Extended results

Note S1

According to the NMR studies Bax 1-2/L-6 adopts the same fold as inactive wt Bax. In this state the molecule differs from wt Bax only in the formation of two disulfide bonds that stabilize this state in the region of helix 1 and helix 2 and the loop in between both helices, which is constrained to the tip of helix 6. Structural disulfide bonds like 1-2 and L-6 stabilize folded protein conformations (Frisch et al., 1994; Waschütza et al., 1996). While the 1-2 and L-6 disulfides restrain conformational changes in the N-terminal segment and BH3 domain of Bax, the C-terminal helix 9 (tail) would be predicted and is experimentally validated to be able to change conformations, as in wt Bax. Thus, Bax 1-2/L-6 can undergo conformational changes in the C-terminal segment, while conformational changes in helices 1 and 2 and the extended loop are hindered and predicted to restrict BH3 domain exposure.

Note S2

The formation of heterodimers between isolated Bax and Bcl-x_L requires the presence of detergents (Hsu and Youle, 1998). Detergent-induced interaction between wt Bax and Bcl-x_L occurs with recombinant full-length proteins at detergent concentrations above CMC with all tested detergents, while no interaction was observed at detergent concentrations below CMC (Figure S2C, right panels). Similarly tethered Bax shows no interaction with Bcl-x_L at detergent concentrations below CMC, whereas Triton X100, Triton X114 and dodecyl maltoside induce

heterodimerization at concentrations above CMC (Figure S2C, lower left panels and upper left panels lane a, b and d). However, the detergents CHAPS, Tween 20, W-1, sodium deoxycholate, octyl glucoside or NP-40 above CMC concentrations that induce wt Bax interaction with Bcl-x_L failed to induce heterocomplexes between Bax 1-2/L-6 and Bcl-x_L. The intramolecular disulfide bonds prevent interactions that are induced by these detergents between full-length wt Bax and Bcl-x_L, but not interactions induced by Triton X100, Triton X114 and dodecyl maltoside. This may indicate two different states of interactions between Bax and Bcl-x_L. The state that can be inhibited by the tethers in Bax 1-2/L-6 is probably important for the initial control of Bax activity, as Bcl-x_L cannot inhibit Bax 1-2/L-6 (Figure 2A-C). Interactions between Bax and Bcl-x_L that are unaffected by the tethers may occur after more global rearrangements of both proteins perhaps similar to complexes integrated into the membranes of liposomes (Lovell et al., 2008). The differential induction of wt Bax and Bax 1-2/L-6 interactions in certain detergents can also be observed in the formation of homodimers or –oligomers. While both full-length Bax variants migrate as monomers on gel filtration in the absence of detergents, Triton X100 induces oligomerization of both wt Bax and Bax 1-2/L-6 at 1.5 x CMC (Figure S2D). However, other detergents, such as NP-40 and sodium deoxycholate, cause differential complex formations of wt Bax and Bax 1-2/L-6. In these detergents Bax 1-2/L-6 forms lower molecular weight complexes than wt Bax suggesting that tethering Bax diminishes the ability of Bax 1-2/L-6 to oligomerize.

Note S3

Measuring the co-localization of GFP fluorescence and mitochondrial staining following apoptosis induction by the Pearson's coefficient, ranging from 0 for no co-localization to 1 for complete overlap, yields values of about 0.8 for Bax 1-2/L-6 co-localization with mitochondrial staining, while 0.5 to 0.63 was measured for Bax ΔSH and the mitochondria (Figure S3B), further indicating the even coating of Bax 1-2/L-6 on the mitochondria in contrast to foci formation of wt Bax.

Note S4

We examined Bax retrotranslocation after apoptosis induction and Bax accumulation on mitochondria using HCT116 Bax/Bak DKO stably expressing GFP-Bax and Omi-mCherry. Within 10 minutes after MOMP, wt Bax retrotranslocation was heterogeneous with cells displaying Bax in foci lacking detectable retrotranslocation and other cells with non-focal mitochondrial Bax retrotranslocating at a low rate (Figure S6B, D). In the presence of the viral Bax inhibitor vMIA (Arnoult et al., 2004) wt Bax translocates to the mitochondria and does not detectably retrotranslocate (Figure S6C, E).

Note S5

We also analyzed retrotranslocation of Bax S184 mutants, which constitutively localize to the mitochondria (Nechushtan et al., 1999). Mitochondrial GFP-Bax S184A fluorescence is

diminished comparable to wt Bax (Figure S5A), while the GFP-Bax S184V displays an 18 % slower retrotranslocation rate (Figure S7B, C), indicating that the predominant mitochondrial localization of Bax S184 variants is due to an increased rate of binding to mitochondria rather than a reduced rate of retrotranslocation.

Figure S1.

(**A**) Single amino acid substitutions used in different Bax variants.

(**B**) Thiol trapping of cysteine residues in recombinant wt Bax and Bax 1-2/L-6 that are accessible to modification by mPEG-maleimide (mPEG-MAL, 10 kDa). After incubation with mPEG-MAL both Bax variants were analyzed by SDS-PAGE and Western blot using 2D2 antibody.

(**C**) Thiol trapping of accessible cysteine residues in GFP-Bax variants using mPEG-MAL. Different GFP-Bax variants were transfected into HCT116 Bax/Bak DKO and lysed in RIPA buffer, incubated with mPEG-MAL and analyzed by SDS-PAGE and Western blot using a rabbit α-GFP antibody.

(**D**) Migration of Bax 1-2/L-6 in the presence of different concentrations of reducing agents in SDS-PAGE in comparison to wt Bax analyzed by Western blot using 2D2 antibody. Bax 1-2/L-6 reduction occurred in 6M guanidinium chloride (GdmCl), 10 mM DTT and iodoacetamide (IAM).

(**E**) Differences of backbone chemical shifts between wt Bax and Bax 1-2/L-6. The weighted average chemical shift difference $\Delta\delta$ for each residue was calculated as $\sqrt{\left\{\left(\Delta\delta_{\mathrm{H}}\right)^2+\left(\Delta\delta_{\mathrm{N}}/5\right)^2\right\}/2}$ in ppm, and plotted as a function of the residue number. The

absence of a bar indicates the presence of a proline or a residue that was not assigned. Relatively large chemical shift differences are observed for the nearest neighbors of the mutations. Modest differences are observed for the residues that are spatially close to the mutations, such as some residues within helices α5 and α8. The rest of the residues showed relatively small differences, indicating that the structure of Bax 1-2/L-6 is similar to that of wt Bax.

(**F**) Transverse relaxation (^{15}N T_2) (Barbato et al., 1992) for the backbone amides of Bax are plotted as a function of residue number.

Figure S2.

(**A**) Spontaneous cyt c release induced by different protein levels of either wt Bax (left) or Bax 1-2/L-6 (right) dependent on the DNA concentration (50, 100, 200 and 400 ng repectively) used for transfection of HCT116 Bax/Bak DKO cells.

(**B**) Western blot analysis of wt Bax and Bax 1-2/L-6 levels in HCT116 Bax/Bak DKO cells transfected with 50, 100, 200 and 400 ng plasmid DNA per well using rabbit anti-GFP antibody. In addition to the GFP-Bax variants a second protein band is unspecifically bound by the antibody (*).

(**C**) Bax1-2/L-6 tethers inhibit detergent induced Bcl-x_L interactions. Recombinant Bax wt and Bax 1-2/L-6 were incubated with full-length purified recombinant Bcl-x_L in the presence of Triton X-100 (lane a), Triton X-114 (lane b), CHAPS (lane c), dodecyl maltoside (lane d), Tween 20 (lane e), W-1 (lane f), sodium deoxycholate (lane g), octyl glucoside (lane h) or NP-40 (lane i) at 2 CMC (upper panels) and ½ CMC (lower panels). Bax wt/Bcl-x_L interaction in Triton X100 (2x

CMC) is shown as control (*). α-Bcl-x_L antibody (rabbit) was used to immunoprecipitate Bcl-x_L and Western blot analyses was performed using α-Bcl-x_L (2H12) antibody and α-Bax (2D2) antibody.

(**D**) Western blot analysis of wt Bax and Bax 1-2/L-6 migration on a Superdex 200 (10/30) column in the absence and presence of Triton X100, sodium deoxycholate, NP-40 and CHAPS at 1.5 times CMC using 2D2 antibody. On the right two chromatograms of wt Bax (blue) and Bax 1-2/L-6 (red) are depicted from the migration of both Bax variants in the absence (top) and presence of sodium deoxycholate (bottom).

Figure S3.

(**A**) Confocal imaging of HCT116 Bax/Bak DKO transfected with Bcl-x_L and either GFP-Bax ΔSH or GFP-Bax 1-2/L-6 with or without 1 μM ActD treatment for 2 h. Q-VD was used to prevent caspase activation. GFP-fluorescence is depicted in the second panels and in green in the merge and detail on the right, whereas α-Tom20 staining is shown in the left panels and in red in the merge and detail images. In the merged and detail images co-localization is shown in yellow. The white line in the low right corner of every image is the scale of 10 μm. White broken lines in the merge images show the section analysed in the line scans. The white box in the merge indicates the section depicted in the detailed image.

Line scans show the fluorescence intensities of GFP-Bax 1-2/L-6 signals (green) and mitochondria stained by α-Tom20 (red) for the sections in the merged images.

(**B**) Comparison of the co-localization between GFP fluorescence and α-Tom20 staining of the mitochondria defined by the Pearson's coefficient of HCT116 Bax/Bak DKO cells transfected

with GFP-Bax ΔSH or GFP-Bax 1-2/L-6. The confidence range is depicted as box with the most extreme data points as dots and mean (□ □) of the data set. n ≥ 15 cells. The p value according to the unpaired students t-test comparing GFP-Bax ΔSH and GFP-Bax 1-2/L-6 is depicted.

Figure S4.

(**A**) FLIP of GFP-Bax in the cytosol (●) and on the mitochondria (○) of HCT116 Bax/Bak DKO cells. Fluorescence of the neighboring cell is shown as control (△). Data represent averages ± SEM from 20 ROI measurements.

(**B**) Time course recorded for the influence of MG132 on GFP-Bax in absence (top) and presence (bottom) of over-expressed Bcl-x_L in FLIP experiments. Mitochondrial GFP-Bax fluorescence is diminished by FLIP in the presence of 10 µM MG132 in a comparable time course compared to the absence of MG132 (Figure 4B).

(**C**) FRAP experiment studying mitochondrial and cytosolic GFP-Bax (i) by bleaching cytosolic GFP-Bax once completely (ii). The regain of cytosolic fluorescence due to Bcl-x_L-dependent retrotranslocation of unbleached GFP-Bax molecules is monitored over time (iii).

(**D**) Mitochondrial GFP-Bax fluorescence recorded after a single bleach (in Figure 4F) was not different in the presence and absence of Bcl-x_L over-expression prior to FRAP in the cytosol. Data are normalized to the initial fluorescence and represent averages from at least 16 measurements ± SEM.

(**E**) Measuring association of GFP-Bax on the MOM by bleaching half of a targeted GFP-Bax expressing HCT116 Bax/Bak DKO cell including the mitochondria. After recovery of the GFP-Bax

fluorescence in the previously bleached part of the targeted cell for 1 to 10 min the cytosolic Bax fluorescence was diminished by repeated bleaching in the nucleus. Subsequently the recovery of fluorescence was measured on previously bleached mitochondria compared to unbleached mitochondria.

(**F**) FLIP of GFP-Bax wt in the absence (…) and presence (---) of over-expressed Bcl-x_L with additional over-expression of untagged Bax without (●) and with (○) over-expressed Bcl-x_L to test the competition of Bax and Bcl-x_L for a mitochondrial binding site as a possible cause of Bax retrotranslocation. Data represent averages ± SEM from 12 (GFP-Bax wt + Bax wt) and 15 (GFP-Bax + Bax wt + Bcl-x_L) ROI measurements.

Figure S5.

(**A**) Time course recorded for Bax S184A (top), Bax 1-2/L-6 S148A (center) and Bax 1-2/L-6 S184E in FLIP experiments. FLIP diminishes GFP-Bax in the cytoplasm of a targeted cell (circled) quickly, but mitochondrial signals (arrows) of Bax S184A and Bax 1-2/L-6 S184A remain more stable. Cells transfected with Bax 1-2/L-6 S184E lack a mitochondrial Bax pool. Time points in seconds are displayed above the images.

(**B**) Expression of Bax wt, Bax 1-2/L-6 S184A, Bax 1-2/L-6 S184E and Bax ΔSH by SDS PAGE in the absence of BME analyzed by Western blot using anti-GFP antibodies used in Figure S5A.

Figure S6.

(**A**) FLIP experiments with GFP-Bax in absence (•••) and presence of over-expressed Bcl-x_L (---), Bcl-2 (red, ●), Mcl-1 (blue, ○) and Bim (grey, ▼). Measurements for the influence of endogenous

Bak were performed using HCT116 Bax KO cells (green, △). The fluorescence of the neighboring cell serves as control (—). Data represent averages ± SEM from 20 (Bax KO), 22 (+Bcl-2), 24 (+Mcl-1) and 10 (+Bim) ROI measurements.

(**B**) Changes in mitochondrial fluorescence obtained from two measurements per analyzed cell (different grey tones for each individual cell) in FLIP after MOMP as depicted in (**D**).

(**C**) vMIA prevents Bax retrotranslocation. FLIP of mitochondrial GFP-Bax in the absence (•••) and presence of over-expressed vMIA (red, ○) or with over-expressed vMIA and Bcl-x_L (blue, ●). Data represent averages ± SEM from 20 ROI measurements per condition.

(**D**) Time course recorded for GFP-Bax wt FLIP after MOMP. HCT116 Bax/Bak DKO expressing GFP-Bax wt (green) and Omi-mCherry (red) to monitor the occurrence of MOMP were treated with 1 µM STS. Less than 10 min. after the cells lost their mitochondrial Omi-mCherry fluorescence they were analyzed by FLIP for the retrotranslocation of mitochondrial Bax. Typically focal Bax fluorescence remained unaffected by FLIP (arrows, **S6B**). The time following FLIP initiation is displayed above each image in s.

(**E**) GFP-Bax fluorescence on mitochondria in the presence of over-expressed vMIA does not diminish (arrows) during FLIP. Time points in seconds following initiation of FLIP are displayed above the pictures.

Figure S7.

(**A**) Bax retrotranslocation (%) in presence and absence of Bcl-x_L and with or without the low molecular weight inhibitor ABT-737.

(**B**) FLIP experiments of Bax wt (•••) with over-expressed Bcl-x_L (---), Bax D68R (green, ▼), Bax S184V (blue, ○) and Bax S184A (red, ●) over-expressed in HCT116 Bax/Bak DKO cells. The fluorescence of the neighboring cell is shown as control (□ □). Data represent averages ± SEM from 20 (S184A), 16 (S184V) and 12 (D68R) ROI measurements.

(**C**) Retrotranslocation rates measured for GFP-Bax wt and different variants. Data represent averages from at least 10 ROI measurements ± SD.

(**D**) Retrotranslocation rates of Bcl-x_L, Bax and a chimeric protein based on Bcl-x_L with helices 2 and 3 of Bax substituting the corresponding helices in Bcl-x_L (George et al., 2007).

(**E**) Confocal imaging of HCT116 Bax/Bak DKO cells transfected with GFP-Bcl-x_L (center, green in the merge). Mitochondria are stained for Tom20 (left, red in the merge). The white line in the low right corner of every image is the scale of 10 µm.

(**F**) Retrotranslocation rates measured for GFP-Bcl-x_L without and with Bax over-expression or administration of ABT-737. Data represent averages ± SD.

Movie S1.

FLIP of GFP-Bax in the absence of over-expressed Bcl-x_L (Figure 4B, upper panels).

Movie S2.

FLIP of GFP-Bax in the presence of over-expressed Bcl-x_L (Figure 4B, lower panels).

Movie S3.

FLIP of GFP-Bax in the absence of over-expressed Bcl-x_L (Figure 5A, upper panel).

Movie S4.

FLIP of GFP-Bax in the presence of over-expressed Bcl-x_L (Figure 5A, lower panels).

Extended experimental procedures

Cyt c release from isolated mitochondria

Bax/Bak DKO MEFs were harvested with PBS containing 1 mM EDTA, centrifuged at 750g for 10 min., washed in PBS, and resuspended in isotonic MIB (Mitochondrial Isolation Buffer MIB: 210 mM mannitol, 70 mM sucrose, 1 mM EDTA, and 10 mM Hepes (pH 7.5)), supplemented with protease inhibitors. Next, cells were broken on ice by 10 passages through a 25G1 0.5 x 25 needle fitted on a 10 ml syringe, and the suspension was centrifuged at 2000g in an Eppendorf centrifuge at 4° C. Supernatants from each step were pooled before centrifugation at 13,000g at 4° C for 10 min. The resulting pellet was resuspended in 2 ml of MIB and layered on top of a discontinuous sucrose gradient consisting of 20 ml of 1.2 M sucrose in 10 mM Hepes, (pH 7.5), 1 mM EDTA, and 0.1 % BSA on top of 17 ml of 1.6 M sucrose in 10 mM Hepes, (pH 7.5), 1 mM EDTA, and 0.1 % BSA. Then, samples were centrifuged at 27,000 rpm for 2 h at 4° C in a Beckman SW28 rotor. Mitochondria were recovered at the 1.6–1.2 M sucrose interface, washed in MIB, centrifuged at 13,000g at 4° C for 10 min, and resuspended in MIB. Finally protein concentration was estimated. To study in vitro cytochrome c release in isolated mitochondria, MOMP was induced with recombinant tBid (R&D Systems, 10 nM) in association with recombinant wt Bax or Bax 1-2/L-6 (100 nM). Mitochondria (50 μg) were incubated with

recombinant proteins in 200 µl of KCl buffer (125 mM KCl, 4 mM $MgCl_2$, 5 mM Na_2HPO_4, 5 mM succinate, 0.5 mM EGTA, 15 mM Hepes–KOH (pH 7.4), 5 µM rotenone), for 15 min at 30° C and then centrifuged for 5 min at 13,000g at 4° C. Mitochondrial pellets corresponding to 5 µg proteins and the corresponding volume of the supernatant fractions were resolved by SDS–PAGE (10–20 % Tricine gels; NOVEX) and transferred to a nitrocellulose membrane. Their respective contents of cytochrome c were assessed by immunoblotting with mouse anti-cytochrome c (BD Pharmingen, clone 7H8.2C12) (dilution 1:2000 in PBS with 5 % nonfat milk supplemented with 0.1 % Tween 20). Equal loading of the mitochondrial pellet is verified using an antibody against VDAC (Calbiochem, clone 31HL) (1:6000) and the presence of Bax was assessed using anti-Bax (Bax NT, Upstate Biotechnology) (1:2000).

Gel shift assays for Bax variant redox state and immonoprecipitation experiments

Bax variants were transfected into HCT116 Bax/Bak DKO for 6-8 hours. Cells were either harvested in SDS-PAGE sample buffer in the presence and absence of β-mercapto-ethanol (BME) TCA precipitated, resuspended and analyzed by Western blot using rabbit α-GFP antibodies (Invitrogen) or lysed for thiol trapping. After cell lysis 5 µg cell proteins were incubated with a maleimide derivate with a 10 kDa mPEG fusion (mPEG-MAL, 5 mM) for 1 h at 4° C. Subsequently samples were resolved on a 4-12% SDS-PAGE gel and analyzed by Western blot using rabbit α-GFP antibodies (Invitrogen).

For the titration of the intramolecular disulfide bonds Bax 1-2/L-6 was incubated for 30 min. in 0.5 mM GSSG and at 4 or 10 mM GSH or 10 mM DTT in 10 mM Tris (pH 7.9, 150 mM NaCl). Then the protein was precipitated with TCA and analyzed by Western blot.

For immonoprecipitation experiments rabbit anti-Bcl-x_L antibody (Santa Cruz) were immobilized onto dynabeads protein A (Invitrogen). Purified proteins were pre-incubated for 30 min. either in the absence of detergent or in the presence of ½ CMC of Triton X-100 (0.01%), Triton X114 (0.005%), CHAPS (0.25%), dodecyl maltoside (0.005%), Tween 20 (0.005%), W-1 (0.005%), sodium deoxycholate (0.25%), octyl glucoside (0.35%) or NP-40 (0.01%) or 2-fold CMC of Triton X-100 (0.04%), Triton X114 (0.02%), CHAPS (1.0 %), dodecyl maltoside (0.02%), Tween 20 (0.02%), W-1 (0.02%), sodium deoxycholate (1.0 %), octyl glucoside (1.4 %) or NP-40 (0.04%) and mixed with 25 µl of antibody-coupled beads and further incubated for 2 h at 4°C. Unbound proteins were removed by washing the beads 3 times with 1 ml of 10 mM Tris, pH 7.9, and 150 mM NaCl or with the same buffer containing the according detergent concentration and analyzed by SDS-PAGE and Western blot.

NMR

^{15}N-labeled recombinant protein for Bax 1-2/L-6 was obtained using the method described previously for the wildtype Bax (Suzuki et al, 2000). The NMR sample contained 0.1mM protein in 50mM potassium phosphate, pH 6.0, 100mM KCl, 1mM EDTA, 10% D_2O. NMR spectra were acquired at 32°C on a Bruker DMX600 spectrometer equipped with a cryoprobe, processed using NMRPipe (Delaglio et al., 1995), and analyzed with PIPP (Garrett et al., 1991).

Proton homonucelar NOEs were obtained from three-dimensional ^{15}N-edited NOESY (Bax and Grzesiek, 1993) with a 90-ms mixing time. The spectra were recorded using 2048×180×60 complex data points in F3, F2 (^{1}H), and F1(^{15}N) dimensions. The spectral widths were 8417.5, 7812.5, and 1600 Hz in the F3, F2, and F1 dimensions, respectively.

The sequential assignment for Bax 1-2/L-6 was carried out using the 3D NOESY spectra. The pattern of crosspeaks between aliphatic protons and amide proton, with reference to the chemical shifts previously obtained from the wild type Bax (Suzuki et al., 2000), were used for the assignment. H^N-H^N crosspeaks observed for some of α-helical regions were also used for sequential connectivity. The sequential assignment was not complete due to substantial chemical shifts change associated with the mutation. However, a majority of resonances were identified to allow for the analysis. The tryptophan sidechain Nε1-Hε1 chemical shifts were identified from the ^{1}H-^{15}N HSQC spectra. The strips from 3D NOESY data were generated using nmrWish application within the NMRPipe package.

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