Molecular Details of Bax Activation, Oligomerization, and Membrane Insertion*

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Bax and Bid are pro-apoptotic members of the Bcl-2 protein family. Upon cleavage by caspase-8, Bid activates Bax. Activated Bax inserts into the mitochondrial outer membrane forming oligomers which lead to membrane poration, release of cytochrome c, and apoptosis. The detailed mechanism of Bax activation and the topology and composition of the oligomers are still under debate. Here molecular details of Bax activation and oligomerization were obtained by application of several biophysical techniques, including atomic force microscopy, cryoelectron microscopy, and particularly electron paramagnetic resonance (EPR) spectroscopy performed on spin-labeled Bax. Incubation with detergents, reconstitution, and Bid-triggered insertion into liposomes were found to be effective in inducing Bax oligomerization. Bid was shown to activate Bax independently of the stoichiometric ratio, suggesting that Bid has a catalytic function and that the interaction with Bax is transient. The formation of a stable dimerization interface involving two Bcl-2 homology 3 (BH3) domains was found to be the nucleation event for Bax homo-oligomerization. Based on intermolecular distance determined by EPR, a model of six adjacent Bax molecules in the oligomer is presented where the hydrophobic hairpins (helices $\alpha_5$ and $\alpha_6$) are equally spaced in the membrane and the two BH3 domains are in close vicinity in the dimer interface, separated by >5 nm from the next BH3 pairs.

Members of the Bcl-2 protein family are essential players in the complex regulation of apoptosis (1, 2). They are divided into three subgroups: the anti-apoptotic Bcl-2-like proteins, the pro-apoptotic multidomain proteins (Bax and Bak), and the pro-apoptotic BH3-only proteins. To keep programmed cell death under control, Bax activation needs to be strictly regulated, as abnormal cell death is disadvantageous for multicellular organisms.

Following cleavage by caspase-8, the BH3-only protein, Bid, is known to activate Bax (3–6). Recently the events involved in Bax-Bid interaction were investigated by fluorescent techniques (7). Bax is activated through a cascade of conformational changes from being inactive and cytosolic to an oligomeric, membrane-inserted state. In the mitochondrial outer membrane (8, 9) activated Bax is responsible for cytochrome c release and apoptosis initiation (10). Bax oligomerization has been shown to occur also in vitro by incubation with detergents (10–14).

The structures of monomeric Bax and Bid were solved by NMR (14–16). Bax has a globular fold composed of nine $\alpha$-helices ($\alpha_1$ to $\alpha_9$), with $\alpha_2$ representing the BH3 domain and $\alpha_5$/$\alpha_6$ the hydrophobic hairpin (see Fig. 1A). Similarities in structure and function with the monomeric inactive form of the channel-forming domain of bacterial colicins or diphtheria toxins are evident (14, 17). The BH3-only protein, Bid, shows a similar globular fold but lacks $\alpha_8$ and $\alpha_9$ and has an additional short helix ($\alpha_1/2$) between $\alpha_1$ and $\alpha_2$. The structure of active Bax is still unknown, but helices $\alpha_5$, $\alpha_6$, and $\alpha_9$ are reported to insert into the outer mitochondrial membrane (see Fig. 1C) (18–21).

A number of models have been proposed to describe interactions between Bcl-2 proteins. (i) an NMR study reports an activation mechanism based on the interaction of a stabilized helix of the BH3-only protein Bim with $\alpha_1$ and $\alpha_6$ of Bax, initiating conformational changes in Bax (22). (ii) The Bax homolog Bak is reported to homodimerize by an interaction of a fused $\alpha_2/\alpha_3$ helix with the $\alpha_2/\alpha_3$ helix of a second Bak monomer (Fig. 1B) (23). (iii) A heterodimerization-based survival mechanism has been proposed in which the BH1–3 domains of anti-apoptotic Bcl-2 proteins form a hydrophobic groove to sequester the BH3 domain of pro-apoptotic family members (24–26). However, the molecular details of Bax activation by the BH3-only proteins and the structure of oligomeric/membrane-inserted Bax and Bid are pro-apoptotic members of the Bcl-2 protein family.

**References**

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3. The abbreviations used are: BH3, Bcl-2 homology 3; AFM, atomic force microscopy; EM, electron microscopy; MTSSL, methanethiosulfonate spin label; DDM, n-dodecyl $\beta$-D-maltoside; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MES, 4-morpholinethanesulfonic acid; mT, mitofusins; NiEDDA, nickel(ethylenediaminediacetic acid); DEER, double electron-electron resonance; SEC, size exclusion chromatography; WT, wild type; ECL, E. coli polar lipid; Tempo, 2,2,6,6-tetramethyl-1-piperidinyloxy.
sputed Bax are still unclear. These areas were investigated here using several biophysical techniques such as AFM, cryo-EM, electrophoresis, mass spectrometry and chromatography, and in particular, site-directed spin-labeling EPR techniques.

The latter technique introduces a nitroxide radical probe, methanethiosulfonate spin label (MTSSL), into a protein by covalent bonding to cysteines (the resulting unnatural side chain is referred to as R1 herein). The spin label EPR spectra can be analyzed in terms of the available conformational space of the side chain and the water exposure of the radical. Moreover, the oligomer formation can be followed by the appearance of spin-spin interactions between singly labeled Bax monomers (EPR is sensitive to distances in the 1.5–6 nm range for membrane proteins).

In this work, the formation of Bax oligomers was monitored after (i) incubation with detergents, (ii) reconstitution into lipid bilayers, and (iii) insertion into liposomes in the presence of cleaved Bid. We found that similar oligomers were formed in the presence of high detergent concentration and in liposomes. The EPR-derived kinetics of Bid-induced Bax activation and pore formation showed that the interaction between Bid and Bax has a transient nature and that Bid is not a structural element in the oligomer. Distance measurements performed on assembled Bax oligomers gave insights in the structure of active Bax.

**EXPERIMENTAL PROCEDURES**

Additional details regarding cell culture, protein preparation, and biophysical techniques used are provided under the supplemental “Experimental Procedures.”

**Expression and Purification of Bax and Bid**—The procedure to express and purify Bax is essentially that described by Suzuki et al. (14). Protein purity was about 99% analyzed by SDS-PAGE and liquid chromatography/mass spectrometry.

The plasmids pTYB1-BaxC62 (C126A) and pTYB1-BaxC126 (C62A) were cloned by site-specific mutagenesis and controlled by DNA sequencing. Mouse Bid was expressed and purified according to Desagher et al. (27). Caspase-8 (Calbiochem) was used for Bid cleavage. The protein fragments obtained (P15/7-Bid) were further purified by nickel affinity chromatography and analyzed by SDS-PAGE and liquid chromatography/mass spectrometry. Bid was cleaved exclusively before Gly-68 (Gly-60 in the untagged protein). P15-Bid was eluted with buffer C (20 mM Tris, 150 mM NaCl, pH 7.5) containing 2% octyl glucoside (Anatrace Inc., Maumee, OH). The detergent was removed afterward by dialysis.

**Bax Oligomerization Techniques**—In this work, several methods were used to induce oligomer formation. (i) Bax (monomeric form) was mixed with 1–2% (w/v) n-dodecyl-β-D-maltoside (DDM) (Anatrace Inc.) in buffer C. The high (1% w/v) and low (0.1% w/v) detergent concentrations correspond to DDM-to-Bax monomer ratios of >200 and <20, respectively (with 110–140 DDM molecules/micelle according to Sanders and Sönnichsen (28) and Liang et al. (29)). (ii) Bax was reconstituted into lipid vesicles formed by Escherichia coli polar lipid extract or bovine heart total extract (Avanti Polar Lipids, Inc., Alabaster, AL). (iii) Bax (monomeric form) was incubated at room temperature with preformed liposomes in the presence of different triggering factors, such as full-length Bid, p15/7-Bid, p15-Bid, low pH (150 mM NaCl, 100 mM MES, pH 5.4), and CaCl₂.

**Spin Labeling of Bax**—The spin label MTSSL (Toronto Research Chemicals, Toronto, Canada) was covalently attached to the cysteine residues of the monomeric Bax mutants in water solution by overnight incubation at 4 °C with 10-fold MTSSL molar excess in buffer C. Excess label was removed by extensive dialysis. Spin-labeled wild type Bax (25% w/w) was mixed with BaxWT labeled with the diamagnetic MTSSL analog (1-acetoxy-2,2,5,5-tetramethyl-3-pyrroline-3-methyl)methanethiosulfonate (Toronto Research Chemicals) to suppress intermonomer distances in the oligomeric state.

**Size Exclusion Chromatography**—All size exclusion chromatography experiments were performed on a Superdex 200 PC 3.2/30 using a SMART chromatography system (GE Healthcare).

**Cross-linking Experiments**—Protein samples were adjusted to a concentration of 0.5 mg/ml in the presence or absence of detergent. 10 μl of the sample was mixed with 1 μl of ammonium peroxydisulfate (25 mM) and 2 μl of ruthenium(II) tris-bipyridyl dication (5 mM; Sigma-Aldrich) in the dark and then exposed to light (400–700 nm generated by a Xenon lamp (100 watts) and Leica filters (KG4, GG 395 nm)). By a light-induced reaction, neighboring residues form covalent bonds (59). The photoinduced reaction was stopped by the addition of 5 μl of SDS-PAGE loading buffer.

**Atomic Force Microscopy**—AFM was performed using a NanoScope MultiMode III (Veeco) in tapping mode in solution with OMCL-TR800-PSA cantilevers from Olympus (nominal force constant, 0.57 N/m; excitation frequency, ~20 kHz). Bax molecules were adsorbed on mica in buffer C at room temperature. Volumes were calculated with the equation: \( V = \frac{1}{n} \sum \frac{m_i}{\rho_i} \), where \( V \) is the volume of the solution, \( \rho_i \) is the density of the solution, and \( m_i \) is the mass of the solute.
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4/3 II (d/2)² × (h/2), with d representing the mean value of the diameter and h the height of the particle.

Blue Native PAGE—Blue native PAGE was performed according to Schägger and von Jagow (30).

Membrane Poration Assays—In this work, several methods were used to follow the membrane poration in liposomes formed by E. coli polar lipid extracts. The lipids in chloroform solution were dried in a rotary evaporator or under nitrogen flux and resuspended in buffer C containing 2 mg/ml cytochrome c or 5 mM 4-phosphonooxy-TEMPO at a final lipid concentration of 20 mg/ml. Large unilamellar vesicles were formed by repeated freezing and thawing cycles. The large unilamellar vesicles were extruded through a 400-nm filter. The cytochrome and 4-phosphonooxy-TEMPO present in the bulk water were removed by dialysis or ultracentrifugation. The extruded liposomes containing cytochrome or 4-phosphonooxy-TEMPO only in their lumen were mixed to a final concentration of 10 mg/ml E. coli polar lipid (ECL) extract with 0.1–0.3 mg/ml Bax and Bid-derivatives (1:1 stoichiometric ratio) and incubated at 37 °C for 3 h. The samples were diluted with buffer C before ultracentrifugation (about 5–10 times), and liposome fractions were separated from the supernatant fractions. In the case of 4-phosphonooxy-TEMPO experiments, EPR spectra of supernatants and pellets were detected, whereas for the cytochrome c experiments the pellet fractions were analyzed by SDS-PAGE and the supernatants by UV spectroscopy to detect light absorption at the maximum cytochrome c absorption (410 nm). For the cryo-EM experiments, large unilamellar vesicles were extruded through a 100-nm filter, mixed to a final concentration of 13 mg/ml ECL with BaxΔC62R1 (10 μM) and p15/7-Bid in a 1:1 stoichiometric ratio, and incubated at 37 °C for 1 h.

For each sample (absence and presence of Bax and Bid derivative) 3.5-μl aliquots were applied to a lacy carbon grids (Plano, Germany) supported on a copper grid. These grids were blotted for 3 s and plunge-frozen in ethane at liquid nitrogen temperatures in a humidity (95%)- and temperature (25 °C)-controlled chamber using a Vitrobot automated blotting device (FEI Co., Eindhoven, The Netherlands). The frozen grids were transferred into the electron microscope using a Gatan cryostage, which maintains the grids at a temperature below −170 °C. Transmission electron microscopy was performed using low dose methods on a CM12 microscope (FEI Co., Hillsboro, OR) operating at 120 kV and nominal magnifications between ×10,000 and ×30,000. Images were recorded using a Gatan slow scan charge-coupled device camera.

Continuous Wave EPR Experiments—Room temperature continuous wave EPR spectra were recorded using a Magne-tech Miniscope MS200 X-band spectrometer equipped with a rectangular TE102 resonator, with the microwave power set to 1 milliwatt and the B-field modulation amplitude adjusted to 0.15 mT.

For the kinetic experiments, samples containing 5 mg/ml ECL, 1 mg/ml BaxWT-R1, and Bid derivatives with different concentration (see SDS-PAGE in supplemental Fig. 3) were prepared. EPR spectra were recorded at 37 °C using a Bruker E500 X-band spectrometer equipped with a super high Q cavity, with the microwave power set to 0.5 milliwatt and the B-field modulation amplitude adjusted to 0.15 mT. Spectra were recorded at time intervals of 43 s with a 10-mT width, 512 points, 2 scans, and conversion time 40.96 ms. The intensity of the central line was plotted versus time in a logarithmic scale to obtain the half-times of the spectral change.

Pulse EPR Experiments—Samples were shock-frozen in the quartz EPR tube (3-mm outer diameter) in liquid nitrogen in the presence of 10% (v/v) deuterated glycerol. Dipolar time evolution data were recorded at 50 K with a dead time-free four-pulse sequence (double electron-electron resonance (DEER)) (Pannier et al. (40)) at X-band frequencies (9.3–9.4 GHz) with a Bruker Elexys 580 spectrometer equipped with a Bruker Flexline split ring resonator, ER 4118X-MS3, and a continuous flow helium cryostat (ESR900; Oxford Instruments, Abingdon, Oxfordshire, UK) controlled by an Oxford Instruments temperature controller (ITC 503S). Data analysis was performed with the software DeerAnalysis 2008 (31).

RESULTS

Characterization of Spin-labeled Mutants—Bax has two endogenous cysteines (Cys-62 in the center of a2, BH3 domain, and Cys-126 at the end of a5), both accessible for spin labeling. The doubly spin-labeled Bax is for simplicity named BaxWT-R1. For spin-spin distance determination in oligomeric Bax, if both positions are labeled, intra- and intermonomer distances are present, which are difficult to disentangle. Thus, to investigate Bax oligomerization steps by EPR, a single spin label was placed per monomeric unit, allowing measurement of only intermonomer distances. The single cysteine mutants were engineered by replacing either cysteine (Cys-62 or Cys-126) by alanine (BaxC62-C126A or BaxC62A-C126). The two singly spin-labeled mutants are for simplicity named BaxΔC62R1 and BaxΔC126R1, respectively.

Mutagenesis and spin labeling were both tolerated in terms of protein folding and ability to form oligomers. Circular dichroism (CD) spectroscopy performed showed a helical content of about 60% for all produced variants (not shown), consistent with previously published data (14, 32, 33). All Bax variants (spin-labeled or not) in buffer solution were predominantly monomeric and eluted in size exclusion chromatography (SEC) at about 1.7 ml with a reproducible small shoulder at 1.5–1.6 ml, suggesting the presence of a minor fraction of dimers in the preparation (Fig. 2A). The shoulder was also present after spin labeling (more than 90% of the protein population was spin-labeled) or if 2 mM diethiothreitol was present (data not shown). Thus, disulfide bridge formation could be excluded as the dimerization trigger. The dimer fraction was shown to increase after storage of Bax for 10 days at 4 °C (Fig. 2A, top panel).

Monitoring and Characterization of Bax Monomer to Oligomer Transition—Size exclusion chromatography was used to check Bax oligomerization in detergent. In the presence of high concentrations (1–2% (w/v)) of DDM the main elution peak of all Bax variants (spin-labeled or not) shifted from 1.7 to 1.2 ml, indicative of oligomer formation (Fig. 2A, top and middle panels, and Table 1). A high DDM concentration means that at least one complete micelle (∼150 molecules, ∼80 kDa (28, 29)) per Bax monomer is present. Under the same conditions, ferri-
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The size of the Bax oligomer is therefore about 20 times larger than the monomer size (20 kDa) strongly indicating that DDM induces Bax oligomerization, even taking into account the detergent contribution. The elution peak of oligomeric Bax was broader than the peak of the monomeric form (Fig. 2A, top panel), indicating a not perfectly homogeneous oligomer size in the sample. By running DDM-embedded samples (50 μl) with a detergent-free buffer on a detergent-free column (2.4 ml), the DDM concentration was gradually reduced, and the elution peak for Bax shifted to 1.4 ml or ~100 kDa (Fig. 2A, top panel), suggesting that detergent removal induced disassembly of oligomers.

In the absence of detergent Bid showed an elution profile similar to that of Bax: a peak at 1.7 ml, attributed to the monomer, with a small shoulder possibly indicating dimer formation. The addition of high DDM concentrations shifted the main elution peak to 1.4 ml (~100 kDa; Fig. 2A, bottom panel), indicating interaction with a detergent micelle. However, the detergent-induced elution shift was much smaller for Bid than for Bax, suggesting that oligomer formation is an intrinsic property of Bax.

Blue native gel electrophoresis was also performed to investigate Bax quaternary structure under different conditions. In the absence of detergent, BaxWt showed mainly monomers and dimers (Fig. 2B, upper panel). The increased fraction of dimers (compared with SEC) was possibly induced by the hydrophobic Coomassie G-250 dye in the running buffer. Bax preincubated with various highly concentrated detergents such as octyl glucoside, n-decyl β-D-maltoside, or DDM showed a ladder-like distribution of mostly even-numbered complexes from dimers to dodecamers. In the presence of CHAPS, which is reported not to activate Bax (11, 13), a ladder of both odd- and even-numbered oligomers was formed. Hence, in the presence of CHAPS the dimeric units were not as stable as in the presence of n-decyl β-D-maltoside, DDM, or octyl glucoside. A comparable experiment was performed with Bid (Fig. 2B, lower panel). Consistent with the SEC experiments, the size of Bid was less affected by detergent; the largest oligomer found was a tetramer. In summary, this showed that in detergent Bax has an intrinsic ability to form large oligomers composed of an even number of monomers, indicating the dimers as the stable subunits.
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These conclusions were confirmed in cross-link experiments. Cross-linking of monomeric BaxWT produced mainly monomers and dimers, but after preincubation with n-decyl β-D-maltoside, octyl glucoside, or DDM, a ladder-like distribution of mainly even-numbered oligomers was found. The largest clearly visible cross-linked oligomers consisted of 10 monomers (supplemental Fig. 1 and Ref. 32).

Bax oligomer formation was further investigated by AFM in solution (supplemental Fig. 2). AFM images of protein monomers revealed particles of 3 nm mean height and 7 nm mean diameter (supplemental Fig. 2, B and C). Taking into account that the height is precisely imaged, whereas the diameter becomes exaggerated by the tip, the mean particle size is consistent with the NMR structure (3–5 nm particle diameter) (14). The imaged particles have a volume of 77 nm³ (or 39 nm³ if the diameter is decreased by 2 nm because of tip exaggeration). In the presence of DDM, AFM imaging was disturbed by strong interactions between the tip and the detergent, and thus data collection of Bax oligomers was performed after partial removal of DDM. The imaged particles had a mean height and a diameter only a little larger than the monomers (+0.4 and +2 nm, respectively; supplemental Fig. 2, A–C). The volume (144 or 87 nm³ after a 2-nm correction) is in line with a dimer size. However, cross-linking of BaxWT in the presence of DDM before adsorption to the mica and subsequent detergent removal led to larger particles of 3–4 nm in height and 10–20 nm in diameter. Moreover, a few particles showed a pore-like structure (supplemental Fig. 2, D–F). In summary, our experiments on Bax in detergent indicated that the protein forms large, even-numbered oligomers, but detergent removal induces a disassembly into smaller oligomers as small as dimers.

Changes in R1 Side Chain Mobility Reflect Bax Conformational Changes—Continuous wave EPR performed on spin-labeled protein reveals information on the molecular environment in terms of the accessibility and motional constraints of the covalently attached spin label (34). The doubly spin-labeled wild type (BaxWT-R1) and the two singly spin-labeled Bax variants (BaxC62R1 and BaxC126R1) were analyzed by continuous wave EPR in the water-soluble state as well as in the DDM-induced or liposome-reconstituted oligomeric state (Fig. 3).

Bax oligomerization produced significant spectral changes, indicating that both spin-labeled side chains are sensitive to the conformational switch of Bax. The spin labels attached at position 62 in the BH3 domain and at position 126 in helix α5 move from a quite dynamic condition, as reflected by narrow EPR lines in the monomeric form, to a motionally more restricted state in the oligomeric form, as indicated by considerable line broadening. For position 126 the spectral effects induced by DDM and liposomes were almost identical, whereas slightly different spectra were observed for position 62, indicating different interactions of the BH3 domains with the micelle or lipid environment.

The accessibility of the spin label toward the bulk water or the lipid phase was monitored with power saturation continuous wave EPR (35, 36) in the presence and absence of the paramagnetic quenchers NiEDDA (highly soluble in the bulk water) and molecular oxygen (highly concentrated in the apolar lipid environment). Water-exposed spin labels are characterized by high NiEDDA accessibility values and lipid-exposed spin labels by high O₂ values, whereas buried spin labels are inaccessible for both.

In the oligomeric state, BaxC126R1 showed a major decrease in water accessibility. A comparison of oxygen and NiEDDA accessibility values suggested that C126R1 is located at the membrane-water interface (Table 1). In the oligomeric state, C62R1 was shielded from the bulk water, with the liposomes inducing the biggest effect. The concomitant low oxygen accessibility suggests that the R1 side chain at position 62 is involved in tertiary interactions (Table 1). The Aeff parameters (ranging from 3.3 to 3.7 mT going from apolar to polar spin label microenvironments (34)) extracted from low temperature continuous wave EPR spectra (Table 1), as well as the changes in the spectral form, are consistent with this interpretation.

Kinetics of Bax Activation in the Presence of Liposomes—The EPR technique was used to investigate the influence of pH, Ca²⁺, and Bid derivatives on Bax activation in the presence of liposomes at room temperature (20 °C). EPR spectra of

FIGURE 3. Room temperature continuous wave normalized EPR spectra of BaxWT-R1, BaxC62R1, and BaxC126R1. Upper panel, spectra of monomeric water-soluble (sol) Bax (black) superimposed over DDM-activated Bax (DDM). The asterisks denote a small fraction of unwashed free label in the DDM samples. Lower panel, spectra of Bax reconstituted (rec) in liposomes from E. coli polar lipids (blue). For comparison the spectra in DDM are superimposed as dotted red lines. For BaxWT-R1, the spectrum of the oligomeric form reconstituted in liposomes formed by bovine heart lipids is also superimposed in gray.
BaxWT-R1 (to monitor both R1 side chains contemporarily) were recorded at increasing incubation times, normalized, and compared with the normalized spectra of the monomeric-inactive and DDM-activated states (Fig. 4). A pronounced difference in the spectral features can be seen in the low field region of the spectra (box in Fig. 4). Therefore, these regions were shown in the superimposed normalized spectra of Fig. 4.

Prolonged incubation of BaxWT-R1 with p15-Bid (also known as tBid) or p15/7-Bid (also known as c8Bid; data not shown) in the absence of liposomes induced no spectral changes at room temperature. Incubation of BaxWT-R1 with liposomes formed with ECL extracts or bovine heart lipid liposomes induced slight spectral changes after 16 h of incubation, indicative of a slow partial insertion of Bax into the liposomes (Fig. 4). The insertion of Bax into ECL and bovine heart lipid liposomes was markedly accelerated by p15-Bid or p15/7-Bid and slightly accelerated of Bax into the liposomes (Fig. 4). The spectral shape of activated Bax is the same as that obtained after detergent activation; thus, this spectral form is assumed to be typical for oligomeric BaxWT-R1. Enhanced Bax insertion into liposomes was induced by decreasing the pH to 5.4 (as reported in Ref. 37) and by increasing the Ca\(^{2+}\) concentration (Fig. 4).

The pro-apoptotic protein Bid is a known trigger of Bax activation; however, its precise role as trigger and/or structural component has not yet been clarified. Moreover, Bax activation can also take place in the absence of any BH3-only proteins (38). To understand Bid-induced Bax activation in more detail, we performed time course experiments at 37 °C on mixtures of BaxWT-R1 with ECL liposomes and various Bid derivatives at varying stoichiometric ratios (Fig. 5).

An increase in temperature from 20 to 37 °C enhanced Bax activation. Even in the absence of Bid, BaxWT-R1 was activated in the presence of ECL liposomes (with a half-time of about 3 h), as indicated by the EPR spectral change (Fig. 5A, upper panel). This indicates an intrinsic ability of Bax to penetrate into the membrane if retention factors are absent. However, in the presence of Bid derivatives, Bax activation was strongly accelerated. In a 1:1 stoichiometric ratio, p15/7-Bid activated Bax with a half-time of 14 min (Fig. 5A, lower panel) and with a data obtained with fluorescent probes (7). The reaction half-time was slower (30 min) when p15-Bid was used at similar stoichiometric ratios (Fig. 5A, upper panel). This slower activation might be explained by an observed tendency of p15-Bid to dimerize and precipitate in solution. Full-length Bid was also found to enhance Bax activation (90-min half-time) but to a lesser extent compared with the cleaved variants (Fig. 5A, lower panel).

Decreasing the p15/7-Bid to Bax stoichiometric ratios from 1:1 to 1:10 or 1:50 increased the half-times of about 2.9- and 5-fold, respectively (Fig. 5A, lower panel). Notably, after prolonged incubation, the EPR spectra were indistinguishable for all conditions (Fig. 5B, lower panel), indicating 100% efficiency of Bax penetration into the liposomes.
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SDS-PAGE of the samples used for the kinetic experiments after 24 h at 37°C showed that BaxWT-R1 was partially cleaved in lipid-containing samples (Fig. 6A). The cleavage site was found to be in the loop between α1 and α2 (mass spectrometry data; not shown). No indication of cleavage was found if Bax and p15/7-Bid were incubated in the presence of DDM (Fig. 6B). Notably, the conditions used in this work to induce an almost complete insertion of Bax in the presence of triggering agents (3 h at 37°C) could be shown to lead to only negligible amounts of truncated Bax (Fig. 6B), thus ruling out their relevance in the data analysis.

Bax Is Able to Form Pores in Liposomes—Three different techniques were used to show that Bax is active and able to form pores in the membrane bilayer under the experimental conditions used. Cryo-EM was performed on ECL liposomes (~100 nm size; incubated for 3 h at 37°C) in the absence (Fig. 7A, upper panel) and the presence of BaxC62R1 and p15/7-Bid (Fig. 7A, lower panel). In the presence of BaxC62R1 and p15/7-Bid, pores with a diameter of ~50 nm were observed, consistent with recently published data (39). Membrane incorporation was also verified by detecting the release of nitroxide 4-phosphonoxy-TEMPO or horse heart cytochrome c from the liposome lumen after the addition of Bax and Bid derivatives (Fig. 7, B and C). After incubation, the liposomes were pelleted to detect 4-phosphonoxy-TEMPO in both pellet and supernatant fractions by EPR (Fig. 7B). Cytochrome c was detected by SDS-PAGE in the pellet and by light absorption in the supernatant fraction (Fig. 7C).

Control experiments with liposomes alone and in the presence of 1% DDM were performed to detect the background level of 4-phosphonoxy-TEMPO and cytochrome c release for 0 and 100% membrane poration, respectively. The impermeability of the liposomes alone was found to be almost unaffected by incubation at 37°C and subsequent centrifugation, whereas the detergent provoked the disintegration of the liposomes resulting in a 100% release of the molecules from the lumen (Fig. 7, B and C).

In the absence of Bax, both the full-length and the truncated variants of Bid provoked only minor release of 4-phosphonoxy-TEMPO or cytochrome c from the liposome lumen. The presence of Bax in combination with the Bid derivatives allowed release of 4-phosphonoxy-TEMPO and cytochrome c (100 and 70%, respectively). Liposomes treated only with Bax were also partially opened (Fig. 7, B and C), in line with the kinetic data.

Notably, SDS-PAGE showed that after the Bid-induced membrane insertion of Bax, the latter was found mainly in the membrane fraction, whereas the Bid variants stayed predominantly in the supernatant (Fig. 6C). Only a small fraction of cleaved Bid was found to be membrane-inserted if Bax was present (Fig. 6C).

Interspin Distances in Bax Oligomers—When Bax monomers carrying a single spin-labeled side chain form oligomers, dipolar interaction between adjacent spin labels can be monitored by pulse EPR techniques (DEER) if the interspin distance is in the 1.5–6-nm range (40). In the presence of a homogeneous distribution of singly labeled proteins in the sample, the DEER trace will show a simple exponential decay. If dimers or oligomers are formed and the spin labels are within a distance range of 1.5 to 6 nm, a dipolar frequency (proportional to 1/r^3) modulates the DEER trace. The dipolar frequency distribution in the DEER traces was analyzed by the software DeerAnalysis 2008 (31) to calculate the distance distribution between the nitroxide groups within the spin label.

The DEER trace of the monomeric BaxC62R1 and BaxC126R1 in water solution showed a simple exponential decay, typical of a homogeneous distribution of monomeric species in the sample (Fig. 8). After the addition of DDM to BaxC62R1, a dipolar oscillation appeared, indicating intermonomer interactions (Fig. 8A). The derived interspin distance was centered at 2.3 nm.
This indicates the presence of at least Bax dimers in the presence of detergent. A similar defined peak centered at 2.4 nm was found for both Bax reconstituted into liposomes and on the pelleted ECL liposomes incubated for 3 h at 37 °C with BaxC62R1 and p15/7-Bid (Fig. 8A). Decreasing the DDM concentration by a factor of 10 led to a small decrease in the main interspin distance (Fig. 8A). A single distance distribution in an oligomeric state suggests that either the oligomers investigated are pure dimers or the second nearest neighbor distance is beyond the sensitivity of the DEER analysis. The second possibility seems to be more likely, as we have already shown that BaxWT forms oligomers under the experimental conditions used.

A single distance distribution in an oligomeric state suggests that either the oligomers investigated are pure dimers or the second nearest neighbor distance is beyond the sensitivity of the DEER analysis. The second possibility seems to be more likely, as we have already shown that BaxWT forms oligomers under the experimental conditions used.

Similar EPR experiments were performed on oligomeric BaxC126R1 (Fig. 8B). At low detergent concentrations a prominent peak centered at 2.3 nm was found, together with a broad distance distribution in the 3–4-nm range. High detergent concentrations as well as reconstitution into liposomes led to the same distance shift toward 3–4 nm, indicating the formation of similar oligomers in the two conditions tested. The DEER data did not allow us to assign 3 and 4 nm to intradimer or interdimer distances. However, they clearly show differences between the oligomers at low or high DDM concentrations, with the latter resembling the liposome-embedded state.

Interspin distances were also determined for BaxWT-R1. The 3.2-nm distance obtained in the monomeric form (Fig. 8C) is consistent with the C62-C126 distances from the NMR structures (2.4–2.6 nm; Protein Data Bank code 1F16), taking into account the length of the spin label side chain (0.4–0.8 nm, depending on the torsional states of the C–S bonds (41)). Analysis of the overall shape of the distance distribution in the presence of DDM points to an invariant 3-nm distance between C62R1 and C126R1 in both the monomeric and oligomeric forms (Fig. 5C). This was confirmed by the distance distribution centered at 3 nm obtained in the sample containing only 25% of the spin-labeled BaxWT-R1 (Fig. 8C).

**DISCUSSION**

In this work, we have presented new structural and kinetic details of Bax oligomerization in detergent and in liposomes, which elucidate some interesting properties of the pro-apoptotic mechanism. Bcl-2 proteins, key factors in the regulation of cell life and cell death, are promising targets in cancer therapy (42). Although sequence conservation within this group is low, the folding of their individual domains is very similar (14, 15, 43). The function of Bcl-2 proteins and their interactions are still not fully understood, and different models for dimeric or membrane-inserted conformations are discussed in the literature (21, 23, 44, 45).

High detergent concentrations, liposome reconstitution, or insertion into liposomes in the presence of cleaved Bid variants induced Bax oligomer formation. In the latter case, we also showed membrane poration, and thus Bid and Bax heterologously expressed were functional. Membrane poration was monitored by release of 4-phosphonoxy-TEMPO or horse heart cytochrome c from the liposomal lumen and proven also by cryo-EM (50-nm pores consistent with the findings of Schaffer et al. (39)).

The molecular size of active Bax oligomers is still under debate. SEC, used extensively to follow oligomerization in Bax
and Bak (8, 13, 23, 32, 46), showed that the effect of the micelle on oligomer size estimation is difficult to quantify. However, comparing Bax and Bid under similar conditions and performing concomitantly blue native PAGE and AFM experiments, we showed that detergent dilution provoked disassembly of Bax oligomers down to dimers and that oligomerization is an intrinsic property of Bax. These results clearly prove that the protein to detergent ratio is crucial for oligomer formation and maintenance, consistent with a recent study (46) performed on a truncated version of Bax (Bax\(^{C_62R1/H_9004C}\), lacking \(^{C_126R1/H_9251}\)).

In this work, the largest identified oligomers consist of 12 (blue native gels) or 10 (cross-linking in detergents) subunits, consistent with a 240-kDa protein complex. AFM images of cross-linked Bax subunits showed particles with a 10–20-nm diameter. Bax oligomers with 10–12 units are not compatible with the formation of a pure proteinaceous pore with a diameter of 50 nm (cryo-EM data). This poses the question as to whether the pore is formed only by proteins or also by lipids and if the overall topology of the oligomers is the same in micelles and liposomes. The EPR data provided evidence only on the short range topology of the oligomer; the dimeric structure (2.4-nm distance between C62R1 sites) and the intra- and inter-dimer spacing between neighboring hairpins (3–4 nm among C126R1 sites) are the same in micelles and liposomes. However, the role of the lipids might not be perfectly mimicked by the detergent micelles, and thus the long range topology of the oligomer could be different, allowing for example a higher number of monomers to be allocated in a purely proteinaceous pore in

![DEER experimental traces](image1)

![Background corrected traces](image2)

![Derived distance distributions](image3)

**FIGURE 8. Interspin distance analysis.** DEER analysis was performed on Bax\(^{C_62R1}\) (A), Bax\(^{C_126R1}\) (B), and Bax\(^{WT-R1}\) (C). Left column, experimental intensity-normalized DEER time traces \(V(t)\) and exponentially decaying background signals arising from a three-dimensional distribution of remote spins (thin black lines) obtained with DeerAnalysis 2008 (31). Black, monomeric spin-labeled Bax; red, incubated with 1% DDM; blue, liposome-reconstituted. The green trace was recorded for the pellet of liposomes incubated with Bax\(^{C_62R1}\) and p15/7-Bid for 3 h. The brown traces are recorded at a low DDM (0.1%) to protein ratio and the gray traces at a high DDM to protein ratio on the sample containing 25% of the spin-labeled Bax\(^{WT-R1}\). The traces have been vertically displaced for clarity. Middle column, background-corrected experimental data \(F(t)\) fit by Tikhonov regularization with an \(\alpha\) parameter 100 (thin black lines). Right column, distance distribution \(P(r)\) obtained by the fit. The asterisk denotes a possible artifact peak related to background subtraction and noise. a.u., arbitrary units.
the membrane. On the other side, in the membrane, Bax oligomers as small as those observed in detergent via SEC, cross-linking, and AFM could also initiate the formation of several small pores that induce the breaking of the vesicles observed by cryo-EM.

Furthermore, if lipids are involved in the pore formation, as suggested by experiments with the α5 peptide of Bax incorporated into a lipidic membrane (47, 48), small oligomers could be sufficient to trigger the formation of a lipidic pore. Clearly, further analysis on the nature of the pore is needed to resolve this issue.

Our data confirmed that Bax activation strictly requires the membrane bilayer. In its absence, a permanent conformational change of Bax induced by Bid was not detected. We found a slow Bax insertion into liposomes in the absence of triggering factors at 37 °C and in the presence of full-length Bid, which was not expected, although it has already been shown in vivo that Bax activation is possible in the absence of Bid and Bim (38). To analyze the self-insertion phenomenon, we performed SDS-PAGE of the samples used in the kinetics experiments (incubated for 24 h at 37 °C), which revealed partial truncation of Bax. The mechanism of Bax truncation is not clear as yet. Notably, truncation appears only after prolonged incubations (>3 h) and is not induced by Bid derivatives or by residual proteases co-purified with the proteins. Moreover, truncation was observed in different liposome preparations, with different batches of lipids.

Mass spectrometry performed on the cleaved Bax showed that the cutting site is located in the loop between α1 and α2. Cleavage in this loop region is known to increase Bax activity (49, 50) and induce a BH3-only protein-like behavior (51). Thus, the observed self-insertion of Bax might be explained by its own induced BH3-only protein activity in vitro. The rate of insertion was, however, strongly accelerated at 37 °C in the presence of all Bid variants.

In the presence of p15- or p15/7-Bid (1:1 ratio with Bax) and liposomes, the EPR-detected conformational change of Bax has a half-time of about 30 and 14 min, respectively. This reaction time is consistent with the time range found in the cytochrome c release experiments (Fig. 7B) and published fluorescence resonance energy transfer (FRET)-based kinetics (half-time for insertion and oligomerization, 10−15 min (7)). Interestingly, decreasing the p15/7-Bid to Bax ratio from 1:1 to 1:50 still induced a 100% insertion of Bax, suggesting a catalytic role for one p15/7-Bid molecule can run several Bax activation cycles. Moreover, this confirmed the transient nature of Bax-Bid interaction and the irrelevant structural role of Bid in the oligomers. This would explain why experiments performed to show this interaction were only partially successful (see for example Refs. 52 and 53).

The question arises as to where most of the Bid molecules are located during and after Bax activation. The p15-Bid fragment was described to be mostly in the membrane fraction (7, 54). In this work, p15/7-Bid was not membrane-inserted after incubation with liposomes in the absence of Bax (3 h, 37 °C). In the presence of Bax only a minor fraction of p15/7-Bid could be found in the pellet, together with most Bax molecules. Treatment of membranes at higher pH did not affect Bax localization, whereas it almost completely removed p15/7-Bid, indicating that p15/7-Bid is only loosely bound to the membrane surface.

The differences between our results and those of Lovell et al. (7) (using p15-Bid instead p15/7-Bid) might be explained by the presence of the p7 fragment in our work, which covers the hydrophobic surface of the p15 fragment (15), thus impairing a strong interaction with the membrane. Additional proteins might stabilize p15-Bid at the membrane in cells (54).

EPR experiments revealed new molecular details of the conformational switch from the Bax inactive monomeric species to the active oligomeric form. On the basis of the EPR accessibility data, side chain 126 in α5 is suggested to be located at the interface between the membrane and the bulk water, whereas side chain 62 is in a protein interface. These findings are in agreement with the model of Bax (21) in which α5, α6, and α9 are inserted into the lipid bilayer as well as with the models of Bid suggesting that α5 lies on the membrane surface (44, 55).

Detailed distance constraints between spin-labeled positions in the active oligomeric state, in particular between the BH3 domains (position 62) and among the loops connecting the hydrophobic α5 and α6 (position 126), allowed us to model three adjacent Bax dimers. Incubation with DDM, reconstitution into liposomes, or activation with cleaved Bid in the presence of liposomes led to a similar 62-62′ interspin distance distributions centered at 2.3 to 2.4 nm, shown to be only marginally affected by detergent dilution. This indicates a stable interaction between two BH3 domains in a dimeric unit. The invariant monomodal distance distribution observed in the active BaxC62R1 highlights the role of the BH3-mediated dimers in the oligomer stability and cannot be ascribed to a population of pure dimers (SEC, blue native PAGE, and cross-linking data confirmed the presence of oligomers) nor to oligomers with all BH3 domains equally spaced at 2.4 nm. In the latter case, the distances between alternating BH3 domains are expected to be in the 4-nm range depending on the diameter of the active oligomer and should therefore be visible in the DEER traces. Thus, from the DEER analysis, we also deduced that in the active oligomer the BH3-BH3 dimer interfaces must be separated from each other by more than 5 nm.

The interactions involving α5 and α6 are less stable and environment-dependent. At low detergent concentrations the hydrophobic patches of the protein promote the formation of dimers or small oligomers where the BH3-BH3 interface is still intact but the hydrophobic α5 and α6 helices are closely packed. Only by increasing the detergent concentration or by insertion into liposomes can a native-like oligomer be formed with 126–126′ intra- and interdimer distances in the 3–4-nm range.

The present data are consistent with the symmetric dimer model based on cross-linking studies on the Bax-analogous protein Bak (23) showing that the two molecules share the same activation mechanism. On the other hand, our data exclude a Bax oligomer arrangement similar to the reported swapped Bcl-xl dimers that interact by fused α5/α6 helices lying side by side and forming the dimer interface (45). If oligomeric Bax had a similar conformation, short 126–126′ and long 62–62′ inter-spin distances would result, which was not observed.
Fig. 9 presents our model of active Bax and the role of Bid derivatives in Bax activation. In solution, Bax and p15/7-Bid interact transiently. In the presence of the membrane bilayer, Bid act as catalytic trigger for Bax membrane insertion and oligomerization. Once Bax is oligomeric and the pore is formed, Bid recruits further Bax molecules from the water solution.

The model shows three adjacent Bax dimers. The membrane topology of the hydrophobic hairpin (of α5 and α6) and of the BH3 domains conforms to the accessibility and polarity data. The BH3 domains of Bax lie on the membrane bilayer and interact with their nearest neighboring BH3 domains, forming a stable dimer interface (62-62’ distance of 2.4 nm). Residue 126 is placed 3 nm away from C62R1 on the other side of the membrane bilayer. The intra- and interdimer distances between the α5 and α6 loops (carrying C126R1) are in the 3–4-nm range.

We modeled the BH3-BH3 interface starting from the NMR structure of Bax (14) and moving α2 to create the elongated helix α2/α3 (supplemental Fig. 3). Two Bax molecules were aligned according to the Bak cross-linking data (23) (supplemental Fig. 4) and the 62-62’ EPR-derived mean distance. This interface could be built either by facing the hydrophobic residues toward each other (Fig. 9, inset, left panel) or with a stable zipper-like salt bridge network (Fig. 9, inset, right panel). Our data support a model in which, after transient activation by Bid, Bax BH3 domains recognize each other, triggering an irreversible dimer formation that is the nucleation event for the oligomerization and subsequent membrane permeabilization. Trace amounts of cleaved Bid molecules are sufficient to trigger complete Bax oligomerization and insertion, thus excluding Bax-Bid hetero-oligomerization for membrane poration.

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