

[14] Monitoring Activity of Caspases and Their Regulators in Yeast *Saccharomyces cerevisiae*

By CHRISTINE J. HAWKINS, SUSAN L. WANG, and BRUCE A. HAY

Introduction

Caspases are a family of site-specific proteases that play important signaling and effector roles in most apoptotic death pathways.¹ Caspases are made as zymogens and become activated after cleavage. Caspase activity is regulated by both activators and inhibitors. Yeast provide a useful system in which to characterize the function of caspases and their regulators because yeast appear to lack endogenous caspases as well as other components of the apoptotic machinery. Also, yeast are genetically the most tractable eukaryote with which to work. Finally, transformation occurs at a high frequency, making them suitable for library screening or large-scale mutagenesis. In addition to caspases, yeast systems have also been used to analyze other cell death pathway components.²⁻¹¹ These approaches are discussed in [27] and [28].

Yeast, Media, and Plasmids

Saccharomyces cerevisiae W303 α (*MAT α can1-100 leu2-3,112 his3-11,-15 trp1-1 ura3-1 ade2-1*) is a convenient strain for most experiments.

Dropout medium: Autoclave yeast nitrogen base without amino acids (6.7 g/liter; Difco, Detroit, MI) (with agar at 20 g/liter for solid medium). After sterilization, add 0.1 vol of a 10 \times filter-sterilized

¹ N. A. Thornberry and Y. Lazebnik, *Science* **281**, 1312 (1998).

² T. Sato, M. Hanada, S. Bodrug, S. Irie, N. Iwama, L. H. Boise, C. B. Thompson, E. Golemis, L. Fong, H.-G. Wang, and J. C. Reed, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 9238 (1994).

³ M. Hanada, C. Aimé-Sempré, T. Sato, and J. C. Reed, *J. Biol. Chem.* **270**, 11962 (1995).

⁴ W. Greenhalf, C. Stephan, and B. Chaudhuri, *FEBS Lett.* **380**, 169 (1996).

⁵ B. Ink, M. Zornig, B. Baum, N. Hajibagheri, C. James, T. Chittenden, and G. Evan, *Mol. Cell. Biol.* **17**, 2468 (1997).

⁶ J. M. Jurgensmeier, S. Krajewski, R. Armstrong, G. M. Wilson, T. Oltersdorf, L. C. Fritz, J. C. Reed, and S. Otilie, *Mol. Biol. Cell* **8**, 325 (1997).

⁷ H. Zha and J. C. Reed, *J. Biol. Chem.* **272**, 31482 (1997).

⁸ W. Tao, C. Kurshner, and J. Morgan, *J. Biol. Chem.* **272**, 15547 (1997).

⁹ Q. Xu and J. C. Reed, *Mol. Cell* **1**, 337 (1998).

¹⁰ C. James, S. Gschmeissner, A. Fraser, and G. Evan, *Curr. Biol.* **7**, 246 (1997).

¹¹ W. Tao, D. W. Walke, and J. I. Morgan, *BBRC* **260**, 799 (1999).

stock of amino acid mix (L, W, H, U; Sigma, St. Louis, MO) with the appropriate additional amino acids or uracil [i.e., W, H, U (20-mg/liter final), L (30-mg/liter final)] and sugar source, either glucose or galactose, to 2% (w/v). For faster growth on inducing medium, raffinose can also be added (1%, w/v). For induction of copper promoters, add copper sulfate (usually 3 μ M).

Complete medium: Autoclave a solution of 1% (w/v) yeast extract (Difco) and 2% (w/v) Bacto-Peptone (Difco) (with agar at 20 g/liter for solid medium). Add either glucose (2%, w/v) or galactose (2%, w/v) after sterilization to give YPglc or YPgal. For induction of copper promoters, add copper sulfate (usually 3 μ M).

Low-copy yeast plasmids based on the pRS31X series¹² are convenient vectors for protein expression. Versions of these containing either the *GAL1/10*, *ADH1*, or *CUP1* promoters to drive gene expression are available.¹³

Small-Scale Yeast Transformation

Yeast can be easily and efficiently transformed by incubation in lithium acetate followed by heat shock. A convenient protocol, based on Ito *et al.*¹⁴ provides enough competent yeast for 10 small-scale transformations. The volumes of the starter culture and expanded culture can be increased for larger numbers of transformations. This method can be efficiently used for introducing up to three plasmids with different nutritional selections simultaneously. To generate transformants bearing four or more plasmids, two plasmids can be initially introduced using this protocol. A starter culture from one double transformant is grown in selective medium and then expanded into complete medium and transformed with the subsequent plasmids as described below.

1. Inoculate 5 ml of YPglc with a single colony of the parent yeast strain and grow to stationary phase: for 12–16 hr at 30° with shaking at 230 rpm in YPglc. *Note:* If starting with previously transformed yeast, inoculate a transformant colony into the appropriate dropout medium with glucose. These cultures require longer growth, about 18 hr.

2. Add the starter culture to 30 ml of YPglc, and grow for another 3 hr.

3. Centrifuge at 4000 rpm for 5 min, and discard the supernatant.

¹² R. S. Sikorski and P. Hieter, *Genetics* **122**, 19 (1989).

¹³ C. J. Hawkins, S. L. Wang, and B. A. Hay, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 2885 (1999).

¹⁴ H. Ito, Y. Fukada, K. Murata and A. J. Kimura, *Bacteriology* **153**, 163 (1983).

4. Resuspend in 35 ml of LiAc/TE [100 mM lithium acetate, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA].
5. Centrifuge as described above; discard the supernatant.
6. Resuspend the pellet in 1 ml of LiAc/TE.
7. For each transformation, mix 10 μ l of carrier DNA (boiled, chilled salmon sperm DNA, 10 mg/ml) with about 0.3 μ g of each plasmid to be transformed. Add 100 μ l of the yeast solution and 600 μ l of PEG/LiAc/TE [40% (w/v) polyethylene glycol 3350, LiAc/TE] per tube. Mix by inversion.
8. Incubate at 30° for 30 min.
9. Add 70 μ l of dimethyl sulfoxide (DMSO) per tube; mix by inversion.
10. Incubate at 42° for 6 min.
11. Pellet the yeast by centrifuging at 14,000 rpm for about 15 sec (start the microcentrifuge and stop it when it gets to full speed).
12. Discard the supernatant and resuspend the pellet in 70 μ l of TE [10 mM Tris-HCl (pH 7.5), 1 mM EDTA] to pellet; resuspend and plate on selective plates.
13. Incubate the plates at 30° for 2–3 days, until colonies are visible.

Monitoring Caspase Activity in Yeast Using a Transcription-Based Reporter

A number of techniques for visualizing the activity of site-specific proteases have been developed based on assays for the presence of a specific protease cleavage event.^{15–19} Two approaches have been developed to monitor caspase activity in yeast: one based on cleavage-dependent activation of a transcription-based reporter, and a second based on caspase-dependent yeast cell killing.^{13,20} The essential feature of a transcription-based reporter is that caspase cleavage leads to transcriptional activation of a reporter such as the bacterial *lacZ* gene.¹³ One version of such a reporter involves the use of a chimeric protein (known as CLB6) that consists of three parts: a type 1 transmembrane protein (human CD4) truncated to remove the cytoplasmic domain, then a linker containing various caspase target

¹⁵ J. O. McCall, S. Kadam, and L. Katz, *Biotechnology* **12**, 1012 (1994).

¹⁶ H. J. Sices and T. M. Kristie, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 2828 (1998).

¹⁷ I. Stagljär, C. Korostensky, N. Johnsson, and S. te Heesen, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 5187 (1998).

¹⁸ X. Xu, A. L. Gerard, B. C. Huang, D. C. Anderson, D. G. Payan, and Y. Luo, *Nucleic Acids Res.* **26**, 2034 (1998).

¹⁹ S. Mizukami, K. Kikuchi, T. Higuchi, Y. Urano, T. Mashima, T. Tsuruo, and T. Nagano, *FEBS Lett.* **453**, 356 (1999).

²⁰ J. J. Kang, M. D. Schaber, S. M. Srinivasula, E. S. Alnemri, G. Litwack, D. J. Hall, and M.-A. Bjornsti, *J. Biol. Chem.* **274**, 3189 (1999).

sites, followed by a transcription factor (LexA-B42). When this molecule is expressed in yeast that also carry a plasmid in which a LexA-dependent promoter drives *lacZ* expression, the transcription of *lacZ*, and thus β -galactosidase activity, depends on cleavage events that liberate the transcription factor from the membrane.¹³ This system has been developed and used for detection of caspase activity, but in theory should be applicable to any protease for which the cleavage/recognition site is known. Because the screens are function based, relying only on the ability of an expressed protein to cleave at the caspase target site linker, it enables cloning of proteases that recognize caspase cleavage sites but that are unrelated by sequence to caspases (such as the serine protease granzyme B).

Caspase-Dependent Toxicity

Enforced expression of some caspases does kill yeast.^{13,20} This discovery formed the basis of a second system to analyze the activity of caspases and their inhibitors, and is discussed below. However, to overcome this complication in a reporter-based assay, replica plating is used. Yeast are grown initially on noninducing plates to allow growth of yeast carrying caspase expression plasmids, and the colonies are then filter-lifted onto inducing plates. After detection of the reporter (β -galactosidase) activity on the filter, the corresponding original yeast colony from the noninducing plate can be located and grown for analysis. β -Galactosidase is a useful reporter for these assays because it is a stable protein and is enzymatically active in the presence of active caspases in dying cells.²¹

Nonspecific Cleavage

For the caspase reporter system to be effective the chimeric transmembrane protein that constitutes the caspase substrate must be translocated efficiently to the membrane and remain intact in the absence of introduced caspases. Background cleavage or failure to translocate can be measured simply by monitoring the level of β -galactosidase produced (as described below) in cells that contain only the transmembrane reporter and the LexA-responsive reporter plasmid. If background is a problem it can be dealt with to some extent by lowering the level of reporter expression through the use of variable-level inducible promoters, such as the copper-inducible *CUP1* promoter.

²¹ M. Miura, H. Zhu, R. Rotello, E. A. Hartwig, and J. Yuan, *Cell* **75**, 653 (1993).

CD4
cleavage sites
LexA

...CVRCRHRRDEV
DG-WEHDG-IEHDG-IETDG-DEHDG-DQMD
GTMKALTARQQ...

FIG. 1. The amino acid sequence of a target site linker region that incorporates consensus sites for all caspases for which specificity information is available, and the flanking CD4 and LexA sequences.

Target Site Linker

To maximize the probability of being able to detect an active caspase in yeast the reporter should contain caspase target sites that bracket the specificities of known caspases. Figure 1 shows the amino acid sequence of a target site linker region that incorporates consensus sites for all caspases for which specificity information is available²² and the flanking CD4 and LexA sequences. This linker contains the four known caspase consensus target sites, a granzyme B site, as well as the pseudosubstrate cleavage site for baculovirus p35, a broad-specificity caspase inhibitor. This combination of target sites should be cleaved by all known caspases, and is therefore likely to be recognized by other, novel caspases. Yeast proteins can undergo ubiquitin-mediated degradation, with the half-life of the protein determined largely by the amino-terminal residue.²³ To ensure that the cleaved LexA-B42 transcription factor does not undergo ubiquitin-dependent degradation, a glycine residue, which acts as a stabilizing residue in the N-end rule pathway of protein degradation in yeast, was introduced after each cleavage site.

Weeding out False Positives

Two major classes of false positives are library proteins that activate transcription by binding the *lexA* operator sequences directly, and proteases that cleave the reporter at noncaspase target sites. Either one of these events will induce *lacZ* expression. These false positives can be eliminated through the use of a modified version of the membrane reporter protein, a "false-positive reporter," in which the essential P₁ aspartate residues of the caspase target sites are replaced by glycines. Such a change should prevent caspase-mediated proteolysis of the substrate. Library plasmids isolated during a caspase screen can be transformed into yeast bearing the *lacZ* reporter and this false-positive reporter and the resulting transformants assayed for β -galactosidase activity. Plasmids that activate *lacZ*

²² N. A. Thornberry, T. A. Rano, E. P. Peterson, D. M. Rasper, T. Timkey, M. Garcia-Calvo, V. M. Houtzager, P. A. Nordstrom, S. Roy, J. P. Vaillancourt, K. T. Chapman, and D. W. Nicholson, *J. Biol. Chem.* **272**, 17907 (1997).

²³ A. Varshavsky, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 12142 (1996).

expression in this assay are unlikely to encode proteases with specificity for caspase target sites.

Library Screening for Caspase-Like Proteases

To screen for proteases that cleave caspase target sites, library plasmids in which insert expression is driven by the inducible *GAL* promoter are transformed into caspase reporter yeast that carry a plasmid with a *GAL*-driven version of the fusion protein reporter and the LexA-responsive transcriptional cassette, with the following modifications. This technique can also be used to assay reporter activation in single yeast clones streaked onto plates.

1. After the heat shock step, resuspend the yeast in 5 ml of TE and plate on selective plates with glucose at 200 μ l/15-cm plate. Also plate 100 μ l of 1:10 and 1:100 dilutions to determine the transformation frequency.

2. After about 36 hr, when small colonies are visible, lay nitrocellulose filters carefully on the plates, and punch asymmetric holes through the filter and the agar using needles, for subsequent orientation and colony identification.

3. Place the filters yeast side up on YPgal plates, and incubate at 30° for 18 hr.

4. Submerge each filter in liquid nitrogen until bubbling stops. Remove and thaw, yeast side up.

5. Place the filters yeast side up on filter paper that has been soaked in stain solution: 15 μ l of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) [20 mg/ml in dimethyl formamide] and 2.5 μ l of 2-mercaptoethanol (2-ME) per milliliter of Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, pH 7.0).

6. Incubate the filters in a dark humidified chamber at 37° for 4 to 20 hr.

7. Line up blue colonies on the stained filters with the original plates, pick the corresponding original colonies and streak out on fresh plates, and repeat the induction and staining process to clone positive transformants.

Liquid β -Galactosidase Assays

Although less sensitive than X-Gal assays, liquid β -galactosidase assays using *o*-nitrophenyl- β -D-galactopyranoside (ONPG) provide a more quantitative estimate of *lacZ* reporter gene activity.

1. Inoculate colonies to be assayed (in triplicate) into 2 ml of noninducing, selective medium and grow for 14–18 hr at 30°, with shaking.

2. Centrifuge the cultures at 14,000 rpm for 15 sec, and resuspend in TE.

3. Centrifuge again, resuspend at 1:10 dilution (in 2 ml) in selective inducing medium (i.e., with galactose and any other inducers such as CuSO_4).
4. Incubate at 30°C, with shaking, for 10 hr.
5. Centrifuge the yeast, resuspend in Z buffer, and recentrifuge.
6. Resuspend in 400 μl of Z buffer, and measure the OD_{600} .
7. Place 100 μl of each sample into a fresh tube and snap freeze in liquid nitrogen to disrupt the cells. Thaw by moving the tube to a 37° water bath for 1 min.
8. Add 700 μl of Z buffer with 2-ME (0.27%, v/v) and 160 μl of ONPG (4 mg/ml in Z buffer, made fresh before the start of the experiment); start the timer.
9. Incubate the tubes at 30° until yellow color develops.
10. When the solution turns yellow, add 400 μl of 1 M Na_2CO_3 and record the time.
11. When all reactions have been stopped, centrifuge all the tubes (10 min, 14,000 rpm) to pellet the contents.
12. Carefully transfer the top 1 ml from each tube to a clean tube and measure the OD_{420} .
13. Correct for the different number of yeast cells in the different samples by calculating β -galactosidase units^{24,25} as follows:

$$\beta\text{-Galactosidase units} = \frac{2500 \times \text{OD}_{420}}{t \times \text{OD}_{600}}$$

where t equals the time until the yellow color appears.

Variations on Transcription-Based Caspase Reporter System

Screening for Caspase Activators

The simplest version of the reporter system permits the detection of autoactivating caspases. Some caspases do not autoactivate at the levels expressed in yeast, and are thus unlikely to be detected unless an activating agent is also provided.^{13,20} Caspase 9 is an example of such a caspase. When expressed alone, caspase 9 does not activate the reporter. However, coexpression of an activated form of Apaf-1, which binds to and promotes caspase 9 activation, results in caspase 9-dependent reporter activation.¹³

²⁴ J. H. Miller, "Experiments in Molecular Genetics." Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1972.

²⁵ J. H. Miller, in "A Short Course in Bacterial Genetics," p. 4. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1972.

Thus the reporter system described above could be used to screen for activating proteins.

Cell death pathways often involve caspase cascades, in which upstream family members cleave and activate downstream caspases. Expression of downstream caspases in yeast does not lead to reporter activation or (as described below) cell killing.^{13,20} This failure to activate is probably because these caspases lack the ability to autoactivate in yeast.²⁰ It may be possible to screen for caspases that do not autoactivate in yeast by providing a known caspase that can promote activation. Clearly for such a system to be effective, the known “activator” caspase must not activate the reporter by itself. It may be possible to achieve a low background of reporter activation by the activator caspase by expressing it at low levels, or by altering cleavage sites in the reporter to remove any recognized by the activator caspase.

The unprocessed forms of many proteases contain sequences that impair their ability to autoactivate. Some caspases, such as caspase 7, are translated with a prodomain that impairs their ability to autoactivate.^{26,27} Granzyme B, an unrelated serine protease that shares target site similarities with some members of the caspase family,²² is also expressed as an inactive precursor with an amino dipeptide whose removal leads to enzymatic activity. It may be possible to screen for active forms of proteases that contain such inactivating prosequences by screening libraries that are unlikely to contain full-length coding sequences. These can be generated by using random primers rather than oligo(dT) during the initial cDNA synthesis.

Substrate Libraries

The transcription-based caspase reporter system described above can also be modified to screen for substrates of known caspases by replacing the target site linker in CLB6G6 with cDNA fragments from random-primed libraries. It should also be possible to carry out screens for preferred peptide substrates by introducing a randomized sequence into the target site linker region. False positives arising from cleavage by yeast proteases can be identified by virtue of their ability to activate the reporter in the absence of an introduced protease. Other false positives can be identified by virtue of their ability to cleave and activate a false-positive reporter that consists of CD4 and LexA-B42 sequences without an intervening target site linker.

²⁶ H. J. Duan, A. M. Chinnaiyan, P. L. Hudson, J. P. Wing, W. W. He, and V. M. Dixit, *J. Biol. Chem.* **271**, 1621 (1996).

²⁷ J. A. Lippke, Y. Gu, C. Sarnecki, P. R. Caron, and M. S.-S. Su, *J. Biol. Chem.* **271**, 1825 (1996).

Identifying Caspase Inhibitors

Known inhibitors of caspase activity should reduce reporter activity in the transcription-based reporter system described above. Baculovirus p35, a pancaspase inhibitor, and *Drosophila* and mammalian members of the IAP family of caspase inhibitors are in fact able to inhibit reporter expression, but this inhibition is incomplete and does not create a strong enough differential signal to be useful for screening purposes.¹³ Caspase inhibitors can be identified, however, by virtue of their ability to inhibit a caspase-dependent growth arrest or cell death phenotype seen when some caspases are expressed at high levels.

Expression of many caspases that autoactivate results in lethality in yeast. However, coexpression of a caspase inhibitor rescues yeast viability and growth, permitting colony formation.^{13,20,28} This fact creates a powerful screen for caspase inhibitors, because in the presence of an active caspase only those cells that also express a caspase inhibitor will produce colonies. False positives can arise in this sort of screen for several reasons: (1) the yeast are mutant in genes required to induce expression from the *GAL* promoter, which drives expression of the caspase; and (2) the caspase expression plasmid has undergone a mutation that renders the caspase nonfunctional or that blocks caspase expression. These false positives can be eliminated by isolating the library plasmid, transforming it into fresh caspase-bearing yeast, and assessing growth of transformants on inducing plates.

Library Screening for Caspase Inhibitors

A variation of the small-scale transformation protocol (above) can be used for library screening for caspase inhibitors.

1. Grow yeast containing the caspase expression plasmid in 10 ml of selective medium with glucose, then expand into 70 ml of YPglc for 3 hr.
2. Transform the yeast, by the small-scale procedure, performing 10 transformations with 5 μ g of each library plasmid.
3. After the heat shock and centrifugation, pool the yeast by resuspension in 250 ml of prewarmed YPglc.
4. Grow the transformed yeast for 4 hr at 30°, with shaking at 230 rpm.
5. To remove the glucose and amino acids, centrifuge the yeast and discard the supernatant. Resuspend the pellet in 250 ml of TE, recentrifuge, and resuspend in 10 ml of TE.
6. To determine the transformation frequency, plate 100 μ l of a 1:10 and 1:100 dilution on dropout plates with glucose to select for cells that carry the library plasmid.

²⁸ P. G. Ekert, J. Silke, and D. L. Vaux, *EMBO J.* **18**, 330 (1999).

7. Plate the remainder of the suspension (200 μ l/plate) on 15-cm drop-out plates with galactose (2%, w/v) and raffinose (1%, w/v).
8. Check the plates daily from the fourth day posttransformation for colony growth.
9. Streak colonies onto dropout medium plates with galactose/raffinose, then grow in 2 ml of liquid dropout selective medium with galactose/raffinose to obtain enough cells for plasmid DNA extraction and retesting.

Verification of Positive Clones

1. Streak colonies onto selective inducing plates. This step verifies that the colonies can survive and turn pink (indicating that they possess the *ade2* mutation that is present in the W303 α strain). This step also selects for the library plasmid that encodes the caspase inhibitor, hopefully enriching for it over other library plasmids that may have been cotransformed.
2. Inoculate 2 ml of liquid selective inducing medium with a colony and grow for 14–18 hr at 30° with shaking.
3. Centrifuge the yeast for 15 sec at 14,000 rpm and resuspend in complete medium (YPglc).
4. Grow the yeast for 3 hr. This step increases the growth rate of the yeast and reduces the strength of the cell wall.
5. Pellet the yeast and discard the supernatant.
6. Add 200 μ l of lysis buffer [2% (v/v) Triton X-100, 1% (w/v) sodium dodecyl sulfate (SDS), 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, pH 8.0], 200 μ l of phenol–chloroform–isoamyl alcohol (25:24:1, v/v/v) and 200 μ l of glass beads.
7. Ensure that the tube is fully closed (and wear gloves). Vortex at top speed for 2 min.
8. Centrifuge for 5 min at 14,000 rpm.
9. Transfer the top layer to a fresh tube, add 20 μ l of 3 M sodium acetate and 200 μ l of isopropanol, and mix.
10. Precipitate by centrifugation (14,000 rpm, 10 min).
11. Wash the pellet in cold 70% (v/v) ethanol, centrifuge for 1 min at 14,000 rpm, and remove traces of ethanol.
12. Resuspend the pellet in 200 μ l of TE.
13. Use 2 μ l to transform *Escherichia coli*.
14. Isolate library plasmid DNA from the transformed bacteria. This can be done either by identifying colonies that contain library plasmids, using colony lifts and a probe for the plasmid auxotrophic marker, or by transforming bacteria that are auxotrophic for the plasmid-encoded nutritional marker. Once plasmid DNA is isolated from *E. coli* it is retransformed into yeast together with the caspase. Transformants are plated onto

selective noninducing medium. Colonies are then streaked onto selective inducing medium plates to determine if the presence of a library plasmid suppresses caspase-dependent death.

Obviously not all library plasmids that suppress caspase-dependent death need encode caspase inhibitors. For example, such plasmids might encode proteins that disrupt expression of the caspase or that suppress caspase-dependent death downstream of caspase cleavage. Other approaches, including *in vitro* assays for caspase inhibitor function using purified proteins, will be necessary to confirm that the library plasmid encodes a caspase inhibitor.

Screens for Molecules that Disrupt Productive Caspase–Caspase Inhibitor Interactions

Members of the IAP (inhibitor of apoptosis) family of proteins are the only known cellular caspase inhibitors.²⁹ Work in *Drosophila* shows that IAP function is essential for cell survival, and that an important mechanism for promoting cell death involves the expression of death-activating proteins such as Reaper, HID, and Grim, which disrupt IAP–caspase interactions.³⁰ Given the general conservation of cell death signaling mechanisms throughout evolution, it seems likely that proteins that induce cell death or sensitize cells to other death-inducing signals by disrupting productive IAP–caspase interactions also exist in mammals.

As a first step, the expression of the caspase inhibitor (e.g., the IAP) is first titrated such that a minimal level required for caspase inhibition and rescue from lethality is achieved. The copper-inducible CUP1 promoter is ideal for these purposes because different levels of activation can be achieved by varying the levels of copper in the medium. Cells that survive because they express a caspase inhibitor as well as a death-inducing caspase can then be used as a background against which to identify inhibitors of IAP function.

To carry out a screen for IAP inhibitors a replica-plating strategy must be used. Cells carrying caspase and IAP expression plasmids are grown on noninducing medium and transformed with library plasmids as described above. Colonies are then replica plated onto inducing medium and scored for growth over the next 3–4 days. Those colonies that fail to grow on inducing medium encode candidate inhibitors of IAP function. Retesting of the library plasmid can be carried out as described below, and illustrated in Fig. 2.

²⁹ L. K. Miller, *Trends Cell Biol.* **9**, 323 (1999).

³⁰ S. L. Wang, C. J. Hawkins, S. J. Yoo, H.-A. J. Muller, and B. A. Hay, *Cell* **98**, 453 (1999).

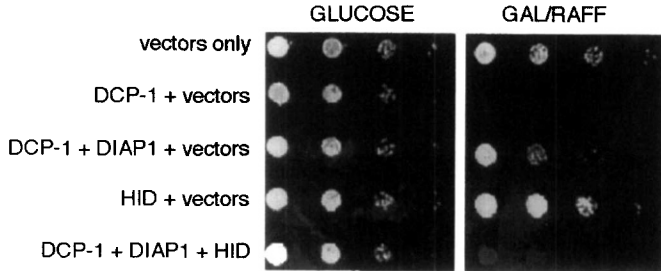


FIG. 2. *Drosophila* HID blocks the ability of DIAP1 to suppress caspase-dependent cell death. Library plasmids that are good candidates to encode specific inhibitors of IAP function have no growth phenotype when present in yeast in isolation (compare *HID* + vectors with empty vectors). DIAP1 protects cells from caspase-dependent cell death (compare *DCP-1* + vectors with *DCP-1* + *DIAP1* + vectors). But HID suppresses colony formation when co-expressed in cells with *DCP-1* and *DIAP1* (compare *DCP-1* + *DIAP1* + vectors with *DCP-1* + *DIAP1* + *HID*).

1. Cells are transformed with different combinations of three expression plasmids with different auxotrophic markers that are either empty vectors, or that express a caspase (*DCP-1*), an IAP (*DIAP1*), or a putative IAP inhibitor, *HID* (see Fig. 2).

2. Single colonies carrying different combinations of plasmids are inoculated into selective medium containing 2% (w/v) glucose and grown at 30° overnight or to an OD₆₀₀ of 1.0–2.0. It is likely that the densities of the cultures will be similar. If not, adjust with TE.

3. Spin down 300 μl of each culture (14,000 rpm, 1 min) and wash the cells free of glucose by resuspending the pellet in TE and spinning as described above. Resuspend the pellet in 200 μl of TE.

4. Serially dilute each sample 10-fold with TE four times. Dilutions may be performed directly in a 32-well frogger tray (Dan-Kar, Wilmington, MA; <http://www.dan-kar.com>). The volume of sample in each well is 100 μl.

5. Plate the dilutions onto selective inductive medium plates (i.e., selective plates containing 2% (w/v) galactose, if using a GAL-inducible promoter) and noninducing selective growth medium plates [2% (w/v) glucose] using a 32-spoke frogger (Dan-Kar). The volume of each spot is about 5–10 μl. Cell growth on the noninducing glucose medium provides an indication of cell number, while growth on inducing medium provides a reporter for cell survival.

6. Allow the spots to dry and incubate the plates at 30° until colonies are visible. Colonies will grow more slowly on galactose-containing medium.

An example of such an assay for an inhibitor of IAP function is shown in Fig. 2, using the *Drosophila* IAP inhibitor *HID* as an example.³⁰

Acknowledgments

C.J.H. was supported by a fellowship from the Human Frontiers Science Program. B.A.H. is a Searle Scholar. This work was supported by grants to B.A.H. from the National Institutes of Health (GM057422-01), the Ellison Medical Foundation and the Burroughs Wellcome Fund (New Investigator Award in the Pharmacological Sciences).