One Step production and purification of full length pro-apoptotic protein Bax. Reconstitution in nanodiscs

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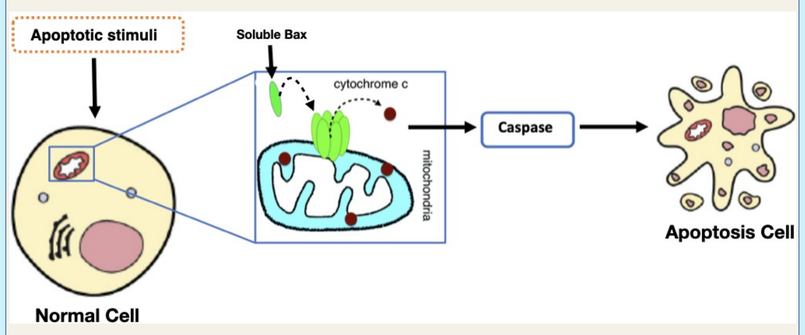
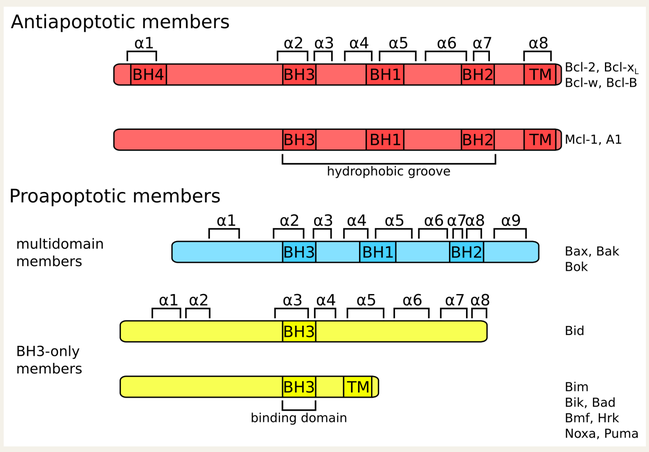
Apoptosis

Apoptosis is the process by which cells initiate self-destruction in response to a death signal, DNA damages induced by cancer therapies for example. These signals activate the mitochondrial pathway of apoptosis, in which the protein Bax is involved.



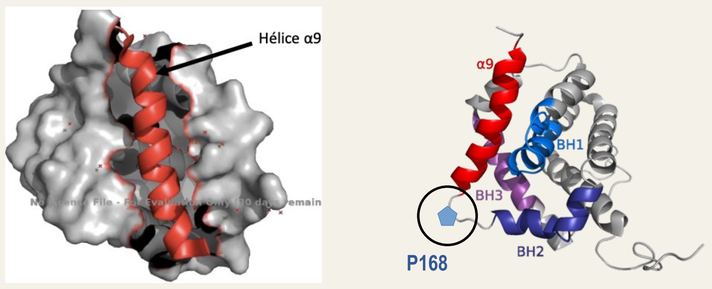
The Bcl-2 family

Bax is member of the Bcl-2 family, that are characterized by 4 domains of homology with Bcl-2, called BH1 to BH4. These domains are essential for the function and regulation of these proteins *via* a network of interactions between the different members. Bcl-2 family proteins are divided into three functional groups: anti-apoptotic proteins (Bcl-2, Bcl-xL...), pro-apoptotic multi-domain proteins (Bax, Bak, and Bok) and BH3-only proteins (Bid, Bad, Puma, Bim…)



Pro-apoptotic protein Bax

Bax is a 21.2kDa protein, structured in 9 α-helices. Under non-apoptotic conditions, hydrophobic α9 helix is stabilized in a groove formed by the rest of the protein, favoring a soluble conformation of the Bax. Under apoptotic conditions, Bax undergoes conformational changes that lead to its insertion and oligomerization in the mitochondrial outer membrane, making it permeable to apoptogenic factors.



It has been shown that the presence of a proline between α8 and α9 helices forms a bend that brings α9 closer to the hydrophobic pocket. A point mutation of this proline to alanine resulted in a Bax mutant (P168A) that is constitutively membrane-bound and active.

Challenges

The structure of soluble Bax is now well known. However, the structure of the membrane-inserted protein remains to be elucidated.

The heterologous expression of Bax in *E.coli* does not yield satisfactory results due to its hydrophobic C-terminus, which has forced investigators to work with a truncated protein.

Because the C- and N-terminal ends of Bax are important for its activation, obtaining the native Bax protein untagged and in a membrane environment remains a major challenge.

We then produced Bax in a cell free protein synthesis system and reconstituted the protein into nanodiscs.

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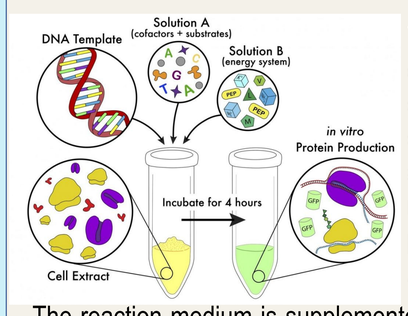
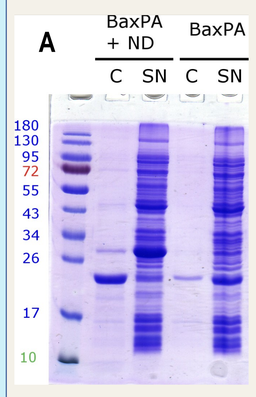
Cell free (CF) protein synthesis



The cell free production is comparable to heterologous expression, but it takes place entirely *in vitro*. The transcription and translation machinery necessary for the production of the protein of interest comes from a *E.coli* lysate.

The reaction medium is supplemented with the essential elements for the synthesis (aminoacids, nucleotides, cofactors, etc..) and an ATP regenerating system. A major advantage of this production mode is that the synthesis of membrane proteins can be done directly in the presence of elements mimicking a membrane environment (liposomes, nanodiscs, bicelles etc).

CF production of Bax in the presence of nanodiscs

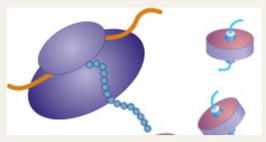


Both Wild-type and P168A mutant were produced *in vitro* in the presence of nanodiscs. The reaction media were centrifuged at 20000xg for 15min.

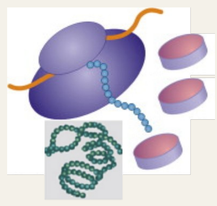
(A) In the absence of nanodiscs, Bax is mostly soluble. Unexpectedly, the addition of nanodiscs leads to the precipitation of more than 80% of the protein. **(B)** Both BaxWT and BaxP168A are precipitated. Furthermore, Bax is almost the only protein in the pellet and contaminants are removed by simply washing the pellet.

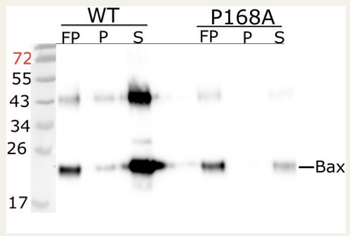
Bax pellet resolubilisation By Brij-58

Expected results



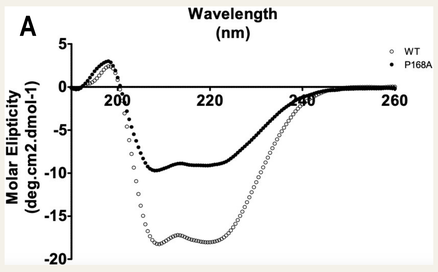
Results obtained



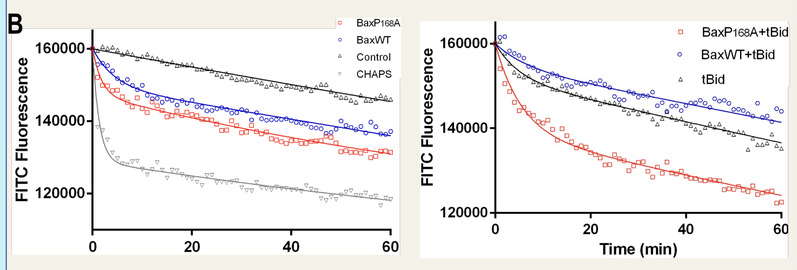


Bax pellet was successfully resolubilised by Brij-58.

Structural and functional characterisation of resolubilised Bax

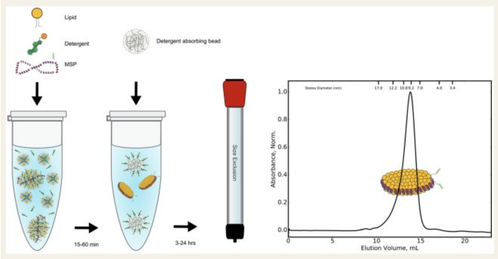


(A) We performed a circular dichroism measurement to check the folding of the protein. The data show that the proteins are correctly folded as α-helices.



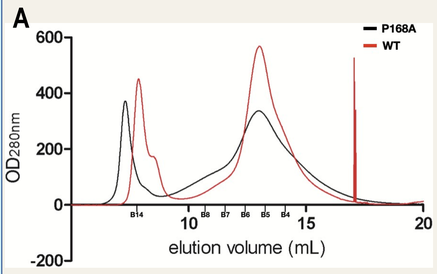
(B) Reconstitution of BaxWT and BaxP168A in liposomes loaded with Dextran-FITC showed that both proteins could form pores with a size compatible with native Bax pores. As expected, BaxP168A was more active than BaxWT, and was further activated by tBid.

Bax Reconstitution in Nanodiscs

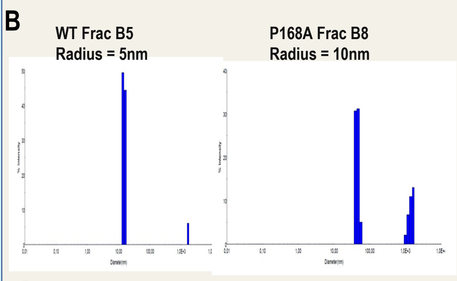


Nanodiscs formation: Dry phospholipids film is solubilized in a buffer containing cholate. The Membrane Scaffold protein (MSP1E3D1) is added and left to equilibrate.

For the reconstitution process, Bax is added at this step. Then the detergents are removed by adding BioBeads. This leads to the formation of phospholipid−MSP1 nanodiscs and possibly some aggregates. The sample is then run over an SEC column to purify the formed nanodiscs.



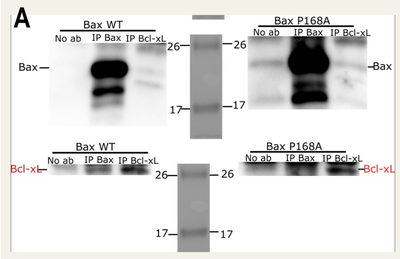
(A) SEC chromatogram of Bax co-formed with nanodiscs. We can notice that there is a more important shoulder on the peak number 2 for Bax P168A compare to Bax WT.



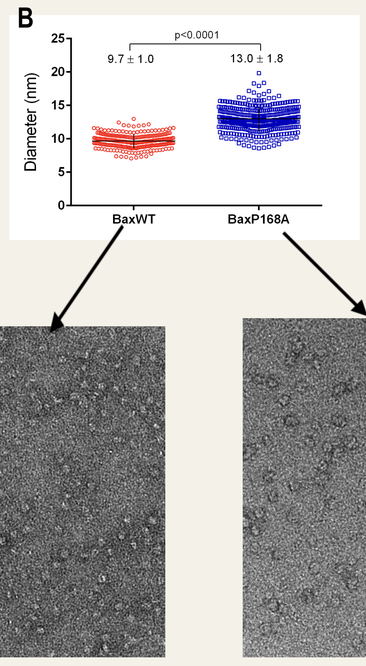
(B) Dynamic Light Scattering (DLS) suggest the presence of larger particles for the mutant P168A than the WT which is consistent with the SEC profiles.

(C) Western Blot shows a difference in elution between BaxWT and mutant P168A. In addition, Bax and MSP1 colocalized. This suggests the presence of Bax in nanodiscs. .

Bax in Nanodiscs



(A) Co-immunoprecipitation of Bax and its main anti-apoptotic partner Bcl-xL. The western blot shows that both Bcl-xL and Bax inmmunoprecipitate each other. Showing that Bax in nanodiscs is under a coformation that allows its interaction with Bcl-xL.



(B) Structural characterization of nanodiscs containing Bax by Transmission Electron Microscopy (TEM). Diameter of each particle was determined manually using Image J. Data show that there is a size difference between nanodiscs containing Bax WT and Bax mutant P168A. Nanodiscs containing Bax WT are around 10nm diameter, which is close to the expected size for empty nanodiscs, while nanodiscs containing the mutant P168A are larger (around 13nm diameter). Theses results are consistent with the previous observations suggesting that mutant P168A is constitutively oligomeric and membrane-inserted.

Perspectives

We plan to use electron microscopy methods to determine the shape of Bax oligomers in nanodiscs.
Fluorescent probes and HDX-MS, combined to site directed mutagenesis, will be use to identified the domains of membrane-inserted Bax involved in the interactions with its partners, namely anti-apoptotic protein Bcl-xL