Detergent-activated BAX Protein Is a Monomer*

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BAX is a pro-apoptotic member of the BCL-2 protein family. At the onset of apoptosis, monomeric, cytoplasmic BAX is activated and translocates to the outer mitochondrial membrane, where it forms an oligomeric pore. The chemical mechanism of BAX activation is controversial, and several in vitro and in vivo methods of its activation are known. One of the most commonly used in vitro methods is activation with detergents, such as n-octyl glucoside. During BAX activation with n-octyl glucoside, it has been shown that BAX forms high molecular weight complexes that are larger than the combined molecular weight of BAX monomer and one detergent micelle. These large complexes have been ascribed to the oligomerization of BAX prior to its membrane insertion and pore formation. This is in contrast to the in vivo studies that suggest that active BAX inserts into the outer mitochondrial membrane as a monomer and then undergoes oligomerization. Here, to simultaneously determine the molecular weight and the number of BAX proteins per BAX-detergent micelle during detergent activation, we have used an approach that combines two single-molecule sensitivity technique, fluorescence correlation spectroscopy, and fluorescence-intensity distribution analysis. We have tested a range of detergents as follows: n-octyl glucoside, dodecyl maltoside, Triton X-100, Tween 20, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid, and cholic acid. With these detergents we observe that BAX is a monomer before, during, and after interaction with micelles. We conclude that detergent activation of BAX is not congruent with oligomerization and that in physiologic buffer conditions BAX can assume two stable monomeric conformations, one inactive and one active.

BAX is a pro-apoptotic member of the BCL-2 protein family. In a simplified apoptosis model, monomeric inactive BAX is localized in the cytoplasm of healthy non-dying cells (1). During apoptosis BAX is activated and translocates to the outer mitochondrial membrane (2) where it inserts as a monomer (3), undergoes oligomerization (4), and forms a pore through which cytochrome c and other apoptotic factors are released into the cytoplasm. Once in the cytoplasm, these apoptotic factors induce the activation of the effector caspases that execute the cell death process. This mechanism, which is generally correct, requires that soluble BAX becomes integrated into the mitochondrial membrane where it forms a functional oligomeric pore capable of cytochrome c release. However, the molecular mechanism of BAX activation remains controversial (5, 6).

It has been understood for some time, but frequently ignored, that activity of the BCL-2 family proteins is exhibited in cells when these proteins are associated with the hydrophobic environment of membranes. Therefore, it has always seemed that attention to the effect of hydrophobic environments on the BCL-2 family proteins would be rewarding. It has been shown that BAX can be directly activated by treatment with nonionic detergents such as n-octyl glucoside, dodecyl maltoside, and Triton X-100 (1, 7). During activation by nonionic detergents, to gain the ability to form pores in a bilayer membrane, BAX needs to undergo a major conformational transition from a globular protein with two pore-forming α-helices 5 and 6 hidden in the protein core (8) to a conformation in which these two helices are exposed and inserted into a lipid membrane (3, 5, 9). The nature of this active conformation of BAX is important for the understanding of the death decision in cells. Most proposals suggest that in a cell this activated form of BAX protein is initiated and maintained by the interactions with other proteins, such as tBID, or by BAX itself as a homooligomer (7, 10).

Nonionic detergents have been commonly used to activate BAX for in vitro studies because they are reliably effective and simple to employ. However, little is known about the detailed molecular mechanism of BAX activation by these detergents and its comparability with in vivo activation of BAX. What is known is that concentrations of detergent above their critical micelle concentration (CMC) are necessary for BAX activation. This suggests that, to be activated, BAX needs to interact with detergent micelles instead of monomeric detergent molecules. For example, in the case of BAX activation by n-octyl glucoside, it has been shown that n-octyl glucoside concentration should be 1% (w/v) (7), which is well above the CMC for this detergent (0.6% w/v) (11). In addition, it has also been shown that above their individual CMC concentrations most BAX-activating detergents produce a change in BAX conformation that can be detected by a conformation-sensitive 6A7 antibody against...
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BAX (1, 12, 13). In cellular experiments this feature of BAX reactivity to 6A7 antibody is commonly associated with the onset of apoptosis (14, 15). However, CHAPS does not generate the antibody-detected conformational change or the activation of BAX. The small micelle size of this detergent (10 kDa) suggests that perhaps BAX cannot adopt an activated state with this detergent. However, cholic acid with even smaller micelle size (4 kDa) can partially activate BAX (1).

Many important detergent properties are associated with micelles. The formation of detergent micelles in solution is concentration-dependent beginning at the CMC. The CMC value for a detergent has practical importance because in most cases only monomers of detergent can be removed by dialysis, and therefore, it is easier to remove detergent monomers for a detergent with high CMC value than for a detergent with low CMC (11). For BAX this same consideration applies to its activation with n-octyl glucoside (CMC ~23 mM) as compared with its activation with Triton X-100 (CMC ~0.25 mM). The ease of dialysis is why, in most cases, OG is used to activate BAX in vitro.

It has been shown by analytical gel filtration that, when incubated with n-octyl glucoside, BAX creates complexes with molecular weight larger than the combined size of a BAX monomer (21 kDa) and an n-octyl glucoside micelle (~26 kDa) (7, 11). It has also been shown that in defined liposomes BAX pore formation requires oligomerization (16). These data combined with the knowledge that oligomerization is important for the biological function of BAX led to a hypothesis that BAX oligomerizes during its detergent activation prior to membrane insertion (7). However, it has been shown that in vivo activated BAX inserts into the outer mitochondrial membrane as a monomer (3), and to create a pore, BAX undergoes oligomerization in this membrane (4). This discrepancy between the oligomeric state of active BAX prior to its insertion into a lipid membrane in vivo (monomer) and in vitro (possibly hexamer or octamer) led us to study the oligomerization state of BAX in detergent micelles. The important issue is whether BAX activation requires protein oligomerization or whether active BAX conformation can be generated from a single protein monomer. To solve this issue we used two single-molecule sensitivity techniques: fluorescence correlation spectroscopy (FCS) (17) and fluorescence-intensity distribution analysis (FIDA) (18). Combined use of FCS and FIDA allows simultaneous determination of the apparent molecular weight and the number of fluorescently labeled BAX monomers per protein-detergent micelle. Our results are consistent with previously established results in which BAX forms high molecular weight protein-detergent micelles with n-octyl glucoside (4) and show that BAX is present as a monomer in these complexes. In addition, we determined the apparent molecular weight and the number of BAX proteins bound per protein-detergent micelles formed by BAX and micelles of five additional detergents (dodecyl maltoside, Triton X-100, Tween 20, cholic acid, and CHAPS). Our data show that BAX is a monomer before, during, and after interaction with the micelles of all tested detergents.

EXPERIMENTAL PROCEDURES

All chemicals used in this paper were from Sigma, unless otherwise stated. All lipids were obtained from Avanti Polar Lipids. Fluorescent dyes for protein labeling were purchased from Molecular Probes.

Protein Constructs, Protein Purification, and Protein Labeling—
The cDNA for human BAX with 19-amino acid truncation on the C terminus (BAXΔC) was fused to the C-terminal intein/chitin-binding domain of the pTYB1 vector (New England Biolabs) (8). Three mutations (G40C, C62A, and C126A) were introduced into each of the DNA plasmids using a QuikChange mutagenesis kit (Stratagene), and the presence of mutations was confirmed by sequencing. The resulting construct and purified protein were dubbed as BAXΔC(G40C). All proteins (human BAXΔC and human BAXΔC(G40C)) were purified from BL21(DE3) Escherichia coli cells without detergent. Briefly, bacterial cultures were grown at 37 °C in Terrific Broth (19) to an A600 of 1.5–2.0, and then the cultures were induced with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside (Research Products International Corp., Mt. Prospect, IL), and the temperature was dropped to 25 °C. After 12–15 h, bacteria were collected via centrifugation; the resulting pellet was resuspended in lysis buffer (phosphate-buffered saline, pH 7.2, 1 mM EDTA, 0.25 mM phenylmethylsulfonyl fluoride), and cells were broken by four passages through a Microfluidizer (Microfluidics) at 1000 bar. Lysate was clarified by centrifugation, and the supernatant containing BAX was incubated with chitin affinity resin (New England Biolabs) overnight at 4 °C on a rocker. Resin was subjected to a high salt wash and then equilibrated in cleavage buffer (10 mM HEPES/NaOH, pH 8.0, 100 mM NaCl, 50 mM dithiothreitol) and incubated for 48 h at 4 °C. The purity of the proteins were assessed by SDS-PAGE. BAXΔC(G40C) was labeled with Bodipy FL c5-maleimide (Molecular Probes) according to the manufacturer’s protocol. Labeled protein was separated from the free dye using a Sephadex G-25 column. The degree of protein labeling was determined using a NanoDrop spectrophotometer (Thermo Scientific) by measuring absorbance at 280 nm (for protein concentration) and 504 nm (for Bodipy FL concentration). Resulting protein was ~ 80% labeled and was stored in EB buffer (10 mM HEPES/KOH, pH 7.2, 100 mM KCl) at 4 °C.

Incubation of BAX with Detergent Micelles—Prior to FCS studies and cytochrome c and carboxyfluorescein release experiments, fluorescently labeled BAXΔC(G40C) or BAXΔC (20–30 μM) was incubated with 2% (w/v) of the indicated detergent in EB buffer for 1 h at 4 °C. For FCS studies of the fluorescently labeled BAXΔC in detergent micelles, after incubation with detergent, protein was diluted to a concentration below 0.5 μM in a solution containing the same detergent concentration as the activated protein. This dilution was done to ensure that the fluorescent signal emitted by the protein sample is within the dynamic range of the detector in the ConFocor 3 (Zeiss, Germany). To remove detergent micelles for the cytochrome c release and for the FCS studies of post-micelle-activated fluorescently labeled BAX, the BAX/detergent mixture was diluted below the CMC of each particular detergent. The disappearance of micelles occurred at different rates (<1 min for OG and longer.
but <60 min for Triton X-100) but was allowed to finish before further studies on the sample (cytochrome c release or FCS) proceeded.

Cytochrome c Release Assay—Mitochondria from HeLa cells were isolated using a previously published procedure (20). Isolated mitochondria were resuspended in 10 mM HEPES/KOH, pH 7.4, 100 mM KCl, 1 mM EGTA, 200 mM sucrose. For the cytochrome c release assay isolated mitochondria were incubated with 100 nM of protein that was detergent-activated but micelle-diluted as described above or inactive protein at 37 °C for 20 min. After the incubation with BAX protein or detergent control solutions, mitochondria were spun down at 10,000 × g for 10 min at 4 °C and then pellet and supernatant fractions were collected and stored at −20 °C. For the cytochrome c release assay we used 0.5 μg of mitochondria in a 30-μl volume. Protein concentration in the preparation of isolated mitochondria was determined by protein assay (Bio-Rad). Cytochrome c content in the pellet and supernatant fractions was determined using TiterZyme EAI human cytochrome c enzyme immunoassay kit (Assay Designs) in combination with Synergy HT plate reader (Bio-Tek Instruments, Inc.).

Surface Plasmon Resonance Studies of BAX Binding to Liposomes—These studies were done using Biacore X instrument (GE Healthcare) at an ambient temperature of 25 °C. Liposomes with lipid composition of DOPC:DOPA:bovine heart cardiolipin (70:20:10 mol %) were prepared using the reverse-phase evaporation method (21) following the procedure described in detail in Ref. 22. The buffer was EB unless otherwise noted. The rest of the experimental conditions, experimental protocol, and data analysis were the same as described previously (22).

Analytical Gel Filtration—Analytical gel filtration experiments were performed on a Superdex 200 HR 10/30 column (GE Healthcare) equilibrated with 20 mM HEPES/KOH, pH 7.5, 300 mM KCl, 0.2 mM dithiothreitol. In the corresponding samples, 2% (w/v) of the indicated detergent was included in the equilibration buffer. Prior to loading the sample on the column, 2.5 nmol of BAXΔC(G40C) were incubated in 2% (w/v) of the corresponding detergent for 1 h at 4 °C. Then samples were loaded into the column and run at 0.5 ml/min. BAXΔC(G40C) elution was detected by light absorption at 280 nm.

Carboxyfluorescein Release Assay—Liposomes containing 50 mM carboxyfluorescein (CF) were prepared following the procedure described previously (22). Incubation with detergent protein was diluted into wells in a black bottom 96-well plate (NUNC, Denmark) using EB buffer. Liposomes (200 nm in diameter, DOPC:DOPA, 70:30 mol %) were added to the wells last. Immediately upon addition of liposomes, measurement of CF fluorescence was done using a Synergy HT plate reader (Bio-Tek Instruments, Inc.). CF releases for all protein samples containing detergent were corrected for the base-line of detergents in the absence of protein.

FCS, FCCS, and FIDA Analyses, Instrumentation, and Measurements—LSM 510 ConFocor 3 system coupled with a Zeiss Axiovert 200 M inverted microscope (Zeiss, Germany) was used for FCS and FIDA experiments. A water immersion C-Apochromat ×40 objective (Zeiss, Germany) focused the excitation light to a diffraction-limited spot. The pinhole size was set to 70 μm for 488 nm excitation laser light. The excitation light of a 25-milliwatt 488 nm argon laser was set at 1% of the acousto-optical tunable transmission. Laser power in the sample was 7 microwatts. In front of the detector LP 530-nm filter was used. For the FCS and FIDA analyses, each sample was measured at least nine times for 50 s. The detection volume was previously calibrated with free Bodipy FL maleimide in solution (diffusion time 22.6 ± 0.5 μs, structure parameter 5.0 ± 0.4).

FCS Analysis—FCS measurements provide three characteristic parameters for interpretation as follows: $\tau_{FP}$, diffusion time of a fluorescent particle (i.e. the average time a particle spends in the detection volume); $N$, number of fluorescent particles in the detection volume, and the counts/particle (cpp) or the average fluorescent intensity per particle. These parameters are extracted by performing a fit of FCS auto-correlation data to one component diffusion model as shown in Equation 1, which takes into account photophysical dynamics of fluorophores,

$$G(\tau) = \frac{1}{N} \cdot \frac{1}{1 - T} \cdot \frac{1}{1 + \frac{T}{\tau_D}} \cdot \frac{1}{1 + \frac{\tau}{\omega^2 D}}$$

(Eq. 1)

In this equation $G(\tau)$ is the auto-correlation function; $\tau$ is the lag time; $T$ is the fraction of molecules in the triplet state; $\tau_T$ is the triplet decay time; $\omega$ is the structure parameter (aspect ratio) of the Gaussian detection volume. Fitting of the FCS auto-correlation curves was done using Equation 1 with software written in MATLAB (Mathworks) using a weighted nonlinear least squares fitting algorithm.

Calculation of Protein-Detergent Micelle Molecular Weight Based on FCS Diffusion Time—For a particle in solution the diffusion time is inversely proportional to the diffusion coefficient as shown in Equation 2,

$$\tau_D = \frac{\omega^2}{4D}$$

(Eq. 2)

In general, protein molecules diffusing in solution are assumed to approximate a spherical shape permitting the Einstein-Stokes relationship to be used in evaluating the diffusion constant as shown in Equation 3,

$$D = \frac{k_BT}{6\pi\eta R}$$

(Eq. 3)
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where, \( k_B \) is the Boltzmann constant; \( T \) is temperature in degrees Kelvin; \( \eta \) is viscosity of solution in which particle is diffusing, and \( R \) is the radius of the spherical particle. The radius of diffusing particle depends on the molecular weight of the particle (\( R \propto (MW) \)). Under these conditions a relationship can be established between the FCS diffusion time of a particle and its molecular weight as shown in Equations 4 and 5.

\[
\tau_D = \frac{\omega^2}{4} \cdot \frac{6 \pi \eta}{k_B T} \cdot R
\]

(Eq. 4)

\[
\tau_D^* = \frac{\omega^2}{4} \cdot \frac{6 \pi \eta}{k_B T} \cdot (MW)^{1/3}
\]

(Eq. 5)

In the case of BAX\(\Delta C\), we know diffusion time of a BAX\(\Delta C\) monomer, \(\tau_{D,\text{monomer}}\), and its molecular weight, \(MW_{\text{monomer}} = 19\) kDa. We also know the diffusion time of BAX\(\Delta C\) protein-detergent micelle, \(\tau_{D,\text{oligomer}}\). Using Equation 5 we can determine the apparent molecular weight of the BAX\(\Delta C\) protein-detergent micelle as shown in Equation 6.

\[
MW_{\text{oligomer}} = MW_{\text{monomer}} \left( \frac{\tau_{D,\text{oligomer}}}{\tau_{D,\text{monomer}}} \right)^3
\]

(FCCS Analysis)—FCCS employing BAX labeled with dyes having nonoverlapping fluorescence spectra and a two-channel collection of data was used to determine the presence or absence of BAX homo-oligomers in detergent micelles (23, 24). Using Bodipy FL maleimide-BAX\(\Delta C\) and Bodipy 630/650 maleimide-BAX\(\Delta C\), we studied cross-correlation in micelle-associated protein. The cross-correlation value, a ratio of the number of the fluorescent complexes containing both proteins of interest to the number of the fluorescent species of one of the proteins, was used to estimate the micelles with more than one BAX molecule. As a positive control and as a calibration sample, the 21-bp-long double-stranded RNA labeled with AlexaFluor 488 and Cy5 on each 3’-end was used (25).

FIDA—Analysis of the fluorescence brightness of a particle can provide an additional measure of the number of fluorescent proteins associated with a detergent micelle. The particle fluorescence brightness determined by FIDA is extracted by fitting the distribution of the number of photon counts and is similar to the cpp value determined by FCS for a monodisperse fluorescent solution. Determination of the particle brightness of the BAX-detergent micelle and comparison with particle brightness of monomeric BAX molecules estimate the number of BAX molecules in detergent micelles. FIDA was performed according to Kask et al. (18). The raw data of photon arrival times were binned to 20 \(\mu s\), and photon counting histograms were constructed. Parameters describing the detection volume were determined in a solution of fluoro-BAX\(\Delta C\) in the absence of detergent. As indicated, histograms were fitted to model functions for one or two components, as described in Kask et al. (18) subtracting a background of 310 Hz for the buffer solution.

RESULTS

Detergent-activated Fluorescently Labeled BAX\(\Delta C\) Can Release Cytochrome \(c\) from Isolated Mitochondria—For the FCS and FIDA experiments, we prepared recombinant, fluorescently labeled human BAX\(\Delta C\) containing a fluorophore at a single cysteine residue (Fig. 1, A and B). Human BAX\(\Delta C\) contains two indigenous cysteines (Cys-62 and Cys-126), which we considered inappropriate for fluorophore conjugation due to structural and functional reasons (8). Previously full-length BAX with G40C, C62A, and C126A mutations has been reported to be functional in vivo (3). Therefore, we removed cysteines, Cys-62 and Cys-126, by mutation to alanine and added an additional cysteine residue in place of glycine 40 creating BAX\(\Delta C\)(G40C) (Fig. 1A). BAX\(\Delta C\)(G40C) labeled with Bodipy FL maleimide fluorophore became fluoro-BAX\(\Delta C\).

To check the biological activity of the fluoro-BAX\(\Delta C\) protein, we studied its ability to release cytochrome \(c\) using mitochondria isolated from HeLa cells (Fig. 1C). Our results show that fluoro-BAX\(\Delta C\) activated with 2% (w/v) n-octyl glucoside releases cytochrome \(c\) from isolated mitochondria similar to BAX\(\Delta C\) but with slightly lower efficiency. For both proteins significant cytochrome \(c\) release required detergent activation, suggesting that the detergent activation of fluoro-BAX\(\Delta C\) was comparable with the detergent activation of the BAX\(\Delta C\) (7, 12, 26). In each cytochrome \(c\) release experiment the final concentration of n-octyl glucoside was 0.005% (w/v) or lower, which is well below the CMC for this detergent (0.6% w/v) (11), so that detergent micelles played no role in the release of cytochrome \(c\) or the maintenance of the active BAX\(\Delta C\) conformation during the assay.

Surface Plasmon Resonance Studies of Membrane Binding and Integration by BAX\(\Delta C\) and Fluor-BAX\(\Delta C\)—Direct analysis of the membrane binding and integration by detergent-acti-
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The obtained results show that in the absence of detergents following the procedure of Antonsson treated with either 2% (w/v) n-octyl glucoside or CHAPS (Fig. 3). The obtained results show that in the absence of detergents BAXΔC(G40C) eluted as a monomer with a molecular mass of slightly less than 25 kDa (Fig. 3A), whereas in the presence of 2% (w/v) n-octyl glucoside, protein eluted at a molecular mass slightly below 440 kDa (Fig. 3C). In contrast, when BAXΔC(G40C) was incubated with and eluted in the presence of 2% (w/v) CHAPS in the column, protein eluted mostly (73%) as a monomer in a broad peak (Fig. 3B). These results are consistent with previously reported analytical gel filtration of BAXΔC (7) and show that mutant BAXΔC(G40C) interacts

FIGURE 2. Surface plasmon resonance data of fluor-BAXΔC and BAXΔC binding to cardiolipin-containing liposomes (200 nm in diameter; DOPC:DOPA:CL 70:20:10 mol %). Increasing concentrations of protein activated with 2% (w/v) n-octyl glucoside was flowed over immobilized liposomes. Protein accumulation on the surface of the liposomes is shown with response units (RU) (28). A, data for fluor-BAXΔC binding. B, data for BAXΔC binding. C, results of concentration dependence analysis of protein integration into liposomes for BAXΔC (squares) and fluor-BAXΔC (triangles).

FIGURE 3. Analytical gel filtration of BAXΔC(G40C) incubated with indicated detergents. BAXΔC(G40C) was incubated with 2% (w/v) of indicated detergent for 1 h at 4 °C. BAXΔC(G40C) incubated with detergent was passed through a Superdex-200 column equilibrated with 10 mM HEPES/KOH, pH 7.0, 300 mM KCl buffer containing 2% (w/v) of the same detergent in which BAX was incubated. The Superdex-200 column was calibrated with two protein standards. A, BAXΔC(G40C) without detergent. B, BAXΔC(G40C) + 2% (w/v) CHAPS. C, BAXΔC(G40C) + 2% (w/v) n-octyl glucoside. D, standards (chymotrypsinogen, 25 kDa, and ferritin, 438.7 kDa).

Analytical Gel Filtration of BAXΔC(G40C) with n-OctylGlucoside or CHAPS Present—To show that fluor-BAXΔC interacts with detergent micelles of n-octyl glucoside and CHAPS comparably with BAXΔC, we performed analytical gel filtration following the procedure of Antonsson et al. (7). We carried out our analytical gel filtration studies using BAXΔC(G40C) incubated with either 2% (w/v) n-octyl glucoside or CHAPS (Fig. 3). The obtained results show that in the absence of detergents...
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with micelles of n-octyl glucoside and CHAPS similarly as BAXΔC.

Activation of BAX with Nonionic Detergents—Compared with the amount of protein required for the analytical gel filtration studies, the amount of fluor-BAXΔC required for the FCS and FIDA analyses is at least 100 times lower. Therefore, we decided to extend the range of the detergents used in our study. We chose n-octyl glucoside, CHAPS, Triton X-100, dodecyl maltoside, and Tween 20 because these detergents have specific and known effects on BAXΔC activity (7, 26). We also chose cholic acid because of its similarity in structure to CHAPS, because it is a physiologic detergent and because it can activate BAX (1). In all cases the 2% (w/v) concentration of the detergent was well above the CMC (11).

Before proceeding to the FCS and FIDA studies, we first tested the ability of all chosen detergents to activate BAXΔC using an assay of carboxyfluorescein release from liposomes (Fig. 4A) (22). For these experiments we used recombinant human BAXΔC purified from E. coli cells without detergent. This BAXΔC protein was monomeric (23 ± 4 kDa as determined by dynamic light scattering) and had very low (<10%) carboxyfluorescein release activity. Upon 1 h of incubation at 4 °C with selected nonionic detergents (n-octyl glucoside, dodecyl maltoside, Triton X-100, and Tween 20), BAXΔC released carboxyfluorescein from liposomes (Fig. 4A), indicating that protein became activated. Incubation with cholic acid, an ionic detergent, also resulted in activation of BAXΔC. However, upon a similar 1-h incubation at 4 °C with CHAPS (detergent known for its inability to activate BAX) no significant carboxyfluorescein release was observed.

Similar carboxyfluorescein release results were obtained for BAXΔC(G40C) incubated with all six detergents (Fig. 4B). Comparison of the maximum carboxyfluorescein release values for both proteins after 90 min presents two instructive observations (Fig. 4C). First, upon incubation with all detergents BAXΔC has almost a 20% higher carboxyfluorescein releasing activity than its mutant, BAXΔC(G40C). Second, for both proteins incubation with Triton X-100 resulted in the most activated form of BAX followed by n-octyl glucoside, dodecyl maltoside, cholic acid, and Tween 20, whereas CHAPS failed to activate either of the two proteins.

CD Spectroscopy on BAXΔC in Detergent Micelles—Circular dichroism measurements were performed to determine whether any significant secondary structure changes occur in BAXΔC during interaction with detergent micelles. BAXΔC without detergent produced CD spectra with strong α-helical pattern (Fig. 5). In the presence of micelles of Triton X-100, Tween 20, or cholic acid, no significant changes in the BAXΔC spectra were observed. However, in the presence of micelles of n-octyl glucoside or dodecyl maltoside, a slight increase in the α-helical content of BAXΔC CD spectra was observed. The CD spectra of BAXΔC in the presence of CHAPS were not collected because of high CD signal from CHAPS (because of presence of amide bond in CHAPS structure).

FCS Detection Volume Is Not Affected by the Presence of Detergents—The maintenance of consistent detection volume is critical for accurate comparison of FCS characteristics of different particles. Size and shape of the FCS detection volume depend on a number of parameters, one of which is the refractive index of solution (29, 30). The latter can be affected by the presence of detergents leading to the distortion of the FCS detection volume.
Commonly AlexaFlour 488 dye is used for calibration of the FCS detection volume (30, 31). During this calibration procedure the diffusion time ($\tau_D$) of the dye molecules and the structure parameter of the FCS detection volume ($\omega$) were obtained by fitting the measured autocorrelation curve using Equation 1. Using such analysis for AlexaFlour 488 diffusing freely in solution in the absence of detergent, we get the following values: $\tau_D = 22.7 \pm 0.9$ μs, $\omega = 5.5 \pm 0.8$. To determine whether the presence of detergent has an effect on our FCS measurements, we determined $\tau_D$ and $\omega$ values for AlexaFlour 488 in solution containing increasing concentrations of $n$-octyl glucoside detergent (Fig. 6). The results of these experiments show that over the range of 0–5% (w/v) of $n$-octyl glucoside, the diffusion time of the dye and the structure parameter of the detection volume do not change indicating that the FCS detection volume is not affected by the detergent presence. Similar experiments were done with the rest of the detergents, and they yielded analogous results (data not shown).

FCS Characterization of Fluor-BAXΔC in Detergent Micelles—
We used fluorescence correlation spectroscopy to confirm the detergent-dependent change in the apparent molecular weight of BAXΔC protein as seen by analytical gel filtration. The diffusion characteristics of fluor-BAXΔC were studied in the absence and in the presence of 2% (w/v) of selected detergents (Fig. 7 and Table 1). The FCS diffusion times were obtained by fitting the autocorrelation curves (Fig. 7) and are shown in Table 1. Fluor-BAXΔC incubated with 2% (w/v) of either $n$-octyl glucoside, dodecyl maltoside, Triton X-100, or Tween 20 had a significant increase in diffusion time, $\tau_D$, compared with the diffusion time of fluor-BAXΔC monomer in the absence of detergent. In contrast, the diffusion time of fluor-BAXΔC in the presence of 2% (w/v) CHAPS or cholic acid did not increase significantly (Table 1).

Using Equation 6 and the diffusion time of the fluorescent particles, we calculated the apparent molecular weight of the BAXΔC-detergent micelle complexes for each detergent (Table 1). The apparent molecular weight of fluor-BAXΔC in the presence of $n$-octyl glucoside and CHAPS calculated from the FCS data are the same as the molecular weight obtained by analytical gel filtration of BAX in the presence of these detergents (Fig. 3). The large molecular weight complexes of BAX generated in the presence of $n$-octyl glucoside, dodecyl maltoside, and Triton X-100 detergents were significantly larger than the sum of BAX and micelle of the respective detergents.

To determine the size of fluor-BAXΔC after interaction with detergent micelles, we removed micelles by diluting detergent concentrations below CMC. In most cases this resulted in disso-
**TABLE 1**
Results of the FCS studies with fluor-BAXΔC-detergent micelles
The molecular weight of the fluor-BAXΔC-detergent micelles was calculated using Equation 6.

| Sample, fluor-BAXΔC+ | Detergent concentration 2% (w/v) | After detergent dilution |
|-----------------------|----------------------------------|--------------------------|
|                       | Molecular mass | τD | Fluorescence brightness for 25 μm BAX | Fluorescence brightness for 3 μm BAX | Detergent concentration, (w/v) | τD | Molecular mass |
| No detergent          | 91 ± 4 μs 4 kDa | 0% | 91 ± 4 kHz | 91 ± 4 kHz | 0.003 | 0% | 100 ± 5 kHz |
| n-Octyl glucoside     | 223 ± 5 μs 280 ± 12 kHz | 0.003 | 100 ± 5 kHz | 100 ± 5 kHz | 0.003 | 100 ± 5 kHz |
| Triton X-100          | 366 ± 8 μs 1236 ± 223 kHz | 0.003 | 158 ± 4 kHz | 158 ± 4 kHz | 0.003 | 158 ± 4 kHz |
| Dodecyl maltoside     | 262 ± 21 μs 453 ± 31 kHz | 0.003 | 90 ± 4 kHz | 90 ± 4 kHz | 0.003 | 90 ± 4 kHz |
| CHAPS                 | 115 ± 4 μs 38 ± 1 kHz | 0.004 | 87 ± 3 kHz | 87 ± 3 kHz | 0.004 | 87 ± 3 kHz |
| Cholic acid           | 124 ± 5 μs 48 ± 1 kHz | 0.003 | 86 ± 2 kHz | 86 ± 2 kHz | 0.003 | 86 ± 2 kHz |
| Tween 20              | 161 ± 12 μs 105 ± 2 kHz | 0.003 | 112 ± 3 kHz | 112 ± 3 kHz | 0.003 | 112 ± 3 kHz |

**TABLE 2**
Analysis of the fluorescence-intensity distribution of the fluor-BAXΔC protein detergent-micelle complexes
Protein (at the concentration indicated in the table) was preincubated with detergent for 1 h at 4 °C. Upon incubation with detergent, the protein was diluted below 0.5 μM into a solution containing the identical detergent concentration as during activation. The measurements were done immediately upon this dilution. Mean and standard deviations of the brightness values were calculated based on nine measurements at 50 s each.

| Sample, fluor-BAXΔC+ | Detergent concentration 2% (w/v) | After detergent dilution |
|-----------------------|----------------------------------|--------------------------|
|                       | Fluorescence brightness | Fluorescence brightness | Detergent concentration (w/v) | Fluorescence brightness |
|                       | kHz                    | kHz | %                  | kHz | GHz | MHz |
| No detergent          | 7.4 ± 0.2 kHz | 7.2 ± 0.3 kHz | 0% | 55 ± 0.1 kHz | 55 ± 0.1 kHz |
| n-Octyl glucoside     | 10.5 ± 0.1 kHz | 10.4 ± 0.1 kHz | 0.003 | 4.7 ± 0.2 GHz | 4.7 ± 0.2 GHz |
| Triton X-100          | 8.5 ± 0.1 kHz | 10 ± 0.1 kHz | 0.004 | 7.1 ± 0.1 GHz | 7.1 ± 0.1 GHz |
| Dodecyl maltoside     | 5.9 ± 0.1 kHz | 5.8 ± 0.1 kHz | 0.003 | 5.0 ± 0.1 MHz | 5.0 ± 0.1 MHz |
| CHAPS                 | 3.49 ± 0.06 kHz | 3.47 ± 0.09 kHz | 0.003 | 5.6 ± 0.1 MHz | 5.6 ± 0.1 MHz |
| Cholic acid           | 4.70 ± 0.24 kHz | 4.71 ± 0.09 kHz | 0.003 | 4.71 ± 0.09 MHz | 4.71 ± 0.09 MHz |
| Tween 20              | 4.70 ± 0.24 kHz | 4.71 ± 0.09 kHz | 0.003 | 4.71 ± 0.09 MHz | 4.71 ± 0.09 MHz |

Comparison of these fluorescence brightness values shows that BAX is a monomer in all cases (Table 2). However, in case of dodecyl maltoside and Triton X-100, the calculated values of fluorescence brightness per protein-detergent micelle were 90% higher than the fluorescence brightness of the fluor-BAXΔC monomer. It is possible that BAX is dimerized in micelles of these detergents. Interestingly, in the presence of cholic acid or Tween 20 protein fluorescence brightness decreased by 36 and 15%, respectively, whereas in the presence of the rest of the detergents protein fluorescence brightness increased or stayed the same as in the absence of detergents. Therefore, because protein fluorescence brightness was clearly affected by the detergent we studied this effect directly.

For three detergents where micelle size was significantly increased by the addition of BAX (n-octyl glucoside, dodecyl maltoside, and Triton X-100), we varied the degree of protein to detergent ratio while holding either detergent or protein concentration constant. First, we varied the concentration of the fluor-BAXΔC from 25 to 3 μM while keeping detergent concentration constant at 2% (w/v). We reasoned that, if fluor-BAXΔC forms oligomers in micelles, the decrease in protein concentration while keeping detergent concentration constant would lead to a change, e.g., reduction, of the oligomeric state of protein in detergent micelles. Such change in the oligomeric state of protein with increasing detergent concentration has been previously demonstrated for some transmembrane peptides in detergent micelles (32). In this case there was no significant change in the fluorescence brightness of the fluor-BAXΔC-detergent micelles (Table 2).

Second, we measured the effect of increasing detergent concentration on the fluorescence brightness of fluor-BAXΔC at constant protein concentration. If protein fluorescence brightness increases because of the presence of detergent, then the total fluorescence intensity of the sample containing constant protein concentration will increase with increasing detergent concentration. As shown in Fig. 8A (empty circles), for constant protein concentration in the presence of increasing n-octyl glucoside concentrations total fluorescence intensity increases. Furthermore, fluorescence brightness per particle of protein-detergent micelles determined by single-component fitting of
resulting FCS autocorrelation curves also increased (Fig. 8A, filled squares). The ratio of the total fluorescence intensity to the fluorescence brightness per particle (also known as cpp) represents the average number of particles in the FCS observation volume. As expected, this number stayed constant for all n-octyl glucoside concentrations clearly showing that increase in n-octyl glucoside concentration leads to increase in protein fluorescence brightness. For n-octyl glucoside the FCS and FIDA yield similar protein fluorescence brightness values (Table 2 and Fig. 8). Such an agreement between FCS and FIDA results further shows that fluor-BAXΔC is present as a monomer in n-octyl glucoside micelles.

Analogous detergent titration experiments were performed for the rest of the detergents giving the same result that protein fluorescence brightness of the fluor-BAXΔC is changing with increasing concentrations of detergent (Fig. 8C). In addition, in the n-octyl glucoside titration experiment gradual increase in the protein diffusion time was observed together with increase in protein fluorescence brightness. This observation suggests that with increasing n-octyl glucoside concentration protein–detergent micelles grow in size (Fig. 8B).

For all tested detergents after micelle removal by dilution, the fluorescence brightness of the fluor-BAXΔC returned to that of a protein monomer. However, upon removal of Triton X-100 protein fluorescence brightness decreased but was still 29% higher than that for the protein monomer in the absence of the detergent. This result suggests incomplete dissociation of the Triton X-100 molecules from BAX and is in accordance with the FCS diffusion time, which shows that upon Triton X-100 dilution below CMC, the apparent molecular weight of the fluor-BAXΔC was higher than that of a protein monomer.

**FCCS Analysis of BAXΔC in Detergent Micelles**—To show the absence of BAX oligomerization in detergent micelles, FCCS analysis was used. FCCS is a variation of the FCS that allows determination of the degree of interaction between two fluorescent molecules or macromolecular assemblies. In these experiments we used two types of fluorescently labeled BAX as follows: fluor-BAXΔC and Bodipy 630/650 maleimide-BAXΔC. The degree of interaction between these two proteins in detergent micelles is proportional to the cross-correlation value that is determined by FCCS analysis. The results of these FCCS experiments show low cross-correlation values between two fluorescent forms of BAXΔC compared with the theoretically predicted cross-correlation value of BAXΔC dimer. These FCCS results suggest the absence of interaction between the two fluorescent variants of BAXΔC in detergent micelles of all tested detergents (Fig. 9).

**DISCUSSION**

**Fluorescently Labeled BAXΔC Is Active**—To apply FCS to the study of BAX, we generated a soluble form of the protein. We employed the BAXΔC in these solution studies of the protein activity and oligomerization because we found that it remained in solution and could be activated by detergent throughout all the manipulations that were used in these studies. To label this protein with a fluorophore, we substituted endogenous cysteines with alanine (C62A and C126A) and converted glycine 40 in an unstructured region of BAX to a cysteine. The position...
of these changes in the \( \text{BAX}^{\Delta C} \) protein are shown not to interfere with the function of the full-length \( \text{BAX} \) in vivo (3), and the resulting protein was well expressed by bacteria. The added cysteine was exposed in the engineered protein and formed disulfide cross-linked \( \text{BAX}^{\Delta C}(G40C) \) dimers at high concentrations (data not shown). After substitution with the fluorescent probe, the disulfide formation did not occur. Therefore, further characterization of the activity of the engineered protein was performed on the fluor-\( \text{BAX}^{\Delta C} \).

To assess the functional capability of fluor-\( \text{BAX}^{\Delta C} \), we tested its ability to promote cytochrome \( c \) release from isolated mitochondria as compared with that of \( \text{BAX}^{\Delta C} \). Because our engineered \( \text{BAX}^{\Delta C} \) was based upon the human protein, we used mitochondria isolated from HeLa cells to study cytochrome \( c \) release (33). In these experiments we observed that fluor-\( \text{BAX}^{\Delta C} \) releases \( 71 \pm 15\% \) of the mitochondrial cytochrome \( c \) when activated with \( n \)-octyl glucoside, whereas \( \text{BAX}^{\Delta C} \) releases \( 100 \pm 30\% \) (Fig. 1). Based on this result we conclude that mutation and fluorophore labeling of \( \text{BAX}^{\Delta C} \) alter pore forming activity of this protein but only in a minor way. In addition, to these experiments, we also studied the ability of various detergents to activate \( \text{BAX}^{\Delta C} \) and its mutant in liposomes (Fig. 4). Again we saw a reduction in pore activation by the mutant protein. However, it is clear that the reduction is consistent across the range of tested detergents (Fig. 4C).

Finally, we compared the integration of \( \text{BAX}^{\Delta C} \) and its mutant into lipid membranes using SPR. We have recently developed methods to quantitatively study binding and integration of \( \text{BAX} \) to membranes using SPR (22). In those studies protein integration into lipid membranes was critical for pore formation and only occurred after protein incubation with \( n \)-octyl glucoside. In the SPR comparison mutant of \( \text{BAX}^{\Delta C} \), \( \text{BAX}^{\Delta C}(G40C) \) was fully functional and integrated into lipid membranes as well as \( \text{BAX}^{\Delta C} \) (Fig. 2). Taken together these studies indicate that \( \text{BAX}^{\Delta C}(G40C) \) is fully functional, but its specific activity for pore formation is slightly lessened by the introduced mutations, possibly due to lower oligomerization rate or changed pore topology.

\textit{Fluor-\( \text{BAX}^{\Delta C} \) Forms High Molecular Weight Protein-Detergent Micelles with Most Activating Detergents—}\( \text{BAX}^{\Delta C} \) has been shown to form high molecular weight complexes with \( n \)-octyl glucoside but not with CHAPS (7). To show that our mutated BAX, \( \text{BAX}^{\Delta C}(G40C) \), can form high molecular weight complexes with \( n \)-octyl glucoside and not with CHAPS, we performed analytical gel filtration studies similar to those in Ref. 7. The results of these analytical gel filtration experiments show that in the presence of 2\% (w/v) of \( n \)-octyl glucoside, \( \text{BAX}^{\Delta C}(G40C) \) elutes at a molecular mass slightly below 440 kDa, whereas in the presence of 2\% (w/v) CHAPS this protein elutes mostly as a monomer (73\%). These results are similar to the previously published results for \( \text{BAX}^{\Delta C} \) indicating that mutations introduced into \( \text{BAX}^{\Delta C} \) do not interfere with formation of high molecular weight complexes of this protein with \( n \)-octyl glucoside.

Next we proceeded to measure the molecular weight of fluor-\( \text{BAX}^{\Delta C} \) in the presence of \( n \)-octyl glucoside and CHAPS using FCS. In the FCS experiments we measured the diffusion time, \( \tau_{Dp} \), of fluor-\( \text{BAX}^{\Delta C} \) molecules in the presence of 2\% (w/v) of these detergents. Then by using Equation 6, we calculated the molecular weight of the fluor-\( \text{BAX}^{\Delta C} \) protein-detergent micelle complexes (Table 1). The molecular weights of \( \text{BAX}^{\Delta C} \) protein-detergent micelle complexes determined by FCS and analytical gel filtration were fairly similar, further demonstrating that mutagenesis and fluorescent labeling of \( \text{BAX}^{\Delta C} \) do not affect the interactions of this protein with \( n \)-octyl glucoside and CHAPS. This result also shows that FCS can be used to determine the apparent molecular weight \( \text{BAX}^{\Delta C} \) with other detergents. Therefore, we extended the range of detergents used in our study to dodecyl maltoside, Triton X-10, Tween 20, and cholic acid. As a result of these FCS studies, we determined that fluor-\( \text{BAX}^{\Delta C} \) forms high molecular weight complexes in the presence of activating nonionic detergents (\( n \)-octyl glucoside, dodecyl maltoside, Triton X-100, and Tween 20). However, in the presence of cholic acid, activating ionic detergent, fluor-\( \text{BAX}^{\Delta C} \) does not form high molecular weight complexes. Fluor-\( \text{BAX}^{\Delta C} \) also did not form high molecular weight complexes with CHAPS, a zwitterionic detergent known for its inability to activate \( \text{BAX} \). In addition, all of the tested activating detergents did not induce significant secondary structure changes in the \( \text{BAX}^{\Delta C} \) protein (Fig. 5).

For the studies of \( \text{BAX}^{\Delta C} \) pore formation in lipid membranes, it is desirable that detergent is removed after fluor-\( \text{BAX}^{\Delta C} \) activation because detergent at concentrations above the CMC can alter the integrity of lipid membranes. Therefore, it is important to know the molecular weight of \( \text{BAX}^{\Delta C} \) after interaction with detergent micelles. To determine the latter, excess detergent was removed from fluor-\( \text{BAX}^{\Delta C} \) by dilution below the CMC. After removal of detergent micelles, the molecular weight of fluor-\( \text{BAX}^{\Delta C} \) decreased to that of the fluor-\( \text{BAX}^{\Delta C} \) monomer for most of the detergents (Table 1). The results for fluor-\( \text{BAX}^{\Delta C} \) treated with Triton X-100 were an exception. Upon Triton X-100 dilution the molecular weight of fluor-\( \text{BAX}^{\Delta C} \) was five times larger than that of fluor-\( \text{BAX}^{\Delta C} \) monomer. There are two possible explanations for this result. The first explanation is based on incomplete dissociation of Triton X-100 molecules bound to fluor-\( \text{BAX}^{\Delta C} \) upon detergent dilution, and the second explanation is possible formation of fluor-\( \text{BAX}^{\Delta C} \) homo-oligomers. To differentiate between these two explanations and to determine the stoichiometry of fluor-\( \text{BAX}^{\Delta C} \) before, during, and after interactions with detergent micelles we performed FIDA.

\textit{\( \text{BAX}^{\Delta C} \) Is Present as a Monomer in Protein-Detergent Micelles—}To further investigate the stoichiometry of the fluor-\( \text{BAX}^{\Delta C} \)-detergent micelles, we performed FIDA on the FCS data (Table 2). The fluorescence brightness of the individual protein-detergent micelles varied \( \sim 90\% \) as a function of the detergent used, but all of them contained one \( \text{BAX} \) protein molecule. The reason for such variation of the fluorescence brightness of the protein monomer in various detergents was because of the enhancement or quenching of the Bodipy FL fluorophore brightness upon transfer into the hydrophobic environment of a detergent micelle. Similar effects of the fluorophore brightness enhancement were reported previously for the fluorescently labeled diphtheria toxin T-domain interacting with detergent micelles (34). In our case there appears to be an enhancement of the fluorescence brightness of fluor-
BAXΔC in the presence of n-octyl glucoside, dodecyl maltoside, and Triton X-100 detergents, and a decrease in protein fluorescence brightness in the presence of cholic acid and Tween 20. Studies of detergent titration into constant protein concentration show that this effect is because of the enhancement of the fluorophore brightness and not because of protein oligomerization (Fig. 8). In addition, detergent dilution studies show that upon detergent dilution, which leads to the dissolution of the fluor-BAXΔC-detergent micelles, the fluorophore brightness of the protein returns to that of the protein monomer prior to the interaction with detergent micelles (Table 2).

The FIDA results mean clearly that before, during, and after interaction with detergent micelles the fluor-BAXΔC protein is a monomer. This observation suggests that BAXΔC interaction with micelles is fundamentally different from the interaction that it establishes in bilayer membranes. In a bilayer membrane BAXC assumes conformation, which allows assembly of homo-oligomers resulting in pore formation. In contrast, in a detergent micelle BAXΔC assumes conformation that in the case of nonionic detergents leads to a dramatic enlargement of the resulting protein-detergent micelle without necessary protein homo-oligomerization (Table 1 and Fig. 3). Because this increase in size can no longer be attributed to additional BAX molecules per micelle, it must be due to the incorporation of additional detergent.

To study the interaction of the fluor-BAXΔC with detergent micelles, we used detergents at 2% (w/v) concentration, which is well above the CMC for all tested detergents. If expressed in molar units, this detergent concentration will be on the order of millimolar for all tested detergents (n-octyl glucoside, 68 mM; Triton X-100, 32 mM; CHAPS, 33 mM; dodecyl maltoside, 40 mM). In contrast, in all of the experiments the concentration of fluor-BAXΔC during incubation with detergent was 20–30 μM. Solution containing 20–30 μM protein and >30 mM detergent has an excess of detergent micelles over the number of protein molecules. Fleming (32) has shown that an excess of detergent micelles moves the protein to a more dissociated state. Fleming (32) also shows that for a glycoporphin A transmembrane α-helix, 40% of dimers of this α-helix are detected for a 40 times lower mole ratio of protein to detergent than was used in our experiments. This demonstrates that if fluor-BAXΔC is forming oligomers in detergent micelles, then we would not have been prevented from detecting them. The above outlined argument together with the FSC results (Fig. 9) led us to the conclusion that fluor-BAXΔC is present as a monomer in detergent micelles.

The outcome of this conclusion is that during and after detergent activation BAXΔC is a monomer. Therefore, this protein has two stable monomeric conformations in physiological buffer conditions, one inactive and one active. Second, this implies that the detergent-activated species of BAX is a monomeric protein, and the large molecular weight in the presence of the micelles of nonionic detergents is a result of the detergent component of the complex.

Consequences for the Physiological Activation of BAX—These studies suggest that detergent activation of BAX is not merely a mimicry of the physiologic BAX activation. The characteristics of the detergent activation indicate two intriguing characteris-

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