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Redesigning apoptotic regulator Bid for thrombin activation

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The apoptotic regulator Bid is a key member of the BH3-only protein family, through which the internal and external apoptotic pathways intercept. In cells, Bid is activated by proteolytic cleavage with caspase 9, which results in the formation of its truncated form tBid. In turn, tBid activates pro-apoptotic members of the Bcl-2 family, such as BAX, leading to the permeation of the outer mitochondrial membrane, considered to be a point of no return in apoptotic programmed cell death. Deciphering the molecular mechanism of this process requires biophysical studies using *in vitro* models. To amend the latter studies, we present here a redesigned version of Bid, in which the caspase cleavage site was replaced with a thrombin-specific cleavage site. We confirm that the tBid generated by thrombin cleavage has functional activity and can target BAX to mitochondria-like membrane causing their permeabilization. Using a combination of FRET spectroscopy with a site-selective fluorescent label we have probed the membrane-induced separation of Bid fragments after thrombin cleavage. We demonstrate that the latter dissociation and tBid-dependent activation of BAX are modulated by the lipid composition of target membranes, specifically by the presence of cardiolipin, which promotes both processes.

K e y w o r d s: apoptotic regulators; Bid; BAX; cardiolipin; Förster resonance energy transfer (FRET); membrane permeabilization.

Introduction

Mitochondria-driven apoptosis is a programmed cell death pathway, regulated by the numerous members of the Bcl-2 family [1, 2]. The permeabilization of the outer mitochondrial membrane by BAX is considered a point of no return in apoptotic regulation. The activation of BAX, in turn, depends on the cleavage of the BH3-only protein Bid with caspase 9, resulting in the active truncated Bid (tBid) and p7 fragments (Fig. 1) [3, 4]. In this

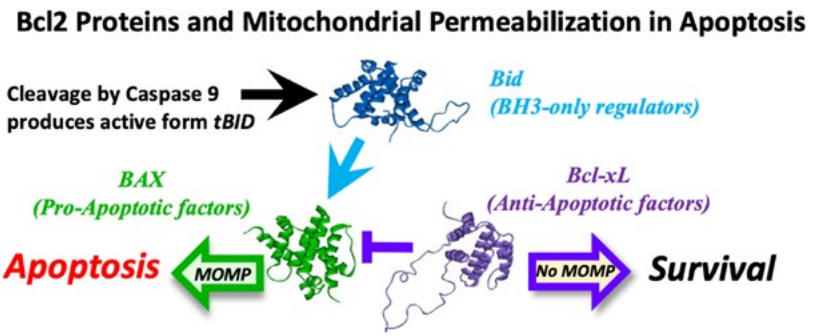

Fig. 1. Simplified scheme of mitochondria-dependent apoptotic regulation. The key role of tBid cleavage by caspase 9 as a central event in the apoptosis cascade activation is underlined.

work, we suggest introducing a thrombin cleavage site in Bid, which yields the same product as the one obtained by caspase 9 cleavage. Redesigning Bid sequence for thrombin activation has several advantages for future biophysical studies of apoptotic regulation in model systems. Highly active thrombin protease is at the same time highly specific and enables Bid cleavage in an hour at low enzyme-protein ratios. Additionally, thrombin is much more accessible than the recombinant caspase 9. The resulting tBid activity was confirmed in the leakage assays where BAX was successfully activated by tBid, leading to the permeabilization of large unilamellar vesicles (LUV) with a preference towards cardiolipin-containing vesicles [5]. We use FRET measurements to demonstrate that tBid and p7 segments, resulted from the cleavage by thrombin, remain associated in solution and dissociated only in the presence of membranes exactly as they do in case of caspase 9 dependent cleavage [6, 7].

Materials and Methods

Materials

Phospholipids used in this study: Palmitoyl-oleoyl-phosphatidylcholine and phosphatidylglycerol (POPC and POPG), as well as tetraoleoyl-cardiolipin (TOCL) were purchased from Avanti Polar Lipids (Alabaster, AL). Fluorescent dyes AEDANS (5-aminoethylnaphthalene-1-sulfonic acid), ANTS (8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt) and the quencher DPX (p-xylene-bis-pyridinium bromide) were from Molecular Probes (Eugene, OR). Thrombin protease was from ThermoFisher Scientific (Hannover Park, IL).

Cloning, expression and purification of BAX and Bid

BAX and Bid proteins were cloned into a pET28b plasmid as described in our previous papers [8, 9]. For the expression of each protein, a single colony of E. coli BL21DEpLysS "Rosetta" was transformed with the corresponding plasmid, inoculated into 5 mL of LB medium supplied with Kanamycin (50 mg/mL) and grown overnight at 37 ºC. Next morning, the bacterial culture was added to 500 mL of LB-Kanamycin and grown at 37 ºC to OD_{600} = 0.6-0.7. At this density, the cells were induced with IPTG (0.8 mM for Bid, or 0.25 mM for BAX). Bid expressing cells were then grown overnight at 24 ºC while BAX expression was performed for 24 h at 20 ºC.

The cells were then harvested and sedimented (5000 g, 15 min) and stored at –20 ºC until protein purification. For protein extraction, the cell pellets were thawed in binding buffer (50 mM Tris-HCl, 300 mM NaCl, 5 mM imidazole, pH 8) with addition of 1 mM PMSF and sonicated. Lysates were clarified by centrifugation (10000g, 30 min), 1 mL of Ni-NTA agarose was then added to the lysate and incubated overnight on a rotator. Next morning the resin was sedimented by brief centrifugation and washed 3 times with the binding buffer. The bound protein was eluted with 0.5 M imidazole in binding buffer. Further purification was performed by gel-filtration on a Superose 12 column in 50 mM HEPES, 100 mM NaCl, pH 7.4 buffer using an AKTA Pure FPLC device. Runs were done at 0.5 mL/min, both Bid and BAX eluted at around 12 mL retention volume. The purity of preparation was inspected by SDS-PAGE, and quantification was performed by absorption measurements at 280 nm using a molar extinction coefficient of 36 000 for BAX and 7000 M^{-1} for Bid [8, 10]. To avoid the nonproductive cleavage of BAX, we have removed the naturally occurring thrombin cleavage site by substituting arginine in position 9 with lysine.

Site-directed mutagenesis

A Q81C mutation was introduced into Bid for site-specific labelling with an AEDANS maleimide dye derivative. Prior to the preparation of the desired mutation, we substituted three native Cysteines in Bid (Cys to Ser substitutions). The naturally occurring thrombin cleavage site in BAX at position 9 (Pro-Arg-Gly) was also eliminated by introducing an R-to-K substitution at the cleavage site. Mutations followed the QuickChange mutagenesis protocol (Stratagene, CA).

Thrombin cleavage

Thrombin digestion was performed in 50 mM sodium-phosphate buffer, pH 7.4 for 1 hour at room temperature using an enzyme:protein ratio of 1:250. Reaction completion was checked by SDS-PAGE in 4–20 % gradient Tris-HCl gels from Bio Rad (Hercules, CA).

Membrane preparation and leakage assay

Large unilamellar vesicles (LUV) were prepared as described in [11, 12] by mixing the appropriate amounts of phospholipids (molar ratios) followed by drying under nitrogen and overnight drying by SpeedVac. The resulting films were resuspended in 50 mM phosphate buffer, 100 mM NaCl, pH 7.4 and extruded 15–20 times through 100 nm polycarbonate filters (Whatman). For leakage assays, the dried phospholipids were resuspended in the same buffer with the addition of 1 mM of the fluorescent dye ANTS and 10 mM of the quencher DPX. The leakage of ANTS/DPX loaded vesicles was conducted in 50 mM phosphate buffer, pH 7.4 at room temperature. Fluorescence was recorded on a SPEX Fluorolog FL3-22 steady-state fluorescence spectrometer (Jobin Yvon, Edison, NJ) equipped with double-grating excitation and emission monochromators. Measurements were performed in a 2x10 mm cuvette oriented perpendicular to the excitation beam. For the kinetic recording of ANTS/DPX release we used the following settings: Excitation 353 nm, emission 520 nm, excitation/emission slits 2/14, respectively. The permeabilization reac-

tion was completed by the addition of 20 μL 20 % Triton-X100, allowing the determination of the signal associated with 100 % content release [13].

FRET measurements

A single Cys Q81C Bid mutant was prepared to perform FRET measurements between the Trp59 donor and AEDANS labeled Q81C as an acceptor. This required the substitution of three native Cys residues in Bid for Ser. Fluorescence measurements were performed using a SPEX Fluorolog FL3-22 steady-state fluorescence spectrometer (Jobin Yvon, Edison, NJ) equipped with double-grating excitation and emission monochromators. We used the following settings for recording excitation spectra: Emission 470 nm, excitation from 270 to 420 nm, and slits 2/5. To ensure the high quality of spectra we recorded and averaged 5 runs.

Circular Dichroism (CD) measurements

CD spectra were recorded using an upgraded Jasco-720 spectropolarimeter (Japan Spectroscopic Company, Tokyo, Japan). Up to 120 scans were run from 260 to 190 nm using a sample containing 4-8 μM of WT or mutant Bid in 20 mM HEPES buffer, pH7.4 in 1-mm optical path cuvette. The background (buffer only) was subtracted from each spectrum.

Results and Discussion

The substitution of the naturally occurring caspase 9 cleavage site in Bid for that selective for thrombin, required the replacements of Thr-Asp pair with Pro-Arg pair (Fig. 2, top). This amounts to total of only three base pair changes in the corresponding DNA sequence (**ACCGAC** to **CCCCGC**), allowing for a single step mutagenesis using primers containing all the desired substitutions. The expression and purification of mutant Bid was performed under the same conditions as those for the WT and no difference in the yield of the two proteins was observed. We have carried out thrombin cleavage of mutant Bid in 50 mM sodium-phosphate buffer, pH 7.4 at room temperature for one hour incubation using varying enzyme-to-substrate ratios (from 1:500 to 1:63) and found the reaction to be completed with the 1:250 ratio (Fig. 2, lower panel).

The published results from several laboratories indicate that after cleavage of Bid with

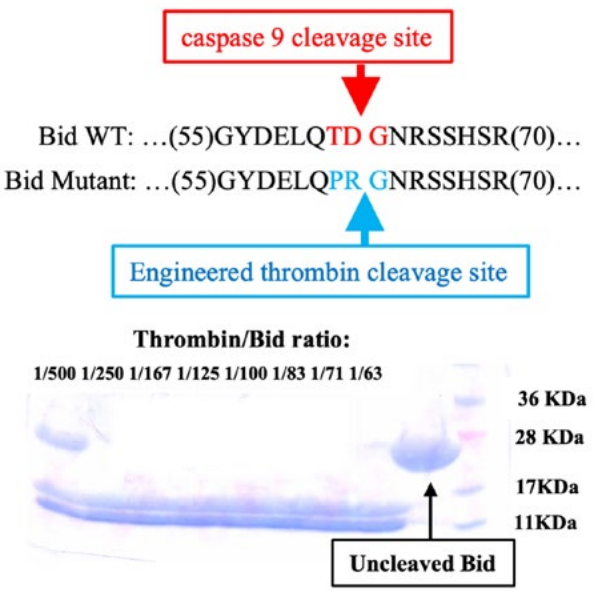

Fig. 2. Alterations to Bid sequence to allow thrombin cleavage. Mutations introduced in Bid to create thrombin site (blue) instead of caspase 9 site (red). Electrophoresis of the cleavage products of Bid indicates that starting from 1:250 enzyme:protein ratio the cleavage is complete in 1 hour at room temperature. The uncleaved Bid appears as a band of 25 kDa, with the cleavage products appearing at the bottom of the gel.

caspase 9, the p7 and tBid segments remain associated until further activation events, such as the membrane interaction of Bid [14, 15]. To determine if the thrombin-cleaved Bid behaves in a similar fashion, we have designed a FRET experiment with donor and acceptor located in the two peptides obtained in cleavage, tBid and p7. We used Bid's only tryptophan W53, located in p7 as the donor, and AEDANS, selectively attached to Q81C Bid mutant, as the acceptor in the context of Cys-less Bid. In order to confirm that replacements of the three native cysteines with serines does not affect the structure of Bid, we have performed CD measurements of the two proteins. Comparison of the CD spectra of WT and Cys-less Bid demonstrates no difference in secondary structure (Fig. 2), which is not unexpected, since cysteines in the WT Bid do not form disulfide bridges.

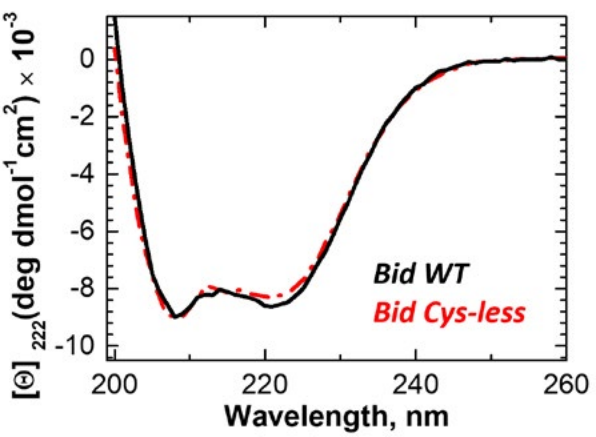

Fig. 3. CD spectra of the Bid WT (solid black line) and its Cys-less mutant (red dashed line). Coinciding spectra indicate that substitution of C5, C17 and C30 with serines does not affect the conformation of Bid.

The distance between the residues in the folded Bid is approximately 17 Å (Fig. 4A), which is sufficiently shorter than the typical

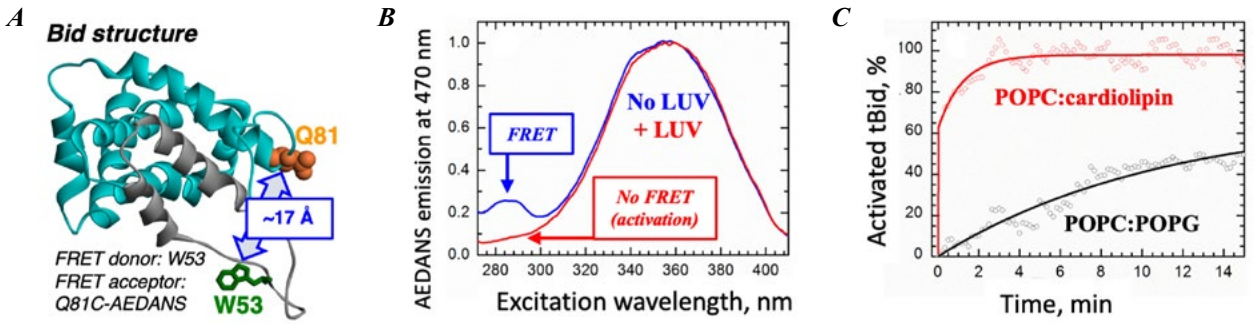

Fig. 4. FRET study of membrane-dependent disassociation of thrombin cleaved Bid fragments. *A* — NMR-derived structure of Bid (PDB ID 2BID [18]) with p7 sequence (residues 1-63) shown in grey and tBid sequence (residues 64-195) shown in cyan. W53 (FRET donor) is shown as green; Q81 (which is mutated to cysteine for subsequent attachment of acceptor AEDANS) is shown in orange. *B* — Excitation spectra of AEDANS-labelled thrombin-cleaved Bid in the absence (blue) and presence of POPC:cardiolipin LUV (red). FRET-associated donor peak at 280 nm is present for Bid in solution and disappears after the addition of LUV, indicating the activation via the dissociation of p7 and tBid. *C* — Time course of the activation of thrombin-cleaved tBid upon addition of LUV of two compositions measured by FRET. Activation of Bid is observed in both LUV lipid compositions, but in cardiolipin-containing membranes the rate of activation is significantly higher. Data for POPC:POPG LUV were fitted to a single kinetic rate (lower solid line) with k=1.5 10^{-3} s^{-1}. Data for POPC:cardiolipin LUV were fitted by a combination of the initial ultrafast increase (k>0.1 s^{-1}) followed by an exponential increase with k=1.4 10^{-2} s^{-1} (upper solid line).

Trp-to-AEDANS Förster distance of 25 Å, insuring the efficient energy transfer. Indeed, the excitation spectra of AEDANS-labeled Q81C mutant of Bid measured for the AEDANS emission band (Fig. 4B), contain a characteristic FRET peak at 280 nm corresponding to Trp (e.g., compare to the results of a similar experiment for diphtheria toxin translocation domain [16]. Note, that similarly to caspase-cleaved WT Bid [4, 17] the thrombin-cleaved Bid (a) retains the association of the two cleavage products in solution (Fig. 4B, blue, FRET peak present) and (b) is dissociated upon addition of the membranes (Fig. 4B, red, FRET peak absent). Confirming the similarity of the thrombin-cleaved version of Bid to that of the caspase 9-cleaved WT Bid (as described above), opens the doors for a systematic biophysical studies of the membrane-dependent activation. The example of such studies is presented in Fig. 4C, where we used the same FRET experimental scheme to study the kinetics of the membrane-induced dissociation of tBid and p7. Our data indicate that the presence of cardiolipin dramatically increases the rate of Bid activation.

The activity of thrombin cleaved Bid was confirmed using a leakage assay where BAX permeabilizes artificial phospholipid membranes upon external activation with cleaved Bid. Release of LUV content (fluorophore: ANTS, quencher: DPX) leads to an increase of fluorescence signal due to quencher dilution in solution, allowing the quantification of sample permeabilization. A mixture of LUV, native Bid and BAX was incubated for 5–7 minutes until the fluorescence baseline stabilized. Thrombin was then added in a 1:250 ratio to Bid which guaranteed full cleavage of Bid.

The following two LUV compositions were used for these experiments: 1POPG:2POPC and 1cardiolipin:2POPC. The lipid to BAX ratio was 500:1 and BAX to Bid ratio was 1:2. After addition with thrombin Bid recruits BAX to the membrane, where the latter forms large pores. The release of the vesicular content via BAX pores can be monitored by the increase of fluorescence due to the dilution of co-encapsulated ANTS/DPX markers (No changes in fluorescence were observed if either BAX, Bid or thrombin were present). As shown in Fig. 5, a maximal leakage of content of 20 % and 60 % was observed from LUV made of

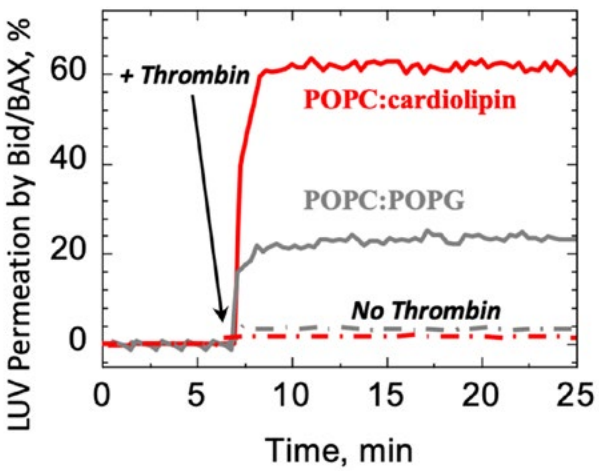

Fig. 5. Measurements of the activity of thrombin-cleaved Bid. Leakage of LUV contents caused by BAX after tBid activation. The experiment was performed using POPC:POPG and POPC:cardiolipin LUV. The components (BAX, Bid and LUV) were premixed and thrombin was added at 7 min incubation timepoint. In the absence of thrombin no pore-forming activity was observed in both LUV compositions (gray for POPC:POPG and red for POPC:cardiolipin LUV, dash-dot lanes). Upon thrombin addition the leakage occurred in both LUV types, in cardiolipin-containing vesicles it was significantly higher at the same BAX:Bid ratio (red versus grey lane).

1POPG:2POPC and 1cardiolipin:2POPC lipid mixtures, respectively. This result is consistent with the higher levels of activation of Bid in the presence of cardiolipin (Fig. 4C).

Conclusions

We conclude that redesigning Bid by introducing a thrombin site instead of the canonical caspase 9 one does not affect Bid-mediated BAX activation. Specifically, the thrombin-cleaved Bid retains its compact structure as seen by FRET experiments, which is only perturbed by interactions with model membranes (Fig. 4B). The rate of the separation of the thrombin-cleaved Bid into two fragments (Fig. 4C) as well as its ability to activate pro-apoptotic factor BAX (Fig. 5), is promoted by the presence of cardiolipin in the target membrane.

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Редизайн регулятора апоптозу Bid для активації тромбіном

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Регулятор апоптозу Bid є ключовим учасником родини білків BH3-only, через який перетинаються зовнішні та внутрішні шляхи апоптозу. У клітині Bid може бути активований розщепленням каспазою 9 у результаті якого формується його усічена форма — tBid. У свою чергу tBid активує інших про-апоптотичних членів родини Bcl-2 таких як BAX, що призводить до формування пор у зовнішній мітохондріальній мембрані, що вважається точкою неповернення у апоптозі — програмованій загибелі клітин. З'ясування молекулярних механізмів цього процесу вимагає біофізичних досліджень з використанням *in vitro* моделей. В цій роботі ми презентуємо перебудовану версію Bid, у якій сайт розщеплення каспази 9 змінено на сайт розщеплення специфічний до тромбіну шляхом сайт-направленого мутагенезу. Підтверджено, що tBid, як продукт протеолізу тромбіном, є функціональним і здатний активувати BAX, що призводить до утворення пор у модельних мембранах. За допомогою комбінації FRET спектроскопії і сайт-селективного мічення флуоресцентними зондами досліджено дисоціацію фрагментів Bid після розщеплення тромбіном. Також показано, що вказана дисоціація та активація BAX, індукована tBid, залежить від ліпідного складу мембран, зокрема від присутності кардіоліпіну, що прискорює обидва процеси.

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