NEW, SENSITIVE BIOLUMINESCENT CASPASE-GLO® 2 AND 6 ASSAYS: USING INHIBITORS TO ACHIEVE SPECIFICITY IN CELLS

MARTHA O'BRIEN[1], CASSANDRA BROUETTE[1], ANDREW NILES[1], MICHAEL SCURRIA[2], LAURENT BERNAD[2], AND WILLIAM DAILY[2],
[1]PROMEGA CORPORATION, [2]PROMEGA BIOSCIENCES, INC.

Introduction

Caspases are cysteine proteinases that cleave their substrates following an Asp residue. They are important regulators of apoptosis and inflammation. The apoptotic caspases can be divided into two types, initiator and effector caspases, a distinction that is both structural and functional. Initiator caspases have long prodomains containing specific protein:protein interaction domains that are critical for recruitment of procaspase zymogens into complexes that lead to autocatalytic activation. The effector caspases do not have a long prodomain and lack the ability to autoactivate. They are typically dimers as zymogens, and cleavage by activated initiator caspases is required for activation (for review see reference 1). Most cellular substrates are cleaved by these downstream "short prodomain" caspases; hence, the designation as effector or executioner caspases. The initiator caspases are typically monomers as zymogens, and cleavage is not obligatory for activation; dimerization is thought to be the critical step for activation (2). In mammals, caspases-8, -9, -10, and -2 are thought to be initiator caspases, and caspases-3, -7, and -6 are effector caspases. We previously developed a bioluminescent assay for the prominent effector caspases (Caspase-Glo® 3/7 Assay[a–c]; 3,4) and for the well defined initiator caspases (Caspase-Glo® 8 and 9 Assays[a–c]; 5). Now we have developed new luminescent assays for the initiator caspase-2 and effector caspase-6, caspases whose physiological roles in apoptosis are less well understood.

Caspase-2 has the structural features of an initiator caspase. Like caspase-9 it has a caspase recruiting domain (CARD) that facilitates dimerization of procaspase-2 molecules and interaction with the adaptor RAIDD protein (1). Caspase-2 has been described as an essential initiator caspase in numerous apoptotic systems (6–10), but its mechanism of activation and downstream targets are poorly understood (1, 11). Caspase-6 is a short prodomain caspase and is thought to function as an executioner caspase, but like caspase-2, its physiological function has not been clearly defined (1,12). Recently, studies have indicated that changes in caspase-6 activity are associated with neurodegenerative diseases such as Huntington disease and Alzheimer disease (13–16). Our new, extremely sensitive, homogeneous luminescent caspase-2 and -6 assays provide unique tools for defining the function of these elusive caspases. An understanding of cross-reactivity between caspases, their substrates, and the various peptide inhibitors is critical for interpreting results from cell studies. Here we provide details on using the Caspase-Glo® 2 Assay[a–c] in cells.

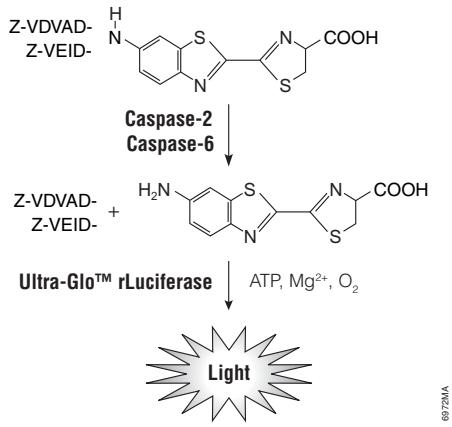

Figure 1. Caspase-2 or -6 cleavage of the luminogenic substrates containing either the VDVAD or VEID sequence, respectively. Following caspase cleavage, a substrate for luciferase (aminoluciferin) is released, resulting in the production of light from the luciferase reaction. Abbreviations: Z = carbobenzoxyl blocking group.

Caspase-Glo® Assay Principle

The Caspase-Glo® 2 Assay is based on the proluminescent substrate Z-VDVAD-aminoluciferin used by caspase-2, and the Caspase-Glo® 6 Assay[a–c] is based on the Z-VEID-aminoluciferin substrate used by caspase-6. These N-terminally blocked, proluminescent caspase-2 or -6 substrates are combined with a thermostable luciferase in a buffer system optimized for caspase and luciferase activity. Adding a single Caspase-Glo® Reagent in an "add-mix-measure" format results in caspase cleavage of the substrate, liberation of free aminoluciferin that is consumed by luciferase, and generation of a "glow-type" luminescent signal (Figure 1). The luminescent signal produced is proportional to the amount of caspase activity present. The caspase and luciferase enzyme activities rapidly reach a steady state so that the luminescent signal peaks in 20–30 minutes and then is maintained for several hours with

minimal loss. Both assays have a half-life greater than 6 hours (17,18). These homogeneous Caspase-Glo® Assays are designed for use with multiwell-plate formats, making them ideal for automated high-throughput screening (HTS) of caspase inhibitors.

Improved Sensitivity and Stable Signal

When compared to fluorescent assays using comparable substrates, the Caspase-Glo® 2 and 6 Assays were significantly more sensitive at all time points. Signal-to-noise ratios (mean signal – mean background / standard deviation of the background) were used to compare the two formats. The luminescent assays have very low background resulting in a broad dynamic range (Figure 2). After an hour, the signal-to-noise ratio is close to 1000-fold higher for the Caspase-Glo® Assays, and the limit of detection is close to 1000-fold lower than the fluorescent assay. Although the sensitivity of the fluorescent assay improves over time due to the accumulation of released free AFC, even after 18 hours, the signal-to-noise ratios still do not approach those of the luminescent assays (Figure 2). The sensitivity of the homogeneous, luminescent assay remains relatively constant over extended periods due to the coupled-enzyme format and the long signal half-life (17,18).

Considerations for Monitoring Caspase-2 and -6 Activities in Cells: Cross-Reactivity Between Caspases and the Proteasome

The buffers for the Caspase-Glo® 2 and 6 Assays contain a lysis reagent that enables use of the assays to measure caspase activities in cultured cells. The sensitivity of the luminescent assays makes them ideal for monitoring caspase activity directly in cells. However, it is critical when using peptide-conjugated caspase substrates to have an understanding of the cross-reacting activities, particularly between caspases. Selectivity for caspases is gained by the presence of Asp (D) in the P1 position of the substrate. The stringent caspase requirement for cleaving protein substrates after an Asp is rare among proteases; granzyme B is the only other known human or mouse protease with a primary specificity for Asp (19). However, selectivity between caspases is more difficult to achieve. Changes in P2–P4 have been used to define consensus sequences for the different caspases, but there is considerable overlap between individual consensus sequences, and caspases are promiscuous on these sequences (19). The various tetrapeptide-conjugated caspase substrates are selective for individual caspases but not uniquely specific. For example, the substrate sequence VDVAD is optimized for caspase-2 because of the unusual preference of caspase-2 for a pentapeptide over a tetrapeptide, but the pentapeptide substrate also contains the consensus sequence DXXD for caspases-3 and -7 and is readily cleaved by these caspases as well (20, Figure 3).

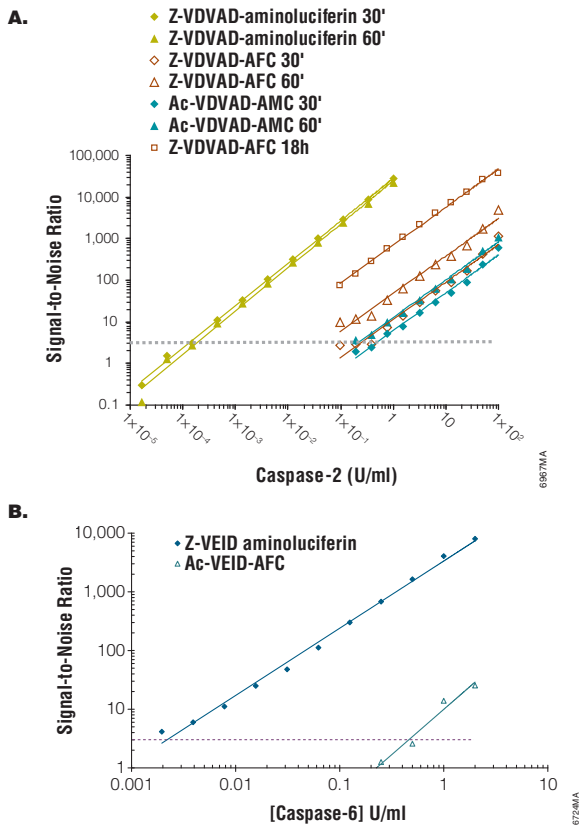

Figure 2. Sensitivity of the Caspase-Glo® 2 and 6 Assays compared to fluorescent assays. Human recombinant caspase-2 and -6 (BIOMOL) were titrated in 10 mM HEPES (pH 7.0), 2 mM DTT and assayed in 96-well plates. **Panel A.** The Caspase-Glo® 2 Assay (Z-VDVAD-Glo™ Substrate) or comparable fluorogenic substrates, Z-VDVAD-AFC and Ac-VDVAD-AMC, were tested with caspase-2. Luminescence and fluorescence were measured at 30 and 60 minutes on a GloMax® 96 Microplate Luminometer or a Labsystems Fluoroskan Ascent plate reader, respectively. AFC fluorescence was also measured at 18 hours. **Panel B.** The Caspase-Glo® 6 Assay (Z-VEID-Glo™ Substrate) or an Ac-VEID-AFC fluorescent substrate were tested with caspase-6. Luminescence and fluorescence were monitored after 60 minutes on a BMG FLUOstar multimode reader. For both graphs, the results are plotted as signal-to-noise ratios. The limit of detection is defined as the amount of caspase giving a signal-to-noise ratio >3 (dashed lines). The signal-to-noise ratio is greater, and the limit of detection is significantly lower for the luminescence assays compared to the fluorescence assays.

Another example of cross-reacting caspase activity is shown for the caspase-6 substrate. The VEID sequence is selective for caspase-6 but can also cross-react to some degree with caspases-3 and -8 (Figure 4). The cross-reactivity is limited with this sequence due to the optimized Val (V) in the P4 position that creates a unique consensus tetrapeptide for caspase-6 (19). Determining if caspase-6 is the effector caspase in a given cell apoptosis paradigm requires

Monitoring Caspases 2 and 6

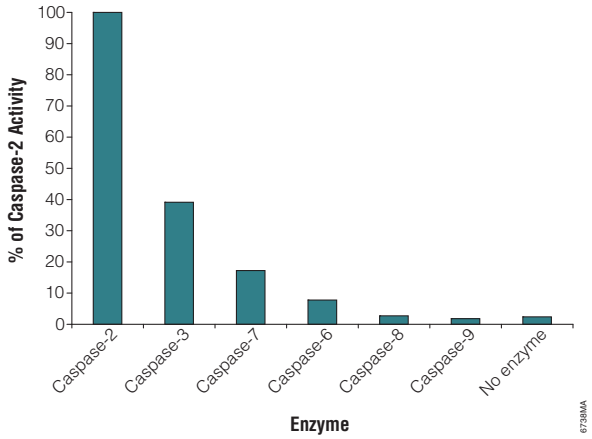

Figure 3. Cross-reactivity of the Caspase-Glo® 2 Assay with other caspases. The caspase enzymes (BIOMOL) were tested at 0.5 U/ml (for caspases-2, -6, -8, and -9) or 0.625 U/ml (for caspases-3 and -7). The caspase enzymes were diluted in 10 mM HEPES (pH 7.2), 2 mM DTT, and 0.1% Prionex® as a carrier protein. Luminescence was recorded as relative light units (RLU) on a GloMax® 96 Microplate Luminometer 30 minutes after adding the Caspase-Glo® 2 Reagent. Results are presented as a percentage of the RLU obtained from the caspase-2 activity. Caspase-3, and to a lesser degree, caspase-7 exhibit significant cross-reactivity with the Z-VDVAD-aminoluciferin substrate. Note that units were kept essentially constant, but this represents 50–100 times more caspase-2 and -9 (by protein quantity) than the other caspases.

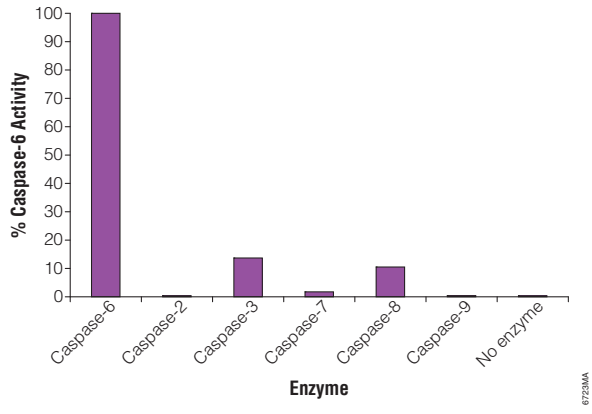

Figure 4. Cross-reactivity of the Caspase-Glo® 6 Assay with other caspases. The Caspase-Glo® 6 Assay was performed comparing human recombinant caspases-2, -3, -7, -8 and -9 (BIOMOL) to caspase-6 (BIOMOL). The purified enzymes were serially diluted in 10 mM HEPES buffer [pH 7.2], 2 mM DTT and 0.1% Prionex® as a carrier protein. Luminescence was recorded as relative light units (RLU) on a GloMax® 96 Microplate Luminometer 30 minutes after adding the Caspase-Glo® 6 Reagent. Caspases were normalized for units of activity (18). Caspases-2 and -9 showed no cross-reactivity even though 100X the amount of protein was added to the reaction.

monitoring for caspase-3 and -8 activity as well as caspase-6 activity. The Ac-VEID-CHO inhibitor has been reported to be 100-fold more selective for caspase-6 over other caspases (21), but others have reported significant inhibition of caspase-8 with Ac-VEID-CHO (22). A combination of assays and inhibitor tests are critical for interpreting caspase activity results from cells.

The proteasome contains a caspase-like activity, and cross-reactivity on the fluorescent Ac-VEID-AMC substrate has been reported (23). We have also found that the proteasome can cleave some of the luminescent caspase substrates in cell-based assays (24). Including a proteasome inhibitor (60µM) in the reagent significantly lowers the nonspecific background contributed from the proteasome (Figure 5). The MG-132 inhibitor has no effect on the caspases at this concentration.

Using Inhibitors to Achieve Specificity for Caspase-2 in Cells

We asked the question whether inhibitors could be added to the Caspase-Glo® 2 Reagent to select for caspase-2 activity. Caspase-2 is particularly challenging because of the similar substrate specificity to that of caspases-3 and -7. The Z-VDVAD caspase-2 substrate cross-reacts substantially with caspases-3 and -7 (Figure 3); likewise, the caspase-2 inhibitor, Z-VDVAD-CHO, also cross-reacts with caspases-3 and -7. The K_i for this inhibitor is nearly identical for caspases-2, -3, and -7 (20; Figure 6, Panel A).

Interestingly, the caspase-3/7 inhibitor, Ac-DEVD-CHO, is significantly more selective for caspases-3 and -7 than for caspase-2 (20,25; Figure 6, Panel B). We exploited the 3 orders of magnitude difference in the K_i of this inhibitor for caspases-3 and -7 versus caspase-2 to select for caspase-2 activity in cells. There are very few confirmed cell models for caspase-2 activation, and to our knowledge there are no caspase-2-specific inhibitors. To determine whether the caspase-3/7 inhibitor, Ac-DEVD-CHO, could be used in combination with the

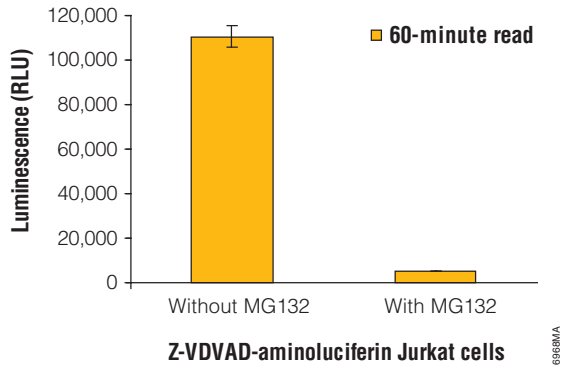

Figure 5. Inhibition of proteasome cross-reactivity in the Caspase-Glo® 2 Assay. Jurkat cells (10,000 cells/well) were plated in 96-well plates. They were tested with the Caspase-Glo® 2 Reagent only or with Caspase-Glo® 2 Reagent in the presence of the proteasome inhibitor MG-132 (60 µM). Luminescence was recorded as relative light units (RLU) on a GloMax® 96 Microplate Luminometer 60 minutes after adding the reagent.

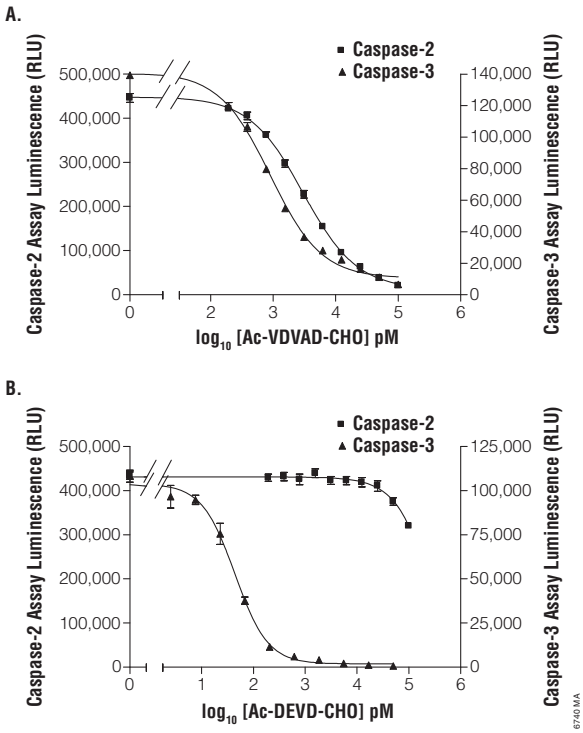

Figure 6. Determination of IC_{50} values for caspase-2 inhibitors. The inhibitor concentrations that result in 50% inhibition (IC_{50}) of caspase-2 activity were determined for the caspase inhibitors, Ac-VDVAD-CHO and Ac-DEVD-CHO, using the Caspase-Glo® 2 Assay. **Panel A.** The competitive inhibitor, Ac-VDVAD-CHO, was resuspended in DMSO, serially diluted, and combined with caspase-2 (0.5 U/ml) or caspase-3 (5 U/ml) in HEPES (pH 7.0), 2 mM DTT and 0.1% Prionex® in 96-well plates. **Panel B.** The competitive inhibitor, Ac-DEVD-CHO, was prepared and incubated with caspase-2 or -3 as described above. The maximum DMSO concentration is <0.01% for both inhibitors. The Z-VDVAD-Glo™ Substrate was used at 20 µM. Luminescence was recorded 40–60 minutes after reagent addition, and GraphPad Prism® software was used to calculate the IC_{50}. The IC_{50} for Ac-VDVAD-CHO with caspase-2 was 3.0 nM and for caspase-3 was 0.9 nM, comparable to the published K_i of 3.5 nM for caspase-2 and 1.0 nM for caspase-3 using fluorescent substrates (20). The Ac-VDVAD-CHO is equally effective on the two caspases, confirming that this inhibitor cannot be used to assign specificity (Panel A). The IC_{50} for Ac-DEVD-CHO for caspase-2 was >500 nM and for caspase-3 was 0.04 nM. The relative potency of the inhibitor for caspase-2 and for caspase-3 is the same as that published using fluorescent substrates (20,25). The Ac-DEVD-CHO inhibitor is highly selective for caspase-3 over caspase-2 and can be used to select for caspase-2 activity (Panel B).

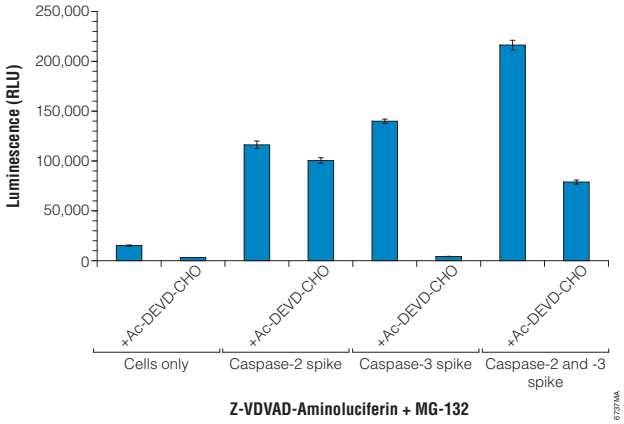

Figure 7. Inhibition of caspases-2 and -3 after addition into cultured cells. Caspase-2 (0.1 U/ml, BIOMOL) or caspase-3 (2 U/ml, BIOMOL) or both were added to 10,000 Jurkat cells/well cultured in RPMI-1640 with 10% fetal bovine serum in 100 µl volume. The proteasome inhibitor MG-132 (60 µM) was added to the Caspase-Glo® 2 Reagent. The reagent with or without added Ac-DEVD-CHO inhibitor (60 nM) was added to the cultured cells along with the added caspases. Luminescence was read on a GloMax® 96 Microplate Luminometer after 90 minutes to allow the inhibitors to equilibrate. The cultured cells alone contained minimal Z-VDVADase activity that was inhibited by Ac-DEVD-CHO. The caspase-3 activity added into the cell culture was completely inhibited by the 60 nM Ac-DEVD-CHO, whereas the caspase-2 activity was not affected. The difference in the signal from cells with added caspase-2 with or without inhibitor can be attributed to endogenous Z-VDVADase activity within the cells.

Caspase-Glo® 2 Assay to counter-select for caspase-2 activity in cell culture, we performed a caspase addition (spiking) experiment. Caspase-2, caspase-3, or a combination of both were added to cultured Jurkat cells (10,000/well; Figure 7). The amounts of caspase added were adjusted to give comparable activity and to be comparable to levels of caspase-3 activity typically detected in apoptotic cells. The proteasome inhibitor, MG-132, was added to the Caspase-Glo® 2 Reagent, ensuring that there was low background VDVADase activity in all the cultured cells. We tested this reagent with and without the selective Ac-DEVD-CHO inhibitor. Using the Ac-DEVD-CHO inhibitor at a relatively low concentration completely inhibited the caspase-3 activity added to the cells (as well as the inherent low-level caspase-3 activity in cultured cells) but did not affect the activity in cells with added caspase-2 activity (Figure 7). The slight reduction in the caspase-2 signal with the Ac-DEVD-CHO inhibitor can be attributed to the reduction in the inherent caspase-3 activity in cells (compare to cells-only results). When caspase-2 and caspase-3 were spiked into cells together, the activities were not precisely additive. However, it is clear that when the two enzymes are combined, the Ac-DEVD-CHO inhibits all the caspase-3 activity, leaving the caspase-2 activity essentially intact. These results demonstrated that it is possible to use inhibitors to select for caspase-2 activity in a cell system. The stable signal of this homogeneous coupled-enzyme system makes it very easy to add inhibitors to the reagent; a new steady state is achieved when inhibition has reached equilibrium.

Testing for Caspase-2 Activity in Heat Shock-Induced Apoptotic Cells

If caspase-2 functions as an initiator caspase and is temporally segregated from the activation of effector caspases, then cross-reactivity would not be such a concern; however, if there is temporal overlap between activation of caspase-2 and caspases-3 and/or -7, then the effector caspases need to be inhibited in order to detect caspase-2.

We applied this counter-selection method of monitoring for caspase-2 activity to a heat-shock-induced apoptosis cell model where there is conflicting information regarding whether caspase-2 is the initiator caspase. In a previous study, one group used the cell-permeant biotin-VAD-FMK to bind and trap apical caspases and reported that caspase-2 is the initiator caspase in heat shock-induced apoptotic Jurkat cells (26). They also found that caspase-2-deficient mouse embryonic fibroblasts (MEFs) were somewhat resistant to heat shock-induced apoptosis. In contrast, another group failed to identify caspase-2 as the initiator caspase in the same Jurkat cell heat-shock paradigm and also found that caspase-2-deficient MEFs were equally sensitive to heat shock-induced apoptosis as wildtype MEFs (27).

We heat-shocked Jurkat cells for 1 hour at 43 ºC and then monitored for caspase activity at various times using the Caspase-Glo® 2 Assay with MG-132 added to inhibit proteasome nonspecific activity (Figure 8). Within 30 minutes after the 1-hour treatment, we detected significant VDVADase activity. This activity increased for at least 4 hours after the heat shock treatment. When Ac-DEVD-CHO at a final concentration of 60 nM was added to the Caspase-Glo® 2 Reagent, the activity was eliminated at all time points, indicating that the VDVADase activity could be attributed to caspase-3 and/or -7. We confirmed this early detection of caspase-3 or -7 using the Caspase-Glo® 3/7 Assay. We checked for both VDVADase and DEVDase activity 5, 10, 15, and 30 minutes after a 30-minute heat shock and were able to detect DEVDase activity within 30 minutes after treatment (data not shown). We did not detect active caspase-2 at any of the time points, supporting the findings of Milleron and Bratton (27) that caspase-2 is not the initiator caspase in heat shock-induced apoptosis. It is possible that there is not enough active caspase-2 to be detected, given how quickly the effector caspases are activated. It should be noted that if the amount of caspase-2 activity in apoptotic cells is dramatically less than that for caspases-3 and -7 and is temporally coincident with activation of these effector caspases, then a peptide-based activity assay would not be suitable for detecting caspase-2 activity because of the masking activity of the effector caspase. The bioluminescent Caspase-Glo® 2 Assay, in combination with the Ac-DEVD-CHO and MG-132 inhibitors, provides a sensitive means of assaying for caspase-2 activity in cells. This should be particularly useful if the caspase-2 initiator activity is temporally distinct from the cross-reacting effector caspases.

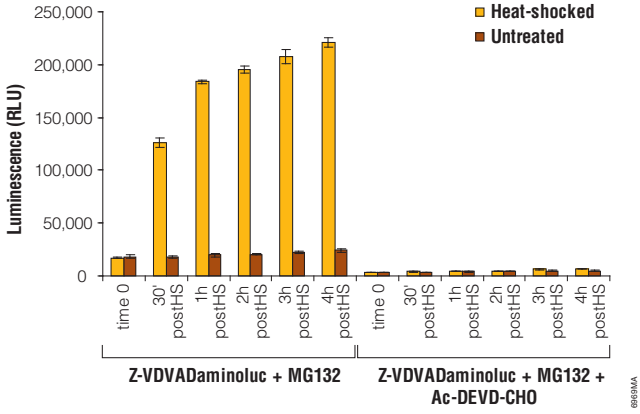

Figure 8. Defining caspase activity in heat shock-induced apoptosis. The Caspase-Glo® 2 Assay was used to monitor caspase activity after a heat shock treatment of cultured cells. Jurkat cells (1 x 10^6 cells/ml) were centrifuged, resuspended in prewarmed culture medium (RPMI-1640 + 10% fetal bovine serum), and incubated for 60 minutes at 43 ºC. Control cells were incubated at 37 ºC. After heat shock or control treatment, cells were diluted in culture medium and added into 96-well plates at 10,000 cells/well and tested with the Caspase-Glo® 2 Reagent with or without Ac-DEVD-CHO added (60 nM final). Both reagents contained MG-132 to inhibit background proteasome activity. The results indicate that the significant caspase response can be attributed to caspases-3 and/or -7 but not caspase-2. The effector caspases are detected as early as 30 minutes after heat shock treatment.

Summary

We have developed ultra sensitive bioluminescent assays for caspases-2 and -6. Similar to our other Caspase-Glo® Assays, the Caspase-Glo® 2 and 6 Assays are significantly more sensitive than assays with comparable fluorescent substrates, give stable signals, and reach maximum sensitivity quickly. The sensitivity and flexibility in read time make them ideal for high-throughput screening of inhibitors using purified caspase enzymes. The physiological roles of caspases-2 and -6 are not as well defined as some other caspases. Improved sensitivity can be critical for helping to better define their functions in cells. As an initiator caspase, active caspase-2 may be present in very low quantities in cells. Our new sensitive bioluminescent assays provide better tools for detecting low levels of active enzyme. We have provided information on cross-reacting caspases and cross-reacting proteasome activity that allows researchers to determine if the assays are suitable for their particular cell system. For the Caspase-Glo® 2 Assay, we have developed a simple method that uses inhibitors to select against nonspecific activities to enable monitoring of caspase-2 activity in cells.

References

1. Kumar, S. (2007) *Cell Death Differ.* **14**, 32–43.
2. Bao, Q. and Shi, Y. (2007) *Cell Death Differ.* **14**, 56–65.
3. O'Brien, M. *et al.* (2003) *Cell Notes* **6**, 13–5.
4. O'Brien, M.A. *et al.* (2005) *J. Biomol. Screen.* **10**, 137–48.
5. Niles, A. *et al.* (2004) *Cell Notes* **8**, 9–12.
6. Lassus, P. *et al.* (2002) *Science* **297**, 1352–54.
7. Lin, C-F. *et al.* (2004) *J. Biol. Chem.* **279**, 40755–61.
8. Wagner, K.W. *et al.* (2004) *J. Biol. Chem.* **279**, 35047–52.
9. Robertson, J.D. *et al.* (2002) *J. Biol. Chem.* **277**, 29803–9.
10. Tinel, A. and Tschopp, J. (2004) *Science* **304**, 843–6.
11. Zhivotovsky, B. and Orrenius, S. (2005) *Biochem. Biophys. Res. Commun.* **331**, 859–67.
12. Slee, E.A. *et al.* (2001) *J. Bio. Chem.* **276**, 7320–6.
13. LeBlanc, A. *et al.* (1999) *J. Biol. Chem.* **274**, 23426–36.
14. Horowitz, P.M. *et al.* (2004) *J. Neurosci.* **24**, 7895–902.
15. Guo, H. *et al.* (2004) *Am. J. Pathol.* **165**, 523–31.
16. Graham, R.K. *et al.* (2006) *Cell* **125**, 1179–91.
17. *Caspase-Glo® 2 Assay Technical Bulletin* #TB365, Promega Corporation.
18. *Caspase-Glo® 6 Assay Technical Bulletin* #TB366, Promega Corporation.
19. Timmer, J.C. and Salvesen, G.S. (2007) *Cell Death Differ.* **14**, 66–72.
20. Talanian, R.V. *et al.* (1997) *J. Biol. Chem.* **272**, 9677–82.
21. Hirata, H. *et al.* (1998) *J. Exp. Med.* **187**, 587–600.
22. Foley, J.D. *et al.* (2004) *J. Biol. Chem.* **279**, 32142–50.
23. Zandy, A.J. and Bassnett, S. (2007) *Inv. Opthal. Vis. Science* **48**, 293–302.
24. Moravec, R. *et al.* (2007) *Cell Notes* **18**, 12–4.
25. Garcia-Calvo, M. *et al.* (1998) *J. Biol. Chem.* **273**, 32608–13.
26. Tu, S. *et al.* (2006) *Nat. Cell Biol.* **8**, 72–7.
27. Milleron, R.S. and Bratton, S.B. (2006) *J. Biol. Chem.* **281**, 16991–7000.

Protocols

Caspase-Glo® 2 Assay Technical Bulletin #TB365
(www.promega.com/tbs/tb365/tb365.html)

Caspase-Glo® 6 Assay Technical Bulletin #TB366
(www.promega.com/tbs/tb366/tb366.html)

Ordering Information

Product	Size	Cat.#
Caspase-Glo® 2 Assay	10 ml	G0940
	50 ml	G0941
Caspase-Glo® 6 Assay	10 ml	G0970
	50 ml	G0971

For Laboratory Use.

[a]U.S. Pat. No. 7,148,030 and other patents pending.

[b]U.S. Pat. Nos. 6,602,677 and 7,241,584, Australian Pat. No. 754312 and other patents and patents pending.

[c]The method of recombinant expression of Coleoptera luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.