Measurement of Caspase Activity in Cells Undergoing Apoptosis

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Summary

Cysteine proteases of the caspase family play key roles in the execution of apoptosis and in the maturation of proinflammatory cytokines. During apoptosis signaling, the latent forms of caspase precursors undergo rapid proteolytic processing and activation. Thus, the measurement of caspase activation provides a quick and convenient mean to assess apoptosis. This chapter outlines the various commonly used assays for determining caspase activity in cultured cells or tissue extracts.

Key Words: Caspase activation; synthetic peptides; immunoblotting; electrophoresis; spectrophotometer.

1. Introduction

Caspases (from cysteine aspases) are a group of cysteine proteases that cleave their substrates after an aspartate residue (1–5). These proteases are the mammalian homologues of the Caenorhabditis elegans death protease CED-3. There are two major functions assigned to caspases. Some caspases, such as caspase-1, -4, -5, and -11, are primarily involved in the processing and activation of proinflammatory cytokines, whereas others, including caspase-2, -3, -6, -7, -8, -9, and -10, have been implicated in the effector phase of
apoptosis (1–6). Caspases are normally present as inactive precursors in cells. Upon receiving an apoptotic signal, the proforms (zymogens) of caspases undergo proteolytic processing to generate active enzyme (1–5). The structural studies on active caspase-1 and caspase-3 predict that the mature enzymes have a heterotetrameric configuration, composed of two heterodimers derived from two precursor molecules (7–10). In addition to the regions that give rise to two subunits, procaspases contain amino terminal prodomains of varying lengths. Based on the length of prodomain, caspases can be divided into two groups: class I, which contain a relatively long prodomain, and class II, which contain a short prodomain (11,12).

From recent studies it has become apparent that the long prodomains in many class I caspases consist of specific protein–protein interaction motifs, such as the caspase recruitment domain and the death effector domain, which play a crucial role in caspase activation. These motifs in the prodomains of class I caspases seem to serve two functions; mediate oligomerization of the procaspase molecules and/or help the recruitment of caspase precursors to specific death adaptor complexes. The clustering of class I procaspase molecules results in caspase activation by autocatalysis by mechanisms that are not entirely understood at present. Class II caspases, which lack a long prodomains, also lack the ability to self-activate and appear to require cleavage by activated class I caspases. For this reason, class I caspases are also referred to as initiator or upstream caspases and class II caspases as executioner or downstream caspases. The activation of class I caspases is of fundamental importance in cell death commitment and, hence, substantial efforts have been devoted to the understanding of mechanisms that underlie the activation of class I caspases (11,12).

Induction of apoptosis is almost always associated with the activation of caspases; therefore, measurement of caspase activity is a convenient way to assess whether the cells are undergoing apoptosis. There are several ways to measure caspase activation. Most common ones, described here, involve use of chromogenic or fluorogenic peptide substrates that release the chromogen or fluorescent tag upon cleavage by a caspase. Other qualitative methods include
monitoring the cleavage of in vitro synthesized $^{35}$S-labeled caspase substrates or measuring the cleavage of endogenous caspase substrates by immunoblotting using specific antibodies. In this chapter, all three techniques are described.

The most direct and quantitative method for measuring caspase activity is by using synthetic peptide substrates. There are 13 mammalian caspases described. Optimal substrate specificities for many of these have been determined using peptide combinatorial libraries expressed in *E. coli* (13,14). The minimum substrate required for a caspase is usually a tetrapeptide sequence with an aspartate residue in P$_1$ position and variable P$_2$ to P$_4$ residues based on cleavage specificity of individual caspases. In some cases, such as caspase-2 and the *Drosophila* caspase DRONC, a P$_5$ residue greatly enhances substrate cleavage (14–16). The most commonly used substrates that are commercially available are listed in Table 1. However, it must be noted that in crude cell extracts containing many caspases, it is not possible to
distinguish which caspases are contributing to activity by using substrates listed in Table 1 because most of the commonly used caspase substrates can be cleaved by more than one caspase, albeit at different efficiencies (13,14). Furthermore, the abundance of individual caspases in a cell type can vary greatly; therefore, the relative contribution of a single caspase to substrate cleavage is always difficult to assess.

2. Materials

1. Cells and tissue samples: Cells and tissue samples in which caspase activities are to be determined are to be supplied by the investigator. For a positive control, mammalian cell lines treated with apoptosis-inducing agents can be used. As a guide, extracts prepared from Jurkat cells treated for 2 h with 200 ng/mL of an anti-Fas antibody (e.g., from Upstate Biotechnology) or for 4 h with 40 µM etoposide, will show significant levels of caspase activity on IETD, DEVD, and VDVAD substrates. Extracts from treated cells can be prepared as described below in Subheading 3.1.1. (see Notes 1–3).

2. Caspase substrates: The fluorogenic substrates, with either acetyl-(Ac) or benzyloxycarbonyl- (z) blocking group and 7-amino-4-trifluoromethylcoumarin (AFC) or 7-amino-4-methylcoumarin (AMC) reporters, and colorimetric substrates, with p-nitroanilide (pNA) reporter, are available from various commercial sources. Two of the largest suppliers are Enzyme Systems Products Inc. (United States) and Bachem (Switzerland), but many commonly used caspase substrates and inhibitors can now be bought from numerous suppliers. In our experience, fluorogenic assays are far more (50- to 100-fold) sensitive than the colorimetric assays. This may be an important consideration when there is a limited availability of starting material (cells or tissue sample). AMC and AFC substrates can be stored at −20°C as 5- to 10-mM stock solution in dimethyl formamide for 1–2 yr. Dissolve pNA substrates at 20 mM in dimethyl formamide and store the same as one would AMC and AFC substrates.

3. Spectrometers: For the measurement of fluorescence, a luminescence spectrometer, such as Perkin-Elmer LS50B fluorometer, preferably equipped with a thermostated plate reader, is required. If using pNA colorimetric substrates, a spectrophotometer, preferably equipped with a thermostated cuvette or plate holder, is required.
4. Caspase assay buffers: Prepare caspase assay buffers containing 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid, pH 7.0; 10% sucrose; 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; and 10 mM dithiothreitol (DTT) and store in aliquots at –20°C. Alternatively, assays buffer without DTT can be stored at room temperature for several months and DTT added to 10 mM from a fresh 1 M stock as required (see Note 1).

5. In vitro translated proteins: A convenient kit for in vitro coupled transcription/translation is commercially available from Promega Corporation. Alternatively, reagents for in vitro transcription and in vitro translation can be purchased separately. For the synthesis of 35S-labeled proteins, follow the instructions provided by the manufacturer. Translated proteins can be stored for up to 2 wk at –70°C.

6. Protein electrophoresis and transfer: A standard protein electrophoresis apparatus and a semi-dry protein transfer apparatus (such as Hoefer™ SemiPhor from Amersham Pharmacia Biotech) are required. Details of protein electrophoresis and transfer protocols can be found in various methods books, such as Molecular Cloning (17).

7. Immunoblotting: Antibodies against many caspase substrates and secondary conjugates are commercially available. The most commonly used caspase substrate is poly(ADP)ribose polymerase (PARP). The anti-PARP antibody supplied by Roche Molecular Biology cleanly detects the 115-kDa PARP precursor and the 89-kDa cleavage product (18). Other common sources of antibodies include Transduction Laboratories, Pharmingen, and Santa Cruz Biotechnology. For the detection of proteins after immunoblotting, enhanced chemiluminescence from Amersham Pharmacia Biotech works well.

3. Methods

3.1. Measurement of Caspase Activity Using Synthetic Peptide Substrates

3.1.1. Preparation of Cell Extracts

For preparation of cell extracts from animal tissue samples, homogenize frozen tissue cut into small pieces in extraction buffer (20 mM piperazine-N,N'-bis[2-ethanesulphonic acid], 100 mM NaCl, 10 mM DTT, 1 mM ethylenediamine tetraacetic acid, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate,
10% sucrose, pH 7.0, containing protease inhibitor cocktail such as, Complete™ from Roche Molecular Biologicals) using a tissue homogenizer prior to cell lysis. For cultured cells in suspension, spin down cells at 200g for 10 min and wash once in ice-cold phosphate-buffered saline (PBS). For adherent cells, gently scrape cells into medium, spin down cell pellet at 200g for 10 min, and wash once in cold PBS. Resuspend cells at approximately 10^7 cells/mL in extraction buffer. Freeze/thaw cells three times and then homogenize by 10 strokes in a glass homogenizer. Spin extracts at 15,000g for 10 min and carefully remove cell extracts leaving the pellet undisturbed. After determining protein concentration the extracts can be frozen at –70°C in small aliquots for several months without any significant loss of caspase activity.

3.1.2. Measurement of Caspase Activity

Caspase assays should be performed continuously if the spectrophotometer is equipped with a regulated temperature chamber that can accommodate cuvettes or 96-well plates; otherwise, the release of AFC, AMC, or pNA can be monitored after a fixed period of incubation. To save reagents, perform assays in a final volume of 50–100 µL. If the fluorometer is equipped with a 96-well plate reader and several assays need to be conducted simultaneously, the reactions can be assembled in a 96-well plate format. If this is not possible, reactions can be conducted in microfuge tubes or cuvettes.

1. Add varying concentrations of the cell lysates to caspase assay buffer supplemented with 0.1 mM of an appropriate caspase substrate and monitor the release or fluorochrome or chromogen at 37°C in the thermostat fitted spectrophotometer (see Notes 1 and 2). For AMC fluorescence detection, adjust the excitation and emission wavelengths to 385 nm and 460 nm, respectively. For AFC, excitation and emission wavelengths are 400 nm and 505 nm, respectively. pNA absorbance should be monitored at 405–410 nm.
2. Monitor the release of the fluorochrome or chromogen for 30 min. The enzyme activities can be expressed as rate of substrate hydrolysis that can be calculated from the linear portion of the progress curves, prior to the time when substrate depletion slows down the
rate of reaction. If substrate depletion occurs too quickly, dilute cell extracts to get a more linear response.

3. If continuous monitoring of fluorochrome or chromogen release is not possible because of the nonavailability of equipment, assays can be conducted for various lengths of time up to 30 min at 37°C in a waterbath. At the end of the incubation, the reactions can be stopped by adding 0.4 mL of ice-cold water and storing tubes on ice.

4. Plot fluorescence (for AFC or AMC) or absorbance (for pNA) and calculate the rate of hydrolysis from the linear part of the curve.

3.2. Assay of Caspase Activity by Cleavage of $^{35}$S Met-Labeled Caspase Substrates

This is a qualitative assay that is suitable for confirming the presence of active caspases in cell extracts. Clone the cDNAs containing caspase cleavage sites, such as PARP (19,20), DNA-PK catalytic subunit (21), or inhibitor of caspase-activated DNase (ICAD) (22), in plasmid vectors that carry either SP6, T3, or T7 promoters (pBluscript, pGEM, and pcDNA3 vectors are all appropriate for this purpose). It is not necessary to clone the entire protein, and truncated coding region containing caspase site(s) that give rise to easily discernible cleavage products work well (23).

1. Purify plasmids using CsCl centrifugation or Qiagen kit and perform in vitro transcription/translation using Promega kit according to the instructions provided by the manufacturer. Typical 50-μL reactions contain 25 μL of TNT lysates, 2 μL of TNT reaction buffer, 1 μL of T3, T7, or SP6 RNA polymerase, 1 μL of amino acid mixture lacking Met, 5 μL of $^{35}$S Met, 1 μL of Rnasin, 1 μg plasmid DNA, and sterile RNase-free water.

2. Incubate reaction tubes at 30°C for 1.5–2 h, spin at maximum speed in a microfuge for 5 min, and remove supernatant to fresh tube. In vitro translated proteins can be stored at −70°C for up to 2 wk.

3. For cleavage assays, 5 μL of labeled protein is incubated at 37°C for 2 h with varying amounts of cell extracts (10–50 μg total protein) in caspase assay buffer in a total volume of 20 μL. In control experiments, cell extracts can be preincubated with caspase inhibitors, such as 50 μM zVAD-FMK, for 30 min before the addition of labeled protein substrate.
4. At the end of incubation period, add 20 µL of 2× protein loading buffer (100 mM Tris-HCl pH 6.8, 200 mM dithiothreitol, 20% glycerol, 4% sodium dodecyl sulfate (SDS) 0.2% bromophenol blue) to each tube, boil for 5 min, and centrifuge at maximum speed in a microfuge for 5 min.

5. Remove supernatant to fresh tube and resolve cleavage products by electrophoresis on 10–15% polyacrylamide/SDS gel.

6. After fixation, gels can be dried. Alternatively, proteins can be transferred to polyvinylidene difluoride or nitrocellulose membranes using a semidyry transfer apparatus before the visualisation of 35S-labeled protein bands by autoradiography (24). This avoids the possibility of gels cracking during the drying process. In most cases, freshly labeled 35S proteins and their cleavage products can be detected after an overnight exposure to a X-ray film.

3.3. Assessing Caspase-Mediated Substrate Cleavage by Immunoblotting

Because caspase activation results in the cleavage of the caspase precursor into subunits, caspase activation can be indirectly observed by immunoblotting using specific antibodies (see Note 4). However, a more direct measure of caspase activity, usually that is contributed by the downstream or effector caspases, such as caspase-3 and caspase-7, is to determine whether endogenous caspase targets are being cleaved (see Note 5). This can be easily achieved by immunoblotting of cell extracts using a specific antibody against a known endogenous caspase substrate. There are close to 300 proteins now known to be cleaved by caspases (25). The most common one, for which good antibodies are available from many commercial suppliers is PARP, a caspase-3 substrate.

1. Prepare samples for electrophoresis by mixing equal volume of protein extract prepared as described in Subheading 3.1.1. and 2× protein loading buffer.

2. Cell pellets or small pieces of tissues can also be directly lysed by boiling in 2× protein loading buffer. However, often the lysates prepared in such a way will be very viscous due to the release of DNA. To reduce viscosity, the samples can be passed through a 22-gauge needle three to four times or sonicated for 30 s to shear DNA.
3. Boil samples for 5–10 min and centrifuge lysates for 5 min at maximum speed in a microfuge to remove any insoluble material. At this stage, if required, the samples can be stored at −70°C indefinitely.

4. Electrophorese 10–20 µg of the protein samples on 10% polyacrylamide/SDS gels.

5. Transfer proteins to polyvinylidene difluoride membrane using the semidry protein transfer apparatus.

6. Block membrane in 5% skim milk in PBS containing 0.05% Tween 20 (PBST) for 1 h at room temperature or overnight at 4°C.

7. Dilute primary antibody as suggested by the manufacturer in PBST and incubate the membrane with the antibody solution for 1 h at room temperature.

8. Wash membrane three times for 10 min each and incubate with the appropriate secondary antibody diluted in PBST.

9. For detection of signals by enhanced chemiluminescence, follow instruction supplied by Amersham Pharmacia Biotech. As an example, in healthy cells PARP will appear as a single band of approximately 115 kDa, whereas in cells undergoing apoptosis a gradual decrease in 115-kDa band and appearance of a 89-kDa cleavage product should be clearly visible.

4. Notes

1. Although most caspases are active at pH 7.0, some have different pH optima. For example, caspase-2 and caspase-9 favor a slightly acidic pH (26). If necessary, the assay buffer containing 0.1 M MES, pH 6.5, can be used instead of 0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid, pH 7.0.

2. If necessary, recombinant caspases expressed in *Escherichia coli* can be used for positive controls. A number of publications describe the preparation of recombinant caspases (21,24,26). Some commercial suppliers, such as Alexis Biochemicals (Switzerland), also provide a number of recombinant purified caspases.

3. To avoid nonspecific hydrolysis of caspase substrates, it is useful to include protease inhibitor cocktail in the cell lysis buffer. Many commercially available protease inhibitor set can be used provided they do not contain caspase inhibitors.

4. To test whether individual caspases are being activated, immunoblot analysis of cell extracts using specific caspase antibodies can be
performed. To do this, prepare cell extract blots as described in **Subheading 3.3.** and probe them with caspase antibodies to determine whether a specific caspase precursor is being cleaved into active subunits. There are numerous commercial sources of caspase antibodies; however, many antibodies on the market are of poor quality. If using a new antibody for the first time, especially when the same antibody has not been used in the published literature, specificity and affinity of the antibody should be empirically established using recombinant caspases. Some antibodies will detect both the precursor and one or more subunits/intermediates, whereas others are specific for either the precursor or the subunits. In some cell types, the half-life of some active caspase subunits is often very short. In such cases a clear decrease in zymogen signal can be seen but not a corresponding increase in the subunit signal.

5. Caspase activation can also be determined *in situ*, in cultured cells, or in tissue sections by immunohistochemistry using antibodies that specifically recognize processed form of caspases. These antibodies are now widely available from various commercial sources.

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**References**


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