Cell-Free Apoptosis in Xenopus laevis Egg Extracts

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1. Introduction

Apoptotic cell death is thought to be mediated by conserved biochemical mechanisms that culminate in the orderly destruction of cellular components. The pathways leading to activation of the effector molecules that direct and carry out the final dismantling of the cell are now partly understood **[reviewed elsewhere in this volume?]**. Central to this process is the caspase family of apoptotic proteases (reviewed, e.g. by Martin & Green 1995; Thornberry 1997; Villa et al., 1997). Caspases can cleave key cellular proteins, thereby either destroying their normal cellular maintenance functions or activating them as death effectors. Caspases can also process and activate each other; thus, one of the key commitment steps of the apoptotic process is the activation of the most upstream member of a caspase cascade. This can occur in two main ways. The first of these involves the binding of ligands to death receptors at the cell surface, leading to the oligomerization and self-activation of proximal caspases (e.g. Caspase-8/FLICE), as occurs in cell death mediated by CD95 (Fas/Apo-1) and the TNF receptor (reviewed, e.g., by Arch et al., 1998; Baker & Reddy 1996; Chinnaiyan & Dixit 1997; Nagata 1998; Peter & Krammer 1998). The second mode of caspase activation involves mitochondria as a point of control. These organelles can mediate cell death through the release of pro-apoptotic proteins, particularly cytochrome c, from the mitochondrial intermembrane space (Liu et al., 1996). Once translocated to the cytosol, cytochrome c binds to the Apaf-1 protein, which then triggers the activation of Caspase-9, in turn leading to the processing of downstream caspases in cascade fashion (Li et al., 1997b; Zou et al., 1997). The release of cytochrome c from mitochondria can be regulated by proteins belonging to the Bcl-2 family (Eskes et al., 1998; Jurgensmeier et al., 1998; Kim et al., 1997; Kluck et al., 1997a; Luo et al., 1998; Rosse et al., 1998; Yang et al., 1997), some of which are pro-apoptotic while others are anti-apoptotic. Bcl-2 relatives may also participate in crosstalk between death receptor- and mitochondria-mediated pathways of cell death (Kuwana et al., 1998; Li et al., 1998; Luo et al., 1998; Scaffidi et al., 1998).

Although Bcl-2 and its relatives have been shown to regulate cytochrome c release, to mitigate the effects of cellular oxidants (Hockenbery et al., 1993; Kane et al., 1993) and to regulate the partitioning of intracellular calcium (Baffy et al., 1993), these effects are probably all indirect, and the precise functions of Bcl-2 family members are not clearly understood. Bcl-2, Bcl-x_L and Bax can form channels in synthetic lipid bilayers (Antonsson

et al., 1997; Minn et al., 1997; Schendel et al., 1997); however, it has yet to be proven that these proteins regulate apoptosis via channel formation in intracellular membranes.

Because of the gaps in our understanding of the nature and sequence of biochemical events in apoptosis, it is desirable to have at our disposal cell-free systems that permit the biochemical dissection of apoptotic pathways. One such system was described by Lazebnik et al. (Lazebnik et al., 1993), who utilized cytosolic extracts from doubly synchronized chicken hepatoma cells ("S/M extracts"). Exogenous nuclei placed in such extracts undergo the morphological changes typical of nuclei in apoptotic cells. This process was shown to depend on caspase activity (Lazebnik et al., 1994). Other investigators have employed apoptotic cell-free systems prepared from mammalian cells (Ellerby et al., 1997; Enari et al., 1996; Liu et al., 1996; Martin et al., 1995; Muzio et al., 1997).

We developed a different apoptotic cell-free system, based on extracts from *Xenopus* eggs (Newmeyer et al., 1994). When nuclei from various sources are placed in "apoptotic" egg extracts, they in time display the morphological changes typical of nuclei in apoptotic cells. Furthermore, these events are inhibited by the addition of exogenous Bcl-2 protein. *Xenopus* egg extracts can be used to study the roles of subcellular organelles, as the particulate components can easily be separated into two fractions: light membranes (LM), largely composed of endoplasmic reticulum and nuclear membrane precursor vesicles, and heavy membranes (HM), which are highly enriched in mitochondria. The LM fraction is required for nuclear membrane formation. In contrast, the HM fraction is dispensable for nuclear membrane formation but essential for the apoptotic activity of the extracts. The *Xenopus* system has proven to be useful in elucidation of the mechanisms of apoptosis and its regulation by Bcl-2 proteins (Cosulich et al., 1996; Cosulich et al., 1997; Evans et al., 1997a; Evans et al., 1997b; Farschon et al., 1996; Faure et al., 1997; Kluck et al., 1997a; Kluck et al., 1997b; Kuwana et al., 1998; Newmeyer et al., 1994). Here we describe in detail the methods needed to prepare and use apoptotic *Xenopus* egg extracts.

2. Basic features of *Xenopus* egg extracts

Due to the developmental strategy of certain amphibians, extracts from *Xenopus laevis* eggs have been proven to be a versatile experimental resource for studies on the function of the cell nucleus (references cited in Newmeyer & Wilson 1991). The eggs of these organisms are very large cells, over a millimeter in diameter, and contain a huge stockpile of all the

materials required for the biogenesis of nuclei and other organelles, except for DNA (Laskey et al., 1979). These stores are mobilized during early development, a period in which the embryos undergo several rounds of cell division without significant new gene expression. The *Xenopus* egg, moreover, is arrested in metaphase of meiosis II, which means that the components of the nucleus (including lamins, pore complex proteins, and nuclear membrane precursor vesicles) are present in their disassembled mitotic form. When the egg is fertilized or artificially activated, endogenous Mos protein and cyclin B are degraded via a Ca^{2+}-dependent mechanism, and the metaphase block is released (Lorca et al., 1993; Minshull et al., 1989; Watanabe et al., 1991; Watanabe et al., 1989). As a result, the stockpiled nuclear components become available for nuclear assembly, as long as a suitable chromatin substrate is introduced into the egg.

Extracts of *Xenopus* eggs can be prepared in either a metaphase or an interphase state, depending on the presence or absence, respectively, of Ca^{2+} chelators and phosphatase inhibitors in the lysis buffer. Interphase extracts support nuclear assembly, whereas mitotic extracts disassemble nuclei. However, the interphase extracts have the capacity to re-enter mitosis, and when prepared carefully, can proceed through several cycles of M and S phases (Murray 1991). This is useful for cell cycle investigations, but a nuisance when the functions of intact nuclei are being studied. To investigate apoptotic events, especially nuclear condensation and fragmentation, the extracts are generally arrested in interphase by including cycloheximide in the egg lysis buffer, thereby preventing the re-synthesis of cyclin B and the subsequent re-entry into mitosis (Newport 1987). However, mitotic extracts also appear to undergo apoptosis (Newmeyer, unpublished.)

Xenopus eggs provide a convenient source of reasonably large amounts of homogeneous and naturally synchronized apoptotic cell extracts suitable for biochemical subfractionation. Thus, this system is well-suited for studying subcellular events in apoptosis. Apoptotic extracts (Newmeyer et al., 1994) are prepared in exactly the same way as extracts used for the study of nuclear assembly and protein import (Newmeyer & Wilson 1991). We formerly believed it important to give the frogs an altered schedule of hormone injections to induce a pre-apoptotic state; however, experience in our laboratory has shown that most extracts (as long as they contain mitochondria) display apoptotic behavior, regardless of the hormone schedules used. The apoptotic character of egg extracts may reflect normal atretic

processes in vivo, which, at least in mammals, are known to be apoptotic (Kaipia & Hsueh 1997). For the care and maintenance of *Xenopus*, see (Wu & Gerhart 1991).

3. Preparation of apoptotic extracts from *Xenopus* eggs

Interphase extract

(Modified from Newport 1987; Wilson & Newport 1988)

All steps are done at room temperature unless otherwise noted.

1. Prime female frogs (available in the U.S.A. from Nasco, Ft. Atkinson, WI, *Xenopus* I, Ann Arbor, MI; or Xenopus Express, Homosassa, FL, USA) 14-28 days prior to egg-laying with 100 Units Pregnant Mare Serum Gonadotropin (PMSG; Calbiochem #367222) in 0.5 ml sterile PBS. Frogs are injected subcutaneously into their dorsal lymph sac.

2. The evening (16 hours) before egg-collection: Inject frogs with 1000 units of human Chorionic Gonadotropin (hCG Sigma # CG-10) in 1 ml sterile PBS and place the frogs in individual containers (preferably clear or translucent non-porous plastic) containing 8 l of salt-water (5 g of food-grade NaCl per l). Often, the frogs will not have finished laying all of their eggs by morning, and an additional quantity can be harvested by squeezing the animal firmly (but not roughly) while holding it in the salt-water. Transfer the frogs to fresh water, and allow the eggs to settle to the bottom of the containers. Pour off most of the salt-water, and pour the eggs into 500-ml beakers. Allow the eggs to settle out, and pour off most of the remaining salt-water.

3. Prepare 4% cysteine solution (ambient temperature), about 100 ml per batch of eggs, and adjust pH to 7.9 with NaOH. Add an equal volume of cysteine solution to the egg suspension, bringing cysteine to 2%. Wait 5-10 minutes, periodically swirling the eggs gently; when the eggs pack tightly with no space between them, the jelly coats have been dissolved. Do not allow the eggs to sit in cysteine solution any longer than necessary.

4. Rinse the eggs several times with MMR. (Prepare about 500 ml MMR per frog). Be gentle and thorough with all rinses and avoid jarring the eggs. Use a Pasteur pipet to remove as much buffer as possible between rinses.

5. Pour each individual batch of eggs into a glass petri dish large enough to spread the eggs in a monolayer, and examine the eggs under a dissecting microscope. Because of the variability that tends to occur between extracts, it is a good idea to keep batches of eggs separate, if possible. Remove debris and all the bad eggs with a glass Pasteur pipet. If a particular batch of eggs contains more than 10% mottled or lysed (white) eggs, it is probably not worth saving. Eggs that are furrowed (pseudocleaved) have been activated by mechanical damage to the outer membrane, but in small numbers are usually not detrimental to the preparation of S-phase extracts. For some reason, batches of eggs that come out of the frog in strings of jelly are usually bad. Occasionally, frogs will lay a small proportion of unmatured oocytes, lacking the white spot indicative of nuclear breakdown. Remove these if M-phase extracts are being prepared.

6. Rinse three times in ELB (prepare about 300 ml ELB per batch of eggs). Remove excess buffer. Add the protease inhibitors, aprotinin and leupeptin, (A/L) to 10 µg/ml final concentration. Transfer the eggs gently to 15 ml Centrifuge tubes (Falcon #2059). Do not fill the tubes above 12 ml. To estimate the yield of extract, measure the volume of good eggs. Experience has shown that if the volume of dejellied eggs is □ V, then the volume of crude cytoplasmic extract is □ V ÷ 3, and the approximate volume of cytosol fraction from a 200,000 x g spin (see below) comes to V ÷ 10. Remove excess buffer. Pack the eggs by centrifuging for 2 min at 2,000 RPM in a Sorvall Centrifuge, with an HB-4 (swinging bucket) rotor or the equivalent. Important: again, remove excess buffer. The idea is to make the extracts as concentrated as possible.

7. Centrifuge the packed eggs at 10,000 x *g* (10,000 RPM in the HB-4 rotor) for 12 min at 4°C. The preparation from this point is shown schematically in Fig. 1. Centrifugal forces lyse the eggs and stratify the egg contents. Dense yolk and pigment granules go to the bottom, and the crude cytoplasmic extract floats above. (Note: for small volumes of eggs, lyse by centrifuging in a 1.5-ml Eppendorf tube for 5 min at maximum speed in a Beckman Microfuge or an Eppendorf centrifuge kept at 4° C, using a horizontal rotor.) After lysis, keep extracts on ice. The cytoplasmic extract is withdrawn from the side of the tube using a 3-ml syringe and 21-gauge needle. Insert the needle, bevel up, just above the pigment layer (see Fig. 1). If the first needle becomes clogged, use a

second syringe with fresh needle and insert it quickly (to prevent leakage) into the hole made by the first one. Transfer the cytoplasm to a clean tube on ice.

8. Repeat the centrifugation to remove remaining yolk, pigment, and lipid. (If preparing small volumes of extract, for this second centrifugation it is best to use long, narrow 0.4-ml polyethylene microfuge tubes (Beckman #314326); after centrifuging for 5 min at top speed, lay the tube on its side, cut off the top of the tube just below the lipid layer with a clean razor blade, and use a Pasteur pipette to remove the cytoplasmic material.) The resulting crude extract is adequate for most kinds of experiments that do not need separate incubations of cytosol and membranous material. Crude extract must be used fresh.

Mitotic extract

(from Newport & Spann 1987)

For optimal separation of light and heavy membrane components and especially the purification of light membranes that can be used for nuclear reassembly, the cell extract has to be arrested in a mitotic state. With S-phase extracts, some of the nuclear membrane vesicles are recovered in the heavy membrane fraction (Wilson & Newport 1988), presumably because they begin to fuse or associate with other extract components after the eggs are crushed. Thus, for experiments which combine cytosol and fractionated membrane components, it is normally necessary to prepare two extracts: an S-phase extract, for cytosol, and an M-phase extract, for membrane components.

The only methodical difference between mitotic and interphase extracts is the buffer in which the eggs are lysed. M-phase Egg Lysis Buffer (MELB) has two ingredients that preserve the meiotic/mitotic state by blocking the calcium-dependent degradation of the Mos protein and cyclin B: □□glycerophosphate, a competitive inhibitor of phosphatase activity; and EGTA, which chelates Ca^{2+}. Alternatively, S-phase ELB containing at least 5 mM EGTA could be used (Kubota & Takisawa 1993). For details of S-phase extract preparation, refer to the previous section.

It has been shown that the addition of staurosporine (3-5 □M) can convert a mitotic extract (prepared with 5 mM EGTA in ELB, rather than MELB) into a pseudo-interphase extract

that assembles nuclei but cannot replicate DNA (Kubota & Takisawa 1993). Hence, if the particular processes being studied are unaffected by the presence of staurosporine (as is the case for apoptotic egg extracts; Newmeyer et al., 1994), it is possible to do fractionation experiments with a single M-phase extract.

4. Subcellular fractionation of crude egg extracts

Ultracentrifugation separates the membranes and soluble components. Each component can be frozen and stored for at least 6 months; however, the light membranes lose some of their activity upon each freeze/thaw cycle and each time they are pelleted.

1. Transfer the crude extract into an ultraclear ultracentrifuge tube, on ice. Centrifuge at 200,000 x g for 1 hour at 4°C, in a swinging bucket rotor. Best: use a Beckman tabletop TL-100 ultracentrifuge with a TLS-55 swinging bucket rotor at 55,000 RPM. Other rotors may not achieve adequate separation in 1 hour.

2. Recover the cytosol fraction from the side using syringe and needle as before, being careful not to disturb the underlying membrane layers (Figure 1). To obtain very pure cytosol, centrifuge again at 200,000 x g for 20 min at 4°C to remove most of the residual membranes. For the best separation of cytosol and membranes add ATP/PC and creatine kinase before recentrifugation. Freeze aliquots in liquid nitrogen and store them at -80°C.

3. To obtain the light membrane fraction, remove and discard the remaining cytosol and lipid remaining above the membrane layers.

4. Using a wide-bore 200-µl micropipet tip (the end chopped off with a razor blade), collect most of the pale yellow light membrane layer, ~ 50 µl at a time. With care, it is possible to avoid contaminating this material with the brown heavy membrane layer underneath.

5. Resuspend in at least 20 volumes of cold membrane wash buffer (MWB). Pellet membranes by centrifugation at 40,000 x *g* for 15 min at 4°C (25,000 RPM in the TLS-55 rotor). Remove and discard the supernatant. Recover membranes in the smallest possible volume of MWB, preferably less than 1/10 of the volume of the cytosol fraction.

6. If long-term storage is desired, supplement the light membranes with sucrose to at least 0.5 M final concentration. Freeze small aliquots in liquid nitrogen and place them at -80°C.

7. Heavy membrane fraction (Newmeyer et al., 1994): After removal of most of the LM fraction, the crude HM material (contaminated with a small amount of LM) is removed with a micropipettor. To isolate a mitochondria-enriched fraction uncontaminated with LM, centrifuge the crude HM material over a Percoll step gradient (modified from Reinhart et al., 1982).

8. Dilute the crude HM with an equal volume of mitochondrial isolation buffer (MIB). Layer up to 100 µl of the diluted HM material over a 1.8-ml Percoll step gradient consisting of four 0.45-ml layers with Percoll concentrations of 25, 30, 37 and 42%, in MIB. Centrifuge at 4°C for 20 min in a TLS-55 rotor, at 25,000 RPM, using a Beckman TL-100 centrifuge, with the brake set at 5.

9. With a Pasteur pipet inserted into the gradient from above, remove the brown material forming the major band at the interface between 30 and 37% Percoll. Try to recover it in as small a volume as possible.

10. To wash out residual Percoll, resuspend the heavy membranes in at least 100 volumes of MIB, centrifuge at 1600 x g for 10 min at 4°C, and resuspend the pellet in an equal volume of MIB. The resulting enriched HM fraction can be frozen in small aliquots in liquid nitrogen and then stored at -80°C.

5. Preparation of demembranated sperm chromatin

(Slightly modified from Lohka & Masui 1983)

All steps are done at room temperature unless otherwise noted. Approximate buffer volumes needed per frog: 4.0 ml of SB; 1.0 ml of SB with 0.05% lysolecithin; and 5.0 ml of SB with 3% BSA.

1. Anesthetize male frogs in 1% tricaine (3-aminobenzoic acid ethyl ester; Sigma) for 15-20 min. When the frogs no longer move and when they are turned belly-up, they are ready.

2. Make an abdominal incision and remove the testes, being careful not to puncture them. Incubate them overnight at 18-22°C in MMR solution containing hCG (10 units/ml) and gentamycin (250 μg/ml).

3. Trim away fat and connective tissue and rinse in SB. Release the sperm by crushing the testes in a 1.5-ml Eppendorf tube containing SB (1 ml per pair of testes). Use broad forceps or , better, a plastic homogenizer pestle designed to fit snugly into the conical bottoms of 1.5-ml Eppendorf tubes (Kontes "Pellet Pestle Mixer", #749520-0000). To avoid contamination with somatic cells, take care not to disintegrate the outer tissue of the testis.

4. Clear debris by centrifugation for 10 sec at setting #3 in a clinical centrifuge or 1,000 RPM in an Eppendorf centrifuge. Transfer the supernatant to a new Eppendorf tube. Add 500 μl SB to the pelleted tissue, mix gently, and recentrifuge. Combine the two supernatants.

5. Pellet the sperm by centrifugation for 2 minutes at 6,000 RPM in the Eppendorf centrifuge. After discarding the supernatant, carefully resuspend the white part of the pellet in SB, 50-100 μl at a time, for a total of ≈ 500 μl. Avoid resuspending the red bottom of the pellet, which contains unwanted erythrocytes and somatic cells. Repeat the pelleting/resuspension procedure once more to eliminate more somatic cells.

6. Pellet the sperm a third time, and resuspend in 100 μl SB. Add 1 ml SB/0.05% lysolecithin. Lysolecithin removes the plasma and nuclear membranes. After a 5-min incubation at room temperature, pipet the suspension into 3 ml of SB/3% BSA and mix gently. The BSA aids in the removal of free lysolecithin.

7. Pellet the demembranated sperm nuclei 8-10 min at 2,000 RPM in a Sorvall centrifuge. Resuspend the pellets in 200 μl each of SB/3% BSA, pool into one tube, and add 1 ml SB/3% BSA.

8. Pellet sperm nuclei for 8-10 min at 2,000 RPM, and resuspend in 50 μl of ice-cold SB. Count the sperm using a hemacytometer.

9. Dilute with SB to get a final concentration of 4 x 10^5 per µl. Freeze 10-µl aliquots in liquid nitrogen, and store at -80°C.

6. Isolation of rat liver nuclei

(From Blobel & Potter 1966; with crucial buffer modifications: Newport & Spann 1987)

Make all solutions in advance and cool to 4°C. Pre-cool ultracentrifuge, SW28 rotor, and buckets to 4°C.

Sacrifice rats, and remove livers, placing them in petri dish on ice. Cut away tough connective tissue between lobes. Record weight (W) of liver tissue.

Transfer tissue to a clean glass plate on ice; mince with razors. Scrape the minced tissue into a chilled homogenizer chamber (≈ 30 ml size). Add (2 x W) ml of chilled Solution A. Homogenize with 12-14 strokes (by Dounce or motorized homogenizer).

Filter the homogenate through 4 layers of cheesecloth in a funnel.

Add (2 x W) ml of Solution B; mix thoroughly by inversion. Pipet into SW28 centrifuge tubes (ultraclear; ≈ 30 ml per tube). Drip 5 ml of Solution B into the tube to form a cushion at the bottom. Balance tubes carefully, and centrifuge 1.5 h at 22,000 RPM at 4°C, with the brake on.

The nuclei form a white pellet. Aspirate and remove all supernatant debris. Use a lint-free paper towel to clean the inside tube wall. Resuspend each pellet gently in 200 µl of Solution A.

Count the nuclei using a hemacytometer. Adjust concentration to 1.6-2.0 x 10^5 nuclei per µl. Freeze small (5-20 µl) aliquots in liquid nitrogen; store at -80°C.

7. Assembly of nuclei around demembranated sperm chromatin

Each egg contains enough nuclear components to construct about 4,000 embryonic nuclei (Newport & Forbes 1987). The amount of extract obtained from one egg is ≈ 0.7 µl. A typical nuclear assembly reaction contains:

1. 100 µl soluble fraction (≈ 70 egg equivalents);

2. 10 µl light membranes (LM) and 2.5 µl of heavy membranes (HM; necessary for apoptotic extracts but not for nuclear assembly);

3. 0.7 µl sperm chromatin (at 400,000 sperm per µl; final sperm concentration ~ 4,000 per egg equivalent);

4. 7 µl of phosphocreatine/ATP (ATP/PC)

5. 3 µl of creatine phosphokinase stock solution (CK);

Nuclear assembly reactions reconstituted from frozen components typically yield decent nuclear envelopes and decondensed chromatin within one hour. An assay for nuclear integrity is described below.

8. Reconstitution and analysis of apoptotic events using fractionated extracts

Cell free fractionated extracts prepared from *Xenopus* eggs offer the advantage that different partial reactions or temporal phases occurring during apoptosis can be studied separately. For example, one can study the activation of caspases by exogenous purified cytochrome c, the response of nuclei to prior events in the cytoplasmic extract, or mitochondrial changes resulting from various treatments. In reconstituted extracts, light membranes are only required if nuclei or chromatin will be added. As most apoptotic events in this system do not require the presence of nuclei, most studies will only require a minimal cell-free system consisting of cytosol and mitochondria (Kluck et al., 1997a).

1. Keep all isolated components on ice before use, and mix gently to avoid damaging mitochondria or nuclei when these are used.

2. Add 3 µl CK and 7 µl ATP/PC to 100 µl clear cytosol. An ATP regenerating system helps provide energy for prolonged apoptotic reactions.

3. For investigations concerning the role of mitochondria, heavy membranes should be added to the reaction mix to a final concentration of ~ 2 – 3% (v:v; in this calculation we refer to the volume of the packed HM pellet *before* dilution 1:1 with buffer.) Nuclei should be used at concentrations around 1000 nuclei per µl.

4. Start the experiment by shifting the samples to room temperature.

5. Take samples at different time points to follow cytochrome c release from mitochondria, caspase activation and nuclear fragmentation.

Caspase activity

Because caspases are the central executioners involved in apoptosis, their activity is a hallmark of this form of cell death. Caspase activation by the Apaf-1/Caspase-9 "apoptosome" complex can also be used as a convenient indirect assay for cytochrome c release from mitochondria. Detection of caspase activity is conveniently done with chromogenic substrates like DEVD-pNA or its more sensitive fluorogenic counterpart, DEVD-AFC. Due to the high caspase concentrations in crude extracts or cytosol preparations, it is possible to perform activity assays in a conveniently small sample volume.

1. Dilute DEVD-pNA or DEVD-AFC into DEVDase assay buffer to 40µM final concentration. (Other tetrapeptide substrates can also be used to assay caspases with different specificities.)

2. Take a 2-µl sample of the extract which is to be assayed and add 200 µl of assay mix.

3. For DEVD-pNA, measure the increase in absorbance at 405 nm for 30 min at room temperature; for DEVD-AFC, measure light emission at 505 nm with excitation at 400 nm.

4. Especially for long time-courses and large experiments, it is convenient to freeze the 2-µl samples in a 96-well plate, placed on dry ice. Cover the plate to avoid condensation. Add the substrate solution after thawing the plate.

Mitochondrial cytochrome c release

The release of cytochrome c from mitochondria has been shown to be a key event during the commitment phase of at least some forms of apoptosis, and this release can be regulated by Bcl-2 family members (Bossy-Wetzel et al., 1998; Ellerby et al., 1997; Eskes et al., 1998; Jurgensmeier et al., 1998; Kim et al., 1997; Kluck et al., 1997a; Kluck et al., 1997b; Kojima et al., 1998; Krippner et al., 1996; Kuwana et al., 1998; Li et al., 1997a; Li et al., 1998; Li et al., 1997b; Liu et al., 1996; Luo et al., 1998; Manon et al., 1997; Rosse et al.,

1998; Stridh et al., 1998; Vander Heiden et al., 1997; Zhivotovsky et al., 1998; Zou et al., 1997). The mitochondrial events leading to cytochrome c release are therefore an important subject of current research. To measure cytochrome c release in the *Xenopus* cell-free system:

1. Take a 10-µl sample of extract and pellet particulate components, including mitochondria, by microcentrifugation for 5 min at 14000 RPM.

2. Separate the supernatant from the pellet and assay for cytochrome c in both fractions by SDS-PAGE and western-blotting. An anti-cytochrome c antibody crossreacting with the *Xenopus* protein can be purchased from Pharmingen (clone 7H8.2C12).

If mitochondria are incubated in buffer instead of cytosol, make sure that the buffer contains at least 80 mM KCl; otherwise, cytochrome c remains associated with the mitochondria even when the outer membrane is permeabilized (Kluck and Newmeyer, unpublished).

Assay of nuclear integrity by fluorescence microscopy

1. To visualize DNA, mix about 4 µl of the nuclear assembly reaction with 0.5 µl of formaldehyde/Hoechst mixture: 100 µg/ml Hoechst 33258 in 37% formaldehyde. View with a fluorescence microscope equipped with filters appropriate for Hoechst or DAPI fluorescence.

2. To visualize membranes, use phase contrast microscopy or use the membrane dye 3, 3'-dihexyloxacarbocyanine (DHCC) and view in the fluorescence microscope using a filter set appropriate for fluorescein. DHCC is light sensitive; the following working solution should be made fresh each day. Dilute the stock DHCC solution, 10 mg/ml in DMSO, into ~ 50 volumes of Hoechst Buffer, centrifuge 2 min in microfuge to pellet insoluble chunks of DHCC, and remove the supernatant as working solution. To reduce the background of membrane vesicles, mix on the microscope slide 1 µl of extract and 4 µl of working DHCC solution.

9. Buffers and reagents

10X PBS: 74.2 g NaCl, 2 g KCl, 2 g KH_2PO_4 and 21.7g $Na_2HPO_4 \bullet 7H_2O$ in 1 l H_2O adjusted to pH 7.4 with HCl.

Dejellying solution: 4 % cysteine, adjusted to pH 7.9 with NaOH (prepare freshly before use)

10X MMR: 1 M NaCl, 20 mM KCl, 20 mM $CaCl_2$, 10 mM $MgCl_2$, 50 mM HEPES-KOH, pH 7.4. Autoclave and store at room temperature. If kept for short periods of time, the 1X solution does not need to be sterilized.

Egg Lysis Buffer (ELB): 250 mM sucrose, 50 mM KCl, 2.5 mM $MgCl_2$, 20 mM HEPES/KOH, pH 7.5, 1 mM DTT, 50 µg/ml cycloheximide, 5 µg/ml cytochalasin B and 10 µg/ml each of aprotinin and leupeptin. Add cycloheximide, aprotinin and leupeptin fresh from 10 mg/ml stocks in water, cytochalasin B from a 10 mg/ml stock in DMSO.

M-Phase Egg Lysis Buffer (MELB): 240 mM □-glycerophosphate, pH 7.4, 60 mM EGTA, 45 mM $MgCl_2$, 1 mM DTT.

Mitochondrial Isolation Buffer (MIB): 210 mM mannitol, 60 mM sucrose, 10 mM KCl, 10 mM succinic acid, 5 mM EGTA, 1 mM ADP, 0.5 mM DTT, 20 mM HEPES/KOH (pH 7.5)

Membrane Wash Buffer (MWB): 250 mM sucrose, 50 mM KCl, 2.5 mM $MgCl_2$, 20 mM HEPES-NaOH pH 8.0, 1 mM DTT, 1 mM ATP, 1 □g/ml leupeptin, 1 □g/ml aprotinin. Store aliquots at -20° C. Add DTT, ATP and protease inhibitors just before use (ATP interferes with the BCA protein assay).

A/L: The protease inhibitors aprotinin and leupeptin are used at 10 mg/ml in water as a 1000X stock. Store in aliquots at -20° C.

CK: 5 mg/ml Creatine kinase in 50% glycerol, 20 mM Hepes-KOH pH 7.5.

ATP/PC: 100 mM ATP, 200 mM Phosphocreatine in 20 mM Hepes-KOH pH 7.5.

DEVDase substrate stock: 20 mM DEVD-pNA in DMSO.

DEVDase assay buffer: 250 mM sucrose, 50 mM KCl, 20 mM HEPES-KOH (pH 7.5), 2.5 mM $MgCl_2$.

Hoechst Fixative: 100 µg Hoechst 33258 (bisbenzimide) in 37 % formaldehyde. A 100x H33258 stock (10 mg/ml in DMSO) can be used to prepare it.

Hoechst Buffer: 200 mM sucrose, 5 mM MgCl2, 1X Buffer A salts, 10 µg/ml Hoechst 33258. Option: add formaldehyde to 3.7%. Store in the dark at 4°C.

10X Buffer A salts: 800 mM KCl, 150 mM NaCl, 50 mM EDTA, 150 mM PIPES-NaOH, pH 7.4.

Solution SB: 1X Buffer A salts, 0.2 M sucrose, 7 mM $MgCl_2$.

Solution A: 250 mM sucrose, 0.5 mM spermidine, 0.2 mM spermine, 1 mM DTT and 1 mM PMSF in 1X Buffer A salts. (Add DTT and PMSF freshly, PMSF from a 100 mM stock in ethanol.)

Solution B: 2.3 M sucrose, 0.5 mM spermidine, 0.2 mM spermine, 1 mM DTT, and 1 mM PMSF in 1X Buffer A salts.

Figure Legend

Figure 1. Schematic illustration of egg extract fractionation procedure. Eggs are crushed by centrifugation at 10,000 x g; the crude cytoplasmic extract is then removed from the side of the tube, using a syringe. This material may be fractionated further by high-speed centrifugation, yielding cytosol and light and heavy membrane fractions. The cytosol and light membrane fractions are recombined to produce a nuclear assembly extract depleted of dense organelles, and lacking apoptotic activity. The HM fraction is enriched further by Percoll gradient centrifugation and added back to the HM-depleted extract to restore apoptotic activity.

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