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Isolation of Synthetic Antibodies Against BCL-2-Associated X Protein (BAX)

Zhou Dai¹, Jonathan R. Lai²

¹Department of Biochemistry, Albert Einstein College of Medicine, Bronx, NY, USA

²Department of Biochemistry, Albert Einstein College of Medicine, Bronx, NY, USA

Abstract

The BCL-2 protein family plays central roles in the mitochondrial pathway of cell apoptosis. The BCL-2-Associated X protein (BAX), along with other proapoptotic proteins, induces cell death in response to a variety of stress stimuli. Upon receipt of killing signals, cytosolic BAX is activated and translocates to mitochondria where it causes mitochondrial outer membrane permeabilization (MOMP) and initiates a series of cellular events that eventually lead to cell destruction. Despite recent progress toward understanding the structure, function, and activation mechanism of BAX, detailed information about how cytosolic BAX can be inhibited is still limited. Here we describe a method of selecting synthetic antibody fragments (Fabs) against BAX using phage display. Synthetic antibodies discovered from the selection have been used as structural probes to gain novel mechanistic details on BAX inhibition. This synthetic antibody selection method could be potentially applied to other BCL-2 proteins.

Keywords

BAX; Apoptosis; Synthetic antibodies; Fab; Phage display; Antibody selection

1 Introduction

Cell apoptosis is essential in maintaining normal tissue homeostasis in multicellular organisms; its deregulation in either proapoptotic or antiapoptotic directions contributes to disease states such as cancer and tissue infarction [1, 2]. Decision of cellular life or death at the mitochondrial pathway is delicately controlled by molecular interactions among the BCL-2 family proteins [3, 4]. A complex network of protein–protein interactions between proapoptotic and antiapoptotic BCL-2 proteins regulate the mitochondrial outer membrane permeabilization (MOMP) process, a key event during the mitochondrial pathway of cell apoptosis [5, 6].

Extensive investigations have yielded significant information about the structure, function and regulation mechanisms of BAX. BAX locates predominantly in cytosol as a monomer and its apoptotic activity is inhibited by antiapoptotic BCL-2 proteins such as BCL-2, BCL-

X_L, and MCL-1 [7, 8]. Upon activation under cellular stress, BAX undergoes a series of conformational changes that lead to its oligomerization and insertion into the mitochondrial membrane to trigger the MOMP process [5, 6]. The resulting release of cytochrome *c*, caspases, and other apoptotic proteins into cytosol leads ultimately to cell death. Despite advances in understanding the BAX activation process, we still lack complete view of the possible BAX conformations in cytosol and in mitochondrial membranes [9–11]. For example, BAX changes its conformation and oligomeric state during the apoptotic signal cascade from cytosolic monomer to oligomer, and eventually to membrane-associated proapoptotic complex [10, 12, 13]. Molecular mechanisms of how BAX maintains various conformations and interacts with other proapoptotic and antiapoptotic BCL-2 proteins in those distinct structural and oligomeric states require further elucidation. Such mechanistic details are prerequisite for developing effective pharmacological approaches to modulate cell death in diseases with either excess of cell survival or loss of vital cells [6].

Antibodies have been commonly used as structural probes to gain molecular insights into proteins of therapeutic interests. For example, a human immunodeficiency virus type 1 (HIV-1) envelope protein complex was captured in a mature “closed” state by two antibodies and the solved X-ray structure shed new light on immune recognition and immune evasion of the virus [14]. Synthetic antibody discovery utilizes diversity targeted to the antibody complementarity determining regions (CDRs) that are encoded by rationally designed synthetic oligonucleotides and screened by surface display techniques. Since library construction and antibody selection are performed in vitro, size and diversity of antibody library, state of target molecule, and stringency of selection can be specifically controlled throughout the antibody discovery process [15–18]. Here, we describe a phage display method to discover novel synthetic antibody fragments that selectively target BAX (Fig. 1). Identified synthetic Fabs bind to monomeric BAX with nanomolar affinity and inhibit BAX activation through a novel mechanism, providing new tools for investigating BAX structure and function [19]. We expect that the method described here can also be utilized to discover synthetic antibodies targeting other BCL-2 family proteins for structural analysis and/or biomedical applications.

2 Materials

2.1 Phage Selection with Biopanning

1. Monomeric wild-type, full-length BAX was expressed and purified as described previously [19].
2. Phosphate-buffered saline (PBS), pH 7.0.
3. 96-well EIA/RIA plate (Corning Incorporated, Corning, NY).
4. 5% nonfat dry milk in PBS.
5. 100 mM glycine, pH 2.0.
6. 2 M Tris buffer, pH 8.0.

2.2 Phage Population Amplification, Purification, and Titration

1. XL1 Blue cells (Stratagene, La Jolla, CA).
2. 2× YT medium: add 10 g yeast extract, 16 g tryptone and 5 g Purification, NaCl in water to make 1 L medium. Then adjust pH to 7.0 and Titration with NaOH.
3. M13K07 helper phage (NEB, Ipswich, MA).
4. Tetracycline: 5 µg/mL.
5. Carbenicillin: 50 µg/mL.
6. Kanamycin: 25 µg/mL.
7. PEG/NaCl: 20% PEG-8000 (w/v) and 2.5 M NaCl.
8. 10% (v/v) ultrapure glycerol.
9. PBS with 5% BSA and 0.05% Tween 20.
10. Nonbinding 96-well plate.
11. LB carbenicillin agar plate.
12. 250 mL autoclaved, baffled flask.

3 Methods

3.1 Phage Selection with Biopanning (See Note 1)

1. Add 1 µg BAX per well (in 100 µL phosphate-buffered saline, PBS, pH 7.0) to ~10 wells on a 96-well EIA/RIA plate. Incubate at room temperature for 2 h or at 4 °C for overnight. Also include a control well that contains 100 µL PBS (*see* Notes 2 and 3).
2. Block all wells with 5% nonfat dry milk in PBS (200 µL per well). Shake at 200 rpm at room temperature for 1.5 h.
3. Wash all wells 2–3 times with PBS (*see* Note 4).
4. Add phage solutions (200 µL per well) to all wells. Shake at 200 rpm at room temperature for 1–1.5 h.
5. Wash all wells 5–6 times with PBS.

¹When sorting for BAX specific Fabs, phage pools representing a phage-displayed synthetic antibody library (Library F) were cycled through several rounds of binding selection with BAX immobilized on 96-well EIA/RIA plate as the capture target. The number of selection rounds needed is usually determined based on the output and input phage population for a particular round. Increase in the ratio of output phage titer/input phage titer through successive rounds suggests enrichment of select phage-displayed antibody clones, implying identification of specific target binders.

²BAX protein is immobilized either through direct binding to the plate, or through biotinylation and subsequent binding to streptavidin-coated plate. BAX biotinylation can be carried out using the EZ-Link[®] Maleimide-PEG2-Biotin Kit (Thermo Scientific, Pierce Biotechnology, Rockford, IL) following vendor instructions.

³To increase the possibility of identifying binders, the amount of immobilized target during selection, especially for initial rounds, can be increased (e.g., 5 or 10 µg target per well may be used for first or second round of selection if necessary). On the contrary, to increase selection stringency, it is common practice to lower the amount of immobilized target during later rounds of selection (e.g., 0.5 or 0.1 µg target per well may be used for third or fourth round of selection if necessary).

⁴The wash step after blocking should not be harsh to avoid loss of target in the wells.

6. Add 100 μ L/well of 100 mM glycine, pH 2.0 to all wells. Shake at 200 rpm at room temperature for 5 min (*see Note 5*). Pipette out and combine elution solution from wells immobilized with BAX, and add 30 μ L 2 M Tris buffer (pH 8.0) per 100 μ L elution of to neutralize. The collected phage population is commonly referred to as “output” for this round of selection. Also add 30 μ L 2 M Tris buffer, pH 8.0 to the control well to neutralize the elution (*see Note 6*).

3.2 Phage Population Amplification and Purification

1. Grow XL1 Blue cells at 37 °C in 25 mL 2 $\times$ YT medium (with 20 μ L tetracycline) in a 250 mL autoclaved, baffled flask to OD₆₀₀ = 0.6–0.8 (*see Note 7*).
2. Transfer 5 mL of XL1 Blue cells into a culture tube and infect with half of the output phage population. Shake at 200 rpm in a 37 °C incubator for 1 h.
3. Add M13K07 helper phage (10¹⁰ PFU/mL) to the culture. Shake at 200 rpm in a 37 °C incubator for 1 h.
4. Add the whole culture mixture to 20 mL 2 $\times$ YT medium with 20 μ L carbenicillin (selecting for library phage) and 20 μ L of kanamycin (selecting for K07 phage) in a 250 mL autoclaved, baffled flask. Shake at 200 rpm in a 37 °C incubator for overnight (16–20 h).
5. Pour the overnight culture into a 50 mL conical tube and centrifuge at 4000 rpm (2800 $\times g$) for 20 min. Amplified phage will be in the supernatant.
6. Pour the supernatant into centrifuge tubes and add PEG/NaCl solution to precipitate phage. Incubate the mixture on ice for at least 20 min (*see Note 8*).
7. Centrifuge the mixture at 14,000 rpm (23,400 $\times g$) for 20 min. The phage pellet should be apparent on side of the centrifuge tubes (*see Note 9*).
8. Carefully pour off supernatant without disturbing phage pellet. Resuspend phage in 1.5 mL PBS with 5% BSA and 0.05% Tween 20.
9. Centrifuge resuspended phage at 8000 rpm (7600 $\times g$) for 5 min to remove cell debris.
10. Collect phage in a clean Eppendorf tube and use for the next round of selection. This phage population is referred to as “input” for the subsequent round of selection (*see Note 10*).

⁵Incubation of bound phage in 100 mM glycine, pH 2.0 should not be longer than 10 min to avoid damage to phage population.

⁶It is good practice to check the pH with pH paper after adding 2 M Tris buffer to neutralize the 100 mM glycine, pH 2.0 elution solution.

⁷It is important that XL1 Blue cells used for infection are at log phase with OD₆₀₀ = 0.6–0.8. Cell doubling time is usually ~20 min, but could vary with different growing environments.

⁸At the phage precipitation step using PEG/NaCl solution, incubation time on ice could be increased (to up to 2 h if necessary) to improve phage recovery.

⁹After phage precipitation and centrifugation, take care not to disturb phage pellet which might be only loosely attached to centrifuge tubes.

¹⁰Precipitation-purified phage population can be stored at 4 °C for several days during selection. For long-term storage, phage libraries should be stored at 80 °C with 10% glycerol.

3.3 Phage Titration

1. Grow XL1 Blue cells at 37 °C in 25 mL 2× YT medium (with 20 µL tetracycline) in a 250 mL autoclaved, baffled flask to OD₆₀₀ = 0.6–0.8 (*see Note 11*).
2. Add 90 µL 2× YT medium to one row and 90 µL XL1 Blue cell culture to another row on a nonbinding 96-well plate (*see Note 12*).
3. Add 10 µL of phage output solution into the first well containing 2× YT medium, and perform 10 serial dilution of phage solution by adding 10 µL solution to the following well with 2× YT medium (*see Note 13*).
4. Transfer 10 µL of all phage dilutions in 2× YT medium to the wells containing XL1 Blue cells.
5. Incubate the plate at 37 °C for 1 h.
6. Plate 5 µL transfected cell culture onto a carbenicillin agar plate and incubate the plate overnight (14–20 h) at 37 °C.
7. Calculate phage titer based on the number of colonies from wells with certain dilution folds.

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¹¹One round of selection can be typically performed in 1 day, and the same XL1 Blue cell culture could be used for both phage amplification and titration.

¹²Be sure to use nonbinding 96-well plates for the phage dilution and titration, not high-binding EIA/RIA plates that are used for selection and ELISA. This will ensure the phage dilutions can be mixed properly and there is no adherence of the phage to the well.

¹³To prepare phage serial dilutions for titration, it is critical to change tips after each transfer. Carryover contamination could lead to abnormal or inaccurate estimation on phage population.

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Fig. 1.

The antibody phage library is produced using designed oligonucleotides. The library is then exposed to an immobilized target antigen. Nonbinding phages are washed away, and clones bearing affinity for the target are eluted. The population of binding clones is amplified and used in successive rounds of selective enrichment to enhance the population for high affinity binders.