rev-mediated cleavage of pro-caspase-3 in crude cell extracts (data not shown). Thus, caspase-6 processing appears not to be induced by the sequential activation of caspase-3 by either caspase-8 or -10. Likewise, active caspase-6 does not contribute to the processing of the caspase-3 zymogen. These data suggest caspase-6 lies in a distinct proteolytic cascade or that additional proteolytic activities, other than that of mature caspase-8, -10, or -3, are required to initiate caspase-6 zymogen maturation.

Different Caspases Produce Distinct Phenotypes in Yeast—Differences in yeast proteins targeted for cleavage by the mature forms of caspase-8β, -10, or -3 were suggested by differential effects on yeast cell viability (Figs. 1 and 2) and the levels of catalytic activity detected in vitro with various tetrapeptide substrates (Table I). Such differences were also manifest as distinct phenotypes accompanying caspase-8β-, -10/-3-, or -8β/-3-induced lethality (Fig. 8). In these experiments, yeast FY250 ρ- cells lacking mitochondrial DNA were used, as DAPI staining of mitochondrial DNA in ρ+ strains obscured the patterns of
cytoplasmic DNA staining attendant with caspase expression.

FY250 \( r^2 \) strains were prepared by standard methods (28). In all cases, the lack of mitochondrial function in the \( r^2 \) cells had no effect on the pattern or timing of caspase-induced cell death that was observed with the \( r^1 \) cells shown in Figs. 1 and 2. The expression of procaspase-3 or caspase-10 had little effect on FY250 \( r^2 \) cell morphology; the phase and DAPI-stained DNA images were indistinguishable from those of the vector control (Fig. 8, A—D, and data not shown). The cells were distributed throughout the cell cycle, and there was no detectable alteration in DNA staining. In contrast, co-expression of caspases-10/-3 resulted in a more diffuse pattern of DAPI-stained DNA and a pronounced decrease in cell refractility (Fig. 8, G and H). The expression of caspase-8 \( b \) alone produced a distinct terminal phenotype. Some of the cells were lysed, despite the inclusion of an osmotic stabilizer (1M sorbitol) in the fixation buffer. This was evidenced by the presence of non-refractile “ghosts” lacking DAPI-stained material (black arrows, Fig. 8, E and F). The intact cells were often swollen and, although clearly defined nuclear masses were observed, the accumulation of DAPI-stained material around the periph-

**Fig. 6.** Procaspase-6 is not activated or processed by caspase-8\( \beta \) or -10. Yeast cells co-transformed with the indicated YCP-GAL1-caspase constructs were induced with galactose at \( t = 0 \). A, at the times indicated, aliquots were serially diluted and plated onto selective media containing dextrose. The average number of viable cells forming colonies (relative to \( t = 0 \)) were determined (\( n = 3 \)). B, as described in the legend to Fig. 2, 100 \( \mu \)g of protein from crude extracts prepared 8 h following galactose induction were resolved by SDS-PAGE, and caspase-6, -8\( \beta \), and -10 bands were visualized by immunostaining with the T7 mAb followed by chemiluminescence. The identity of specific bands are diagrammed at left (for caspase-8\( \beta \) and -6) and at right (for caspase-10 and -6).

**Fig. 5.** Substitution of Ser for the active site Cys in procaspase-8\( \beta \) abolishes the activity and autocatalytic processing of the caspase zymogen. Extracts of yeast cells, induced to express wild-type procaspase-8\( \beta \) or procaspase-8\( \beta \)C345S for 0, 1, 3 and 6 h, were prepared as described under “Experimental Procedures.” A and B, as described in the legends to Figs. 3 and 4. 100 \( \mu \)g of protein from the crude cell extracts of cells expressing caspase-8\( \beta \) or caspase-8\( \beta \)C345S were resolved by SDS-PAGE, and the caspase-8\( \beta \)-specific bands (diagrammed on the right) were visualized by immunostaining with the T7 mAb and chemiluminescence. A contains caspase-8\( \beta \)C345S extracts following 0, 1, 3 and 6 h induction, and B contains the indicated cell extracts prepared 6 h after induction. C, caspase activity in the 6-h extracts was detected by the cleavage of the fluorometric tetrapeptide substrate Ac-DEVD-AMC as under “Experimental Procedures.” Lane C indicates buffer control; lane V refers to extracts of cells transformed with the vector control; lane I indicates samples co-incubated with the caspase inhibitor Ac-DEVD-CHO.
ery of the cell was also visible (white arrows). A similar phenotype was observed in cells expressing constitutively active caspase-6-rev (data not shown).

Co-expression of caspases-8β/-3 produced a third pattern of morphological changes. In Fig. 8, I and J, more extensive cell lysis accompanied caspase-8β/-3 co-expression. Yet, the cellular distribution of DAPI-stained DNA in the intact cells was different from that in cells expressing caspase-8β alone. Distinct nuclear masses were visible; however, the punctate pattern of cytoplasmic DAPI staining distinguished the caspases-8β/-3 cells from those expressing caspase-8β alone. The punctate staining correlated with the fragmentation of nuclear DNA, as shown for the genomic DNA resolved in the agarose gel pictured in Fig. 8B. Although these fragments were not enriched for nucleosome sized DNAs as evident in mammalian cells undergoing apoptosis, this level of DNA fragmentation required the activation of both caspase-8β and caspase-3. This in turn suggests that the yeast cellular targets of both caspases contribute to this terminal phenotype, which are distinct from those that produce the terminal phenotype associated with caspase-8β alone or the combination of caspases-10/-3.

**DISCUSSION**

**Pathways of Sequential Caspase Activation in Yeast**—Numerous stimuli initiate apoptotic responses in mammalian cells, including Fas ligand binding, DNA-damaging agents, and deprivation of requisite growth factors (4, 9, 17, 40). Although...
the signals may be transduced through different pathways, they all impinge on a network of caspases, whose activation is required to execute the cell death program. Considerable effort has gone into deciphering the events regulating the sequential proteolytic processing of caspase zymogens (6, 7, 9, 10, 15, 17, 23, 40, 41). In vitro studies of caspase activity and tetrapeptide inhibitors designed to inhibit specific caspases suggest a cascade of procaspase processing (6, 7, 14–16). However, such analyses are complicated by the identification of at least 11 caspase genes in humans coupled with a complex pattern of caspase gene expression in most mammalian cells. To circumvent the problems attendant with endogenous caspase activities, we examined the galactose-induced expression of caspases-3, -6, -8β, and -10 in the yeast S. cerevisiae. As the yeast genome does not encode caspase homologs, the autoactivation and/or sequential processing of specific caspases could be assessed in vivo in the absence of other contaminating activities.

Here we report that the efficient proteolytic processing of the caspase-8β or -10 zymogen was detected, suggesting that either is capable of catalyzing its own activation. This was confirmed with a mutant form of procaspase-8β, where substitution of serine for the active site cysteine abrogated the cytotoxicity and catalytic activity of the caspase and inhibited the efficient processing of the caspase-8β zymogen. Thus, in the case of procaspase-8β, yeast pro teaseas do not contribute to the autocatalytic maturation of zymogen. However, caspase-8β and -10 maturation was distinguished by their cytotoxic effects as only caspase-8β proved lethal in yeast.

Expression of caspase-3 or -6 alone did not induce autoactivation, yet when caspase-10 or -8β was co-expressed with caspase-3, processing of the caspase-3 zymogen was detected. Caspase-8β or -10 was proficient in initiating the maturation of caspase-3; however, neither affected the processing of the caspase-6 zymogen. These results clearly place caspase-8β and -10 upstream of caspase-3 in cascade(s) of caspase processing, consistent with recent in vitro studies establishing pro-caspase-3 as a physiological target of caspase 8 (42). Surprisingly, neither caspase-8β nor -10 promoted procaspase-6 maturation. Moreover, constitutively active forms of caspase-3 and -6 (32), although cytotoxic on their own, proved ineffective in catalyzing the processing of wild-type procaspase-6 and -3, respectively. Although in vitro studies suggest caspase-8 or -10 can catalyze the processing of all known procaspases (7, 14), this may be a consequence of the levels of active caspases assayed and/or the involvement of additional factors. In yeast, caspase-6 appears to require proteolytic activities in addition to, or separate from, those provided by mature caspase-8β, -10, and -3, which suggests caspase-6 may constitute a distinct caspase cascade.

Regulation of Caspase-8β-10 Activation—Caspase-8β transduces the apoptotic signal from the FAS ligand/tumor necrosis factor receptors to more downstream caspases (such as caspase-3 and -6) (7). Homophilic interactions between DEATH domains in FADD and homologous domains in the prodomain of the caspase-8β zymogen presumably recruit the procaspase to the ligated receptor, initiating the autocatalytic processing of caspase-8β, which, in turn, initiates the subsequent processing of other caspase zymogens (7, 19, 21). Recent studies indicate that the increased local concentrations of the partially active zymogen as a result of receptor oligomerization may be sufficient to initiate the autocatalytic processing of the caspase-8 precursor (43–45). A similar mechanism may apply to caspase-10. However, the fact that the cowpox serpin CrmA, which inhibits FAS-mediated apoptosis, is ineffective as a caspase-10 inhibitor argues for the involvement of caspase-10 in the activation of caspase cascades in response to other apoptotic stimuli (14).

The results presented here support a model of concentration-dependent autoprocessing of both caspase-8β and -10zymogens. When overexpressed in yeast, they appear capable of catalyzing their own activation. A similar mechanism of procaspase-2 activation in mammalian and yeast cells was also recently reported (46). Given the dire consequences of unregulated caspase-8 or -10 activation and the fact that, in the case of receptor-mediated events, homophilic protein-protein interactions mediate the assembly of death-inducing signal complex (7), it is tempting to suggest the presence of additional regulatory factors in higher eukaryotes that suppress procaspase-8 or -10 activation through direct interactions with the prodomain DEG sequences until the appropriate apoptotic signal is received. The recent description of the anti-apoptotic effects of FLIP (CASH/FLAME) through interactions with FADD support such a mechanism for the controlled activation of caspase-8 and presumably caspase-10 (47–50).

Different Substrate Specificities Produce Distinct Morphologies—Differences in the substrate specificities of caspase-8β, -10, and -3 were evident in different levels of caspase-8β, -10, and -3 activity detected with DEVD and IETD substrates and by the distinct morphologies of cells expressing various combinations of these cytotoxic enzymes. Moreover, caspase-8β or -10 activities were both detected in yeast, yet only caspase-8β was lethal. This differential in cell killing indicates a lack of critical cellular target(s) for caspase-10 and raises the likelihood that the mature form of caspase-8β contributes more to the mammalian apoptotic program than the simple processing of downstream caspases. This view is supported by recent studies of BID cleavage by caspase-8 (51, 52) and reinforced by the distinct patterns of morphological changes evident in yeast cells co-expressing caspases-8β/3 or caspases-10/3. If the sole function of caspases-10 and -8β was to initiate the processing of downstream caspases, such as caspase-3, the phenotypic consequences of either initiating event would be the same and result from caspase-3. Clearly, in yeast, this is not the case.

Although yeast lack several caspase targets conserved in mammalian cells, such as poly-ADP ribose polymerase, the distinct phenotypes obtained with lethal levels of specific combinations of caspases suggests that relevant cellular targets may be defined in yeast. Query of the Saccharomyces Genome Data base with consensus caspase cleavage sites yields a large number of matches, including the CDC48 gene, mutant strains of which exhibit several morphological features characteristic of mammalian apoptosis (53). Although one may speculate on the relative contribution of caspase-induced cleavage of such proteins, functional assays are required to deduce events essential for caspase-mediated cell death. Yeast genetic screens are currently underway to address these questions.

The targeting of caspase activities for therapeutic applications in the treatment of cancer, AIDS, and neurodegenerative diseases requires a more thorough understanding of the factors regulating the processing and activity of the network of caspases expressed in mammalian cells. With the regulated expression of specific caspase combinations in yeast, we have begun to establish the sequential proteolytic events required for the maturation of specific caspases. Further applications of this genetically tractable system in defining cellular factors, peptides, and drugs that specifically interfere with or enhance the processing of caspase zymogens or the catalytic activity of the mature caspases will also prove beneficial in the development of new therapeutics.

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