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Interaction of the full-length Bax protein with biomimetic mitochondrial liposomes: A small-angle neutron scattering and fluorescence study

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ABSTRACT

In response to apoptotic stimuli, the pro-apoptotic protein Bax inserts in the outer mitochondrial membrane, resulting in the formation of pores and the release of several mitochondrial components, and sealing the cell's fate. To study the binding of Bax to membranes, we used an in vitro system consisting of 50 nm diameter liposomes prepared with a lipid composition mimicking that of mitochondrial membranes in which *recombinant* purified full-length Bax was inserted via activation with purified tBid. We detected the association of the protein with the membrane using fluorescence fluctuation methods, and found that it could well be described by an equilibrium between soluble and membrane-bound Bax and that at a high protein-to-liposome ratio the binding seemed to saturate at about 15 Bax proteins per 50 nm diameter liposome. We then obtained structural data for samples in this saturated binding regime using small-angle neutron scattering under different contrast matching conditions. Utilizing a simple model to fit the neutron data, we observed that a significant amount of the protein mass protrudes above the membrane, in contrast to the conjecture that all of the membrane-associated Bax states are umbrella-like. Upon protein binding, we also observed a thinning of the lipid bilayer accompanied by an increase in liposome radius, an effect reminiscent of the action of antimicrobial peptides on membranes.

1. Introduction

Apoptosis is a highly regulated type of programmed cell death essential to the development and maintenance of tissues and to the defense of organisms against pathogens [1]. Many of the known apoptotic pathways involve mitochondrial damage, where permeabilization of the outer mitochondrial membrane results in leakage of a number of apoptotic factors, including cytochrome c, into the cytoplasm [2]. This important step just upstream of caspase activation and cell death is regulated by the Bcl-2 family of proteins [3–5]. Pro-apoptotic members of this family act to promote the release of apoptotic factors from the mitochondrial intermembrane space, while anti-apoptotic members act to inhibit it. For a significant number of Bcl-2 family proteins, this function is mediated by a direct interaction with lipid membranes – some family members, such as Bcl-2, Bcl-w and CED-9 are constitutively bound to membranes, while others significantly bind to membranes only after an activation step, for example cleavage in the case of Bid, and interaction with cleaved Bid in the case of Bax.

Human Bax is a 22 kDa (192 amino acids) pro-apoptotic Bcl-2 family member of particular physiological relevance because it can form pores in mitochondrial membranes [6], and because these pores are thought to be the functional units that allow for cytochrome c to be released during apoptosis [7]. In growing cells, Bax is mainly found in the cytoplasm, but at the onset of apoptosis it migrates to the outer mitochondrial membrane [8,9] where it undergoes a conformational change [7] and assembles into oligomers [10,11]. These events coincide with cytochrome c release [7]. In addition, many in vitro studies have shown that Bax is able to permeabilize lipid membranes [6,12–16]. The pore-forming activity of full-length Bax can be realized using the C-terminal fragment of Bid (noted as tBid for truncated Bid), which causes soluble monomeric Bax to bind to lipid membranes and oligomerize [15–17].

Abbreviations: wtBax, wild-type Bax; tBid, truncated Bid; SANS, small-angle neutron scattering; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; CL, cardiolipin; DOPS, dioleoyl phosphatidylserine; TOCL, tetraoleoyl cardiolipin; EGFP, enhanced green fluorescent protein; FIDA, fluorescence intensity distribution analysis; FCS, fluorescence correlation spectroscopy; SDD, sample-to-detector distance; FWHM, full-width-at-half-maximum; SLD, scattering length density.

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The insertion of Bax into a membrane results in increased membrane conductivity [18], as well as the release of varying size particles [12,15,19,20] due to the formation of lipidic pores [16,21,22]. In contrast, anti-apoptotic Bcl-2 family members interacting with membranes (e.g. Bcl-2, Bcl-xL, Bcl-w, CED-9) do not seem to form pores – at least not large enough to allow for the release of particles such as cytochrome c. Instead they are thought to influence apoptosis through protein–protein interactions [3,5,23].

The soluble form structures of several Bcl-2 family proteins, including family members which are usually associated with membranes but become soluble when truncated, have been solved, e.g. Bcl-xL [24], Bcl-2 [25], Bcl-w [26], Bax [27], Bid [28,29] and CED-9 [30]. Interestingly, they all have a three-dimensional structure that is highly homologous to the soluble form of pore-forming bacterial colicins, and of diphtheria toxin, with a mainly hydrophobic α-helical hairpin (also containing charged residues) buried amongst six or seven amphipathic α-helices [31]. The structural homology displayed by these Bcl-2 family proteins, in their soluble form, is intriguing given the fact that they have diverging functions, namely anti-apoptotic (i.e. Bcl-xL, Bcl-2, Bcl-w, CED-9) or pro-apoptotic (i.e. Bax, Bid). However, the active membrane associated conformation of these proteins is still not well defined. In particular, the conformation(s) adopted by Bax when bound to a membrane is (or are) still unknown.

Even so, there are a few clues available regarding the membrane conformation(s) of Bax. Firstly, Bax can presumably adopt at least two conformations when binding to membranes. The clearest evidence of this is that in healthy cells a small fraction of Bax is found associated with mitochondria but remains carbonate extractable – corresponding to an inactive loosely-bound form of Bax, while in apoptotic cells Bax bound to mitochondrial membranes is no longer carbonate extractable – corresponding to a pore-forming membrane-inserted form of Bax [10,32]. Secondly, it is likely that the α-helical secondary structure of the monomeric soluble protein is conserved when the protein interacts with lipid membranes [17,33]. Thirdly, the Bax C-terminus (helix 9) has been identified as a transmembrane domain, while in vitro studies of single-point Bax mutants [34–36] and peptide studies [37,38] have implicated helices 5 and 6 in the formation of pores. It was later confirmed – by chemical labeling of single cysteine Bax mutants – that all three helices indeed insert in the outer mitochondrial membrane of apoptotic cells [39]. Finally, the Bax N-terminus (including helix 1), which interacts with tBid [7,32,34,35] and contains an epitope (6A7) that becomes accessible only after activation [40], has been shown to interact with lipid membranes [41,42] and is involved in mitochondrial targeting [34,36]. In conclusion, while helices 1, 5, 6 and 9 should be in contact with the membrane in membrane inserted pore-forming Bax, the configuration adopted by the remaining five helices is unknown. Moreover, the conformation(s) adopted by additional membrane bound forms of Bax, in particular by its predicted loosely-bound form, are unknown. Fig. 1 illustrates two possible pathways in which soluble Bax may insert in the membrane. We refer to Bax as being "inserted" in the membrane if one or several of its helices are transmembrane (e.g. conformations C and E in Fig. 1), and as being "peripherally bound" if interacting with the membrane without any transmembrane helices (e.g. conformations B and D in Fig. 1). Both "inserted" and "peripherally bound" Bax are collectively referred to as either being "bound" or "targeted" to the membrane.

Small-angle neutron scattering (SANS) has proven an ideal technique for studying the structure of liposomal membranes [43]. It is also a very promising technique for studying the conformation and quaternary structure of proteins in membranes, as it allows for the detection of large protein assemblies through the use of contrast variation experiments [43,44]. For example, SANS has been successfully used to study membrane oligomers formed by bacteriorhodopsin [44], SecA [45], and pneumolysin [46]. For this study, we have used a model system consisting of full-length recombinant Bax bound to the membranes

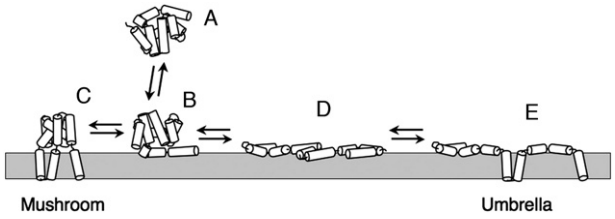

Fig. 1. Schematic representation of two possible pathways for the insertion of Bax into a lipid membrane. The soluble Bax protein (A) is thought to first peripherally associate with the membrane, or with proteins (i.e. tBid or Bax) already inserted in the membrane (B). The peripherally associated Bax protein may then insert into the membrane as follows: (i) it may insert helices 5, 6 and 9 in the lipid bilayer while retaining a globular conformation above the membrane (mushroom conformation, C); (ii) it may adopt an open conformation with all helices roughly parallel to the membrane (D) before inserting helices 5, 6 and 9 in the lipid bilayer (umbrella conformation, E). Interactions with tBid and other Bax molecules, both known to influence the insertion of Bax into the membrane, are not represented here.

of 50 nm diameter liposomes with a composition approximating that of mitochondrial membranes, and we have carried contrast variation SANS experiments to obtain information both on the conformation of Bax when bound to membranes and on the changes in the membrane caused by the binding of the protein. To complement the SANS experiments and support our choice of using small liposomes (made so that higher lipid concentrations and increased signal-to-noise levels could be achieved), we have carried out complementary fluorescence experiments in order to quantify the membrane binding and pore-formation activity of Bax in our model system.

2. Materials and methods

2.1. Liposome preparation

Liposomes were prepared from a mixture of lipids that reflects the overall lipid composition of *Xenopus* mitochondrial membranes, and which has been shown to support pore formation by tBid-activated Bax [15,17], i.e. phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and cardiolipin (CL), with a mass ratio of 48:28:10:10:4. All lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA) either as purified from egg (PC, PE) or bovine liver (PI), or as synthetic lipids in the case of dioleoyl PS (DOPS) and tetraoleoyl CL (TOCL). The lipids were dissolved in chloroform and then mixed in the appropriate mass ratio. For fluorescence fluctuation experiments 0.008% by mass of the fluorescent dye DiD (Invitrogen Canada, Burlington, Canada) dissolved in methanol was added to the lipids. Solvents were then removed by evaporation under a stream of nitrogen gas, followed by incubation under vacuum for 2 h. Dry lipid films were resuspended in assay buffer (10 mM HEPES at pH 7, 200 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA) to a total lipid concentration that varied from 0.5 to 40 g/l. The lipid solutions were then subjected to 10 freeze–thaw cycles and extruded through the pores of a Nucleopore track-etched polycarbonate membrane (Nucleopore, San Diego, CA, USA). Mostly, membranes with 50 nm pores were used, except for a few experiments when membranes with larger pores (100 nm or 200 nm) were used. Although the extruded liposomes are not strictly speaking monodisperse, they do follow a monomodal size distribution with a narrow polydispersity that effectively constitute a homogeneous sample (i.e. spherical unilamellar liposomes). The assay buffer was prepared with the appropriate fraction of H_2O and D_2O (Cambridge Isotopes, Andover, MA). HEPES was from BioShop Canada Inc (Burlington, Ontario, Canada), while all other chemicals were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Estimates for lipid concentrations were obtained by assuming that dry lipid films had been completely resuspended, that the extrusion step had no effect on lipid concentration, and that the average molecular weight of the phospholipids was 770 g/mol [47]. Estimates for