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Liposomal Permeabilization Assay to Study the Functional Interactions of the BCL-2 Family

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Abstract

Apoptosis, a form of programmed cell death that is important for development and homeostasis, is regulated by the BCL-2 family of proteins. Over twenty BCL-2 family members have been classified in three groups based on structural homology and function. The multidomain antiapoptotic proteins promote survival, whereas the multidomain and the BH3-only proapoptotic members induce cell death. Because the interaction among the BCL-2 family members occurs primarily at the mitochondrial outer membrane, biochemical assays using artificial liposomes have been developed to study the functional relationship between these proteins. The liposomal permeabilization assay is a cell-free system that relies on the ability of multidomain pro-apoptotic members to promote membrane permeabilization upon activation. By encapsulating a fluorophore and a quencher into liposomes, the degree of permeabilization can be quantified by the increase in fluorescence intensity as the fluorophore and quencher dissociate. The liposomal permeabilization assay has been used to delineate interactions among BCL-2 family members as well as to characterize peptides, small molecules, and lipids that modulate the function of BCL-2 family of proteins. Here, we describe in detail the permeabilization of liposomes induced by the interaction between BAX and BH3-only activator tBID.

Keywords

BCL-2 family; BAX; BAK; tBID; BH3-domain; Liposomal membrane; Mitochondria; MOMP; Apoptosis

1 Introduction

Programmed cell death, or apoptosis, regulates the critical balance between cellular life and death, and deregulated apoptosis can lead to a variety of human diseases [1]. Apoptosis can occur through two pathways: the TNF/Fas death receptor dependent extrinsic pathway and the BCL-2 protein family dependent intrinsic pathway [1]. The intrinsic pathway is

²²Other BH3-only activators in the form of peptides, such as BIM and PUMA, can be used to stimulate BAX or BAK activation.

characterized by permeabilization of the outer mitochondrial membrane and release of soluble factors from the mitochondria that are important in caspase activation [2]. The BCL-2 family of proteins, which regulate the intrinsic apoptotic pathway, is divided into antiapoptotic, proapoptotic, and BH3-only proteins, and their complex interactions can prevent or promote mitochondrial dysfunction [3]. By sequestering the BH3 helices of both BH3-only and multidomain proapoptotic proteins, antiapoptotic BCL-2 proteins promote cell survival [3]. The BH3-only proteins, on the other hand, function as stress sensors of cellular damage and transmit prodeath signals from various signaling pathways to the core apoptotic machinery [3]. A subset of BH3-only proteins, such as BIM and BID, can directly interact and activate BAX and BAK [4]. Upon activation, proapoptotics BAX and BAK oligomerize and promote mitochondrial outer membrane permeabilization (MOMP) leading to the release of apoptogenic factors (e.g., cytochrome c and Smac/DIABLO) that irreversibly initiate the caspase cascade and ultimately execute the death program [4].

Binding of the BCL-2 members occurs primarily at the mitochondrial outer membrane (MOM) [2]. Using artificial liposomes, a biochemical assay was developed to study the integrity of liposomal membranes' upon BCL-2 family members' interaction [5, 6]. Using the liposome permeabilization assay, as well as other supportive techniques, it was demonstrated for instance that anti-apoptotics such as BCL-2 and BCL-X_L inhibit membrane permeabilization by directly sequestering tBID and/or BAX [5]. Additionally, the liposome permeabilization assay has been adapted as a screening tool to identify and characterize peptides, small molecules or antibodies that specifically bind to BCL-2 family members and modulate their function [7–9]. For instance, a small molecule that directly induces the activation of the BAX trigger site was characterized using this permeabilization assay [10]. Additionally, the inhibitory potential of MCL-1 small molecules binders was determined with this biochemical technique [11]. Synthetic antibodies that bind to the N-terminal activation site of BAX were shown to directly inhibit BAX-mediated liposomal permeabilization [9]. Hence, the liposome permeabilization assay constitutes a simple and well-established cell-free system that explores the function of recombinant BCL-2 family members in the absence of other mitochondrial proteins [5, 12, 13].

In general, liposomes are composed of defined lipids identified in lipid composition studies from solvent extracted *Xenopus* mitochondria that mimic the composition of the MOM [14]. The liposome permeabilization assay relies on the ability of proapoptotic proteins, such as BAX and BAK, to promote membrane permeabilization upon activation. To quantify membrane permeabilization, a polyanionic dye (ANTS: 8-aminonaphthalene-1,3,6-trisulfonic acid) and a cationic quencher (DPX: *p*-xylene-bis-pyridinium bromide) are incorporated within the liposomes [6]. As the liposome is permeabilized by activated BAX [10] or BAK [15], ANTS and DPX diffuse apart, and an increase in fluorescence is detected using a fluorescence plate reader. Here we will discuss in more detail the experimental procedures to measure tBID-induced BAX-mediated permeabilization of liposomes.

2 Materials

2.1 Lipid Composition of Liposomes

See Table 1 (see Note 1)

2.2 Reagents

1. Liposome Buffer (*see* Note 2): 10 mM HEPES pH 7.2, 200 mM KCl, 0.2 mM EDTA, 5 mM MgCl₂.
2. ANTS (8-aminonaphthalene-1,3,6-trisulfonic acid) (*see* Note 3).
3. DPX (*p*-xylene-bis-pyridinium bromide) (*see* Note 3).
4. Sepharose CL2B.
5. Recombinant human BID Caspase-8 cleaved (tBID).
6. Recombinant human BAX.
7. Triton X-100.
8. Liquid nitrogen.
9. Gas nitrogen.

2.3 Equipment

1. Ultrasonic water bath.
2. Gravity-flow chromatography column (14 × 1.5 cm).
3. Mini-extruder.
4. 96-well back plates.
5. 10 mm filter support.
6. Gas-tight syringe (1000 µL).
7. Nuclepore Track-Etch polycarbonate membranes (diameter 19 mm, pore size 0.1 µm).
8. 5 mL glass tubes.
9. Fluorescence plate reader (Excitation wavelength: 355 nm; Emission wavelength: 520 nm).

3 Methods

3.1 Making Lipid Film

1. Under a fume hood, use a pipette to add appropriate amounts of chloroform-solubilized lipids into a glass tube (*see* Note 4) to a total of 1 mg lipid (*see* Table 1, *see* Note 1).

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¹.Chloroform lipid stocks are stored in aliquots of 100 µL in amber glass vials under nitrogen gas or argon and caps sealed with parafilm to reduce lipid oxidation by atmospheric oxygen.

².The liposome buffer solution should be freshly prepared and stored at 4 °C.

³.ANTS and DPX are stored at 4 °C in a desiccator.

⁴.For proper transfer of chloroform-solubilized lipids into a glass tube, pipette lipid stocks up and down to coat tip and then transfer directly to glass tube.

2. After adding each lipid into glass tube, evaporate off chloroform with a “low” stream of nitrogen or argon gas.
3. Protect lipid film from light exposure and place under vacuum for a minimum of 3 h at room temperature to remove any remaining chloroform (*see* Note 5).
4. Dried lipid film can be used immediately for experimentation or for storage cover film with nitrogen gas, seal with parafilm, protect from light and place in -20°C (*see* Note 6).

3.2 Preparing Liposomes

1. Add 12.5 mM ANTS and 45 mM DPX to a 1 mg dry lipid film (*see* Note 7).
2. Hydrate dry lipid film with 1 mL of liposome buffer (10 mM HEPES pH 7.2, 200 mM KCl, 0.2 mM EDTA, and 5 mM MgCl_2). Keep solution on ice and protect from light. Additionally vortex thoroughly for 10 min with intervals of 1 min until ANTS and DPX are completely dissolved (*see* Note 8).
3. To generate unilamellar liposomes, sonicate the lipid film using an ultrasonic water bath sonicator for 10 min (*see* Notes 9 and 10).
4. In order to generate liposomes of a uniform size, the hydrated lipid film is extruded through a filter with 0.1 μm pore size. Assemble the extruder according to the manufacturer’s guidelines (*see* Note 11). Once the extruder is properly assembled, extrude lipid solution 11 times (*see* Note 12).
5. Next, set up a 10 mL bed volume Sepharose CL2B size-exclusion column. This step allows you to separate excess ANTS and DPX in solution from ANTS/DPX encapsulated liposomes. Wash with three column volumes of liposome buffer for three times until all the buffer has run out and the flow has stopped.
6. Carefully add the extruded ANTS/DPX liposome solution to the column and collect flow through as your first fraction (approximately 1 mL); cap the column once the flow has stopped (*see* Note 13).
7. Apply 6 mL of liposome buffer to the column and collect fractions (1 mL each) in glass tubes (*see* Note 13). Liposomes will elute in fractions 4 and 5 (*see* Note

⁵ Lipid film can be stored under vacuum overnight at room temperature if experiment is to be performed on the next day.

⁶ Dried lipid films can be stored for up to 2 weeks at -20°C .

⁷ Equilibrate ANTS and DPX to room temperature before use.

⁸ Store tube on ice between vortexing and protect from light. As you vortex lipid will spontaneously form lipid bilayer vesicles. However, these vesicles are multilamellar and with a size distribution that is not homogenous.

⁹ Ensure that water in bath sonicator does not heat up higher than 37°C . The lipid solution should be completely soluble following water bath sonication.

¹⁰ Alternatively, you can set up a liquid nitrogen bath and water bath. To generate unilamellar liposomes using this method, perform freeze–thaw cycles on liquid nitrogen and warm water ten times.

¹¹ Assemble the extruder according to the manufacturer’s guidelines; one filter support on each side and one 0.1 μm pore size membrane between them. All supports and membranes are prewet in liposome buffer. Additionally, syringes are washed three times in assay buffer. To ensure that the setup is correct, pass syringe with liposome buffer through extruder. If no volume is lost, the setup is correct. If leakiness of buffer occurs, reassemble the extruder, making sure that all the parts are properly secured.

¹² While extruding, some “back-pressure” may occur as lipid solution passes through the membrane. Do not push through quickly as this may tear the membrane. Additionally, if lipid solution passes through the membrane this may indicate that the membrane was not properly set up or has been ruptured; replace membrane if necessary.

¹³ Avoid disrupting the beads interface as you add the liposome buffer or ANTS/DPX liposome solution.

14). Combine the two ANTS/DPX liposome-containing fractions (~0.5 mg/mL lipid). Protect from light and store at 4 °C until use.

3.3 Assessing the Stability of ANTS/ DPX Liposome-Containing Fractions

1. Confirm the stability of ANTS/DPX liposomes before running an experiment. Liposome stability may be compromised if any of the steps provided above were not followed accurately (*see* Note 15).
2. Test ANTS/DPX liposomes stability by comparing the fluorescence intensity of ANTS/DPX exposed to liposome buffer alone or liposome buffer supplemented with 0.2% Triton. It is important to assess if ANTS/DPX liposome are stable over time.
3. For control experiment, on a black 96-well plate, add 90 µL of liposome buffer and 10 µL of ANTS/DPX liposome per well (triplicates). Also, on separate wells, add 90 µL of liposome buffer, 10 µL of ANTS/DPX liposome and 2 µL of 10% Triton X-100 (final concentration 0.2%) (*see* Note 16).
4. Next, read the plate at 30 °C for 1 h using a fluorescence plate reader set to excite at 355 nm (5 nm bandwidth) and emission at 520 nm (12 nm bandwidth) (*see* Note 17).
5. If ANTS/DPX liposomes are stable, there is a four- to fivefold increase in fluorescence between control (no Triton) and burst (Triton) samples over time (*see* Note 18).

3.4 tBID Induced BAX-Mediated Permeabilization in Liposomes

1. To study the permeabilization induced by BAX upon tBID activation ANTS/DPX liposomes are assayed in a 96-well format using a black plate. The total volume per reaction (well) is 100 µL where 10 µL corresponds to ANTS/DPX liposomes (*see* Notes 19 and 20).
2. Next, add 10 µL of a 10× solution of your desired BAX concentration and 10 µL of a 10× solution of your desired tBID concentration. For recombinant full length BAX a concentration ranging from 100 nM to 500 nM is sufficient to detect liposome permeabilization induced after tBID activation. 20 nM tBID and higher concentrations are sufficient to induce BAX activation (*see* Notes 21–23).

¹⁴ Liposome-containing fractions are identified by the slight cloudy appearance due to light scattering by the liposomes. If held against a black background this cloudiness in liposome-containing fractions is easily identified.

¹⁵ Avoid exposing ANTS/DPX liposome-containing fractions to any detergent or Triton. Detergent exposure results in complete disruption of liposomes.

¹⁶ Triton is used to disrupt ANTS/DPX liposome. Alternatively a solution containing 0.5% CHAPS can be used to determine the maximum amount of ANTS/DPX liposomes per assay.

¹⁷ This is the setup for the TECAN M1000 Pro fluorescence plate reader.

¹⁸ Triton disrupts ANT/DPX liposomes during which ANTS (fluorophore) and DPX (quencher) diffuse apart leading to an increase in fluorescence.

¹⁹ You can also set up the assay in a 384-well plate format using 5 µL of ANTS/DPX liposomes.

²⁰ It is important to always incorporate a control for liposomal stability and a control for maximal liposomal release. In the case of maximal liposomal release, samples containing liposomes with 0.2% Triton are used. Liposomes alone or liposomes with vehicle such as DMSO can be used to monitor liposomal stability.

3. Read fluorescence emission of ANTS using a fluorescence plate reader set to excite at 355 nm (5 nm bandwidth) and emission at 520 nm (12 nm bandwidth). Fluorescence emission is recorded every minute for 2.5 h at room temperature (see Notes 17 and 24).
4. After 2.5 h, remove plate from plate reader and add 2 μ L of 10% Triton and read plate again for 10 min every minute. Triton is added to record the maximal liposomal release per well.
5. To normalize the raw data, the percentage release of ANTS/ DPX at every minute is calculated as percentage of fluorescence emission = $((F - F_0)/(F_{100} - F_0)) \times 100$, where F_0 corresponds to the fluorescence intensity at 1 min and F_{100} to maximal fluorescence from Triton treatment and F to the fluorescence intensity at a given time. The normalized data is either plotted as kinetic study (Fig. 1a) or as a single time point (Fig. 1b).

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²¹BAX is able to autoactivate and form oligomers at higher concentrations. It is important to titrate BAX at various concentrations and determine the concentration when no permeabilization is detected in the absence of an activator. The assay can also be adapted for use with other multidomain proapoptotic proteins such as BAK. Similarly, BAK should be titrated to determine the concentration when no permeabilization is detected in the absence of an activator.

²³The liposomal permeabilization assay has been adapted to identify small molecules that modulate the function of multidomain BCL-2 family members. When assaying small molecules, a concentration of the small molecule that does not disrupt ANTS/DPX liposome is determined empirically. Additionally small molecules with fluorescence properties can quench or alter the ANTS fluorescence signal. Proper controls should aim to determine the lowest concentration of small molecule that does not interfere with the assay.

²⁴Fluorescence emission can also be recorded as a single time point after 15, 30, or 60 min.

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Fig. 1. Kinetic (a) or single-time point (b) study of the permeabilization of ANTS/ DPX liposomes upon tBID-induced BAX activation.

Table 1 Composition of lipids for preparation of liposomes (*see* Note 1))

Lipid	Mole %	Chloroform stock (mg/mL)	Volume f mg lipid or 1 film (μL)
POPC	48	25 mg/mL	18.3
POPE	28	25 mg/mL	10.1
PI	10	10 mg/mL	11.7
DOPS	10	10 mg/mL	10.2
TOCL	4	10 mg/mL	7.5