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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[2] 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 nondying 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 homo-oligomer (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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[2] The abbreviations used are: BAX, BCL-2-associated protein x; FCS, fluorescence correlation spectroscopy; FCCS, fluorescence cross-correlation spectroscopy; FIDA, fluorescence-intensity distribution analysis; BAXΔC, BAX with 19 amino acid truncation on the C terminus; BAXΔC(G40C), BAXΔC with G40C, C62A, C126A mutations; fluor-BAXΔC, fluorescently labeled BAXΔC(G40C); CMC, critical micelle concentration; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPA, 1,2-dioleoyl-*sn*-glycero-3-phosphate (monosodium salt); CL, bovine heart cardiolipin; SPR, surface plasmon resonance: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; CF, carboxyfluorescein; cpp, counts/particle.

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 A_{600} 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 c_5-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 fluor-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 fluor-BAXΔC, 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 immunometric assay 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 CF release in the presence of detergents without protein. Data were analyzed in Origin 6.1 (OriginLab Corp.).

Circular Dichroism Spectroscopy—Samples for CD spectroscopy were prepared at 5 μM protein concentration in 10 mM potassium phosphate buffer, pH 7.0. Detergents (*n*-octyl glucoside (2% w/v), dodecyl maltoside (0.6% w/v), Triton X-100 (0.08%), Tween 20 (0.04% w/v), cholic acid (2% w/v)) were added to protein samples 1 h prior to measurements and stored at 4 °C. Samples were measured at 20 °C on Jasco J-715 spectropolarimeter using a 1-mm path length cell. Data were collected every 0.1 nm at 50 nm/min scan speed from 260 to 200 nm, and results were averaged from five scans. Because of high CD signal from Triton X-100 at 200 nm for samples containing this detergent, data were collected from 240 to 205 nm. Spectra 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: τ_D, 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,

$$G(\tau) = \frac{1}{N} \cdot \left(1 + \frac{T \exp(-\tau/\tau_T)}{1 - T}\right) \cdot \left(1 + \frac{\tau}{\tau_D}\right)^{-1} \cdot \frac{1}{\sqrt{1 + \tau/\omega^2\tau_D}} \quad \text{(Eq. 1)}$$

In this equation $G(\tau)$ is the auto-correlation function; τ is the lag time; T is the fraction of molecules in the triplet state; τ_T triplet decay time; ω 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,

$$\tau_D = \frac{\omega^2}{4D} \quad \text{(Eq. 2)}$$

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,

$$D = \frac{k_B T}{6\pi\eta R} \quad \text{(Eq. 3)}$$

where, k_B is the Boltzmann constant; T is temperature in degrees Kelvin; η 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 ($R \propto (\mathrm{MW})$). 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.

$$\tau_D = \frac{\omega^2}{4} \cdot \frac{6\pi\eta}{k_B T} \cdot R \qquad \text{(Eq. 4)}$$

$$\tau_D \propto \frac{\omega^2}{4} \cdot \frac{6\pi\eta}{k_B T} \cdot (MW)^{1/3} \qquad \text{(Eq. 5)}$$

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

$$MW_{\mathrm{oligomer}} = MW_{\mathrm{monomer}} \left(\frac{\tau_{\mathrm{D,oligomer}}}{\tau_{\mathrm{D,monomer}}} \right)^3 \qquad \text{(Eq. 6)}$$

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-BAXΔC and Bodipy 630/650 maleimide-BAXΔC, 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 Alexa-Fluor 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 fluorophore 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 was binned to 20 μs, and photon counting histograms were constructed. Parameters describing the detection volume were determined in a solution of fluor-BAXΔC 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 BAXΔC Can Release Cytochrome c from Isolated Mitochondria—For the FCS and FIDA experiments, we prepared recombinant, fluorescently labeled human BAXΔC containing a fluorophore at a single cysteine residue (Fig. 1, *A* and *B*). Human BAXΔC 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 BAXΔC(G40C) (Fig. 1*A*). BAXΔC(G40C) labeled with Bodipy FL maleimide fluorophore became fluor-BAXΔC.

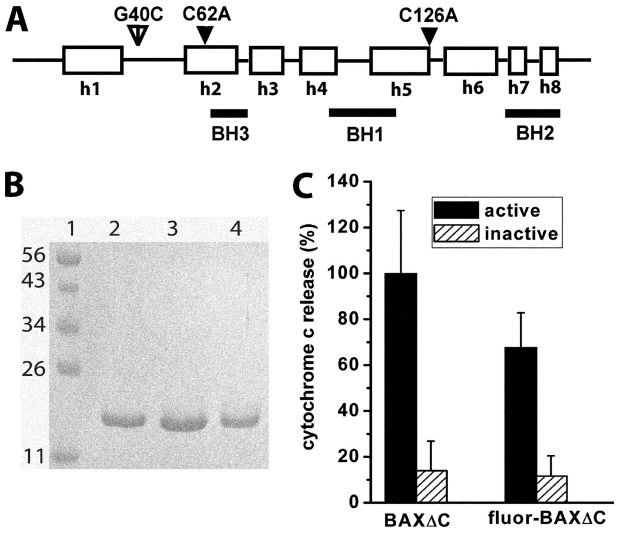

FIGURE 1. *A,* schematic structure of human BAXΔC(G40C). Introduced mutations and their relative position with respect to the helices and BCL-2 homology domains are indicated with *arrows*. *B,* SDS-PAGE of purified recombinant proteins; protein standards with molecular weight indicated (*lane 1*), human BAXΔC(G40C) (*lane 2*), human BAXΔC (*lane 3*), and human fluor-BAXΔC (*lane 4*). *C,* cytochrome *c* release from mitochondria isolated from HeLa cells. Mitochondria isolated from HeLa cells were incubated for 20 min at 37 °C with 100 nM of the indicated recombinant protein. Where indicated, protein was activated by incubation with 2% (w/v) *n*-octyl glucoside for 1 h at 4 °C after which the protein/detergent mixture was diluted to obtain a final detergent concentration of 0.5% (w/v) and the 100 nM protein concentration used in the assays. Cytochrome *c* content of each fraction was normalized for the basal release of cytochrome *c* in mitochondria samples with only detergent added and in mitochondria samples without detergent or protein added. *Error bars* were calculated based on three independent experiments.

To check the biological activity of the fluor-BAXΔC protein, we studied its ability to release cytochrome *c* using mitochondria isolated from HeLa cells (Fig. 1*C*). Our results show that fluor-BAXΔC activated with 2% (w/v) *n*-octyl glucoside releases cytochrome *c* from isolated mitochondria similar to BAXΔC but with slightly lower efficiency. For both proteins significant cytochrome *c* release required detergent activation, suggesting that the detergent activation of fluor-BAXΔC was comparable with the detergent activation of the BAXΔC (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 BAXΔC conformation during the assay.

Surface Plasmon Resonance Studies of Membrane Binding and Integration by BAXΔC and Fluor-BAXΔC—Direct analysis of the membrane binding and integration by detergent-acti-

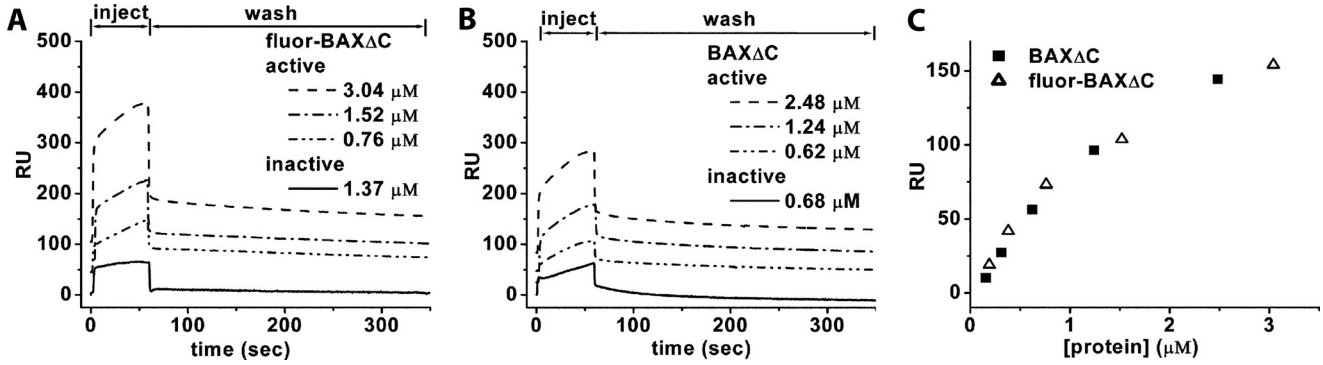

FIGURE 2. **Surface plasmon resonance data of fluor-BAXΔC and BAXΔC binding to cardiolipin-containing liposomes (200 nm in diameter; DOPC: DOPA:CL 70:20:10 mol %).** Increasing concentrations of protein activated with 2% (w/v) *n*-octyl glucoside was flowed over immobilized liposomes. Protein accumulation 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*).

vated fluor-BAXΔC was compared with that of BAXΔC using surface plasmon resonance (SPR) as described previously (22) (Fig. 2). SPR has been applied to the study of membrane binding and integration of a number of pore-forming proteins and peptides (27, 28), including BAX (22). Using this technique it is not necessary to label or chemically modify the protein under study. Therefore, we could use this method to determine the effect of the mutations and the added fluorophore on fluor-BAXΔC membrane binding and integration by comparison with the same properties of BAXΔC.

These SPR experiments were done using cardiolipin-containing liposomes (DOPC:DOPA:cardiolipin 70:20:10 mol %) immobilized on the SPR-chip surface, and the proteins of interest were injected over this surface. Both BAXΔC and fluor-BAXΔC required *n*-octyl glucoside activation to generate significant interaction with liposome membranes (Fig. 2, *A* and *B*).

To determine the concentration dependence of BAX integration into the liposome membranes, we sequentially injected increasing concentrations of BAX over the same liposome-covered surface and determined the amount of BAX integrated into the lipid membrane based on sensorgram response after 300 s of washing. Our results show that, when activated with *n*-octyl glucoside, fluor-BAXΔC and BAXΔC have comparable integration into the liposome membrane (Fig. 2*C*). This similarity in membrane integration suggests that the slight decrease in cytochrome *c* release by fluor-BAXΔC (Fig. 1*C*) is not the result of reduced membrane retention of the labeled protein but possibly a result of reduced stability of the in-membrane open pore conformation. However, despite a possible reduction in the stability of fluor-BAXΔC pores, the cytochrome *c* release experiments (Fig. 1*C*) together with the SPR binding data (Fig. 2) suggest that both BAXΔC and fluor-BAXΔC, when activated with *n*-octyl glucoside, follow a similar mechanism of membrane interaction and pore formation.

Analytical Gel Filtration of BAXΔC(G40C) with n-Octyl Glucoside 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

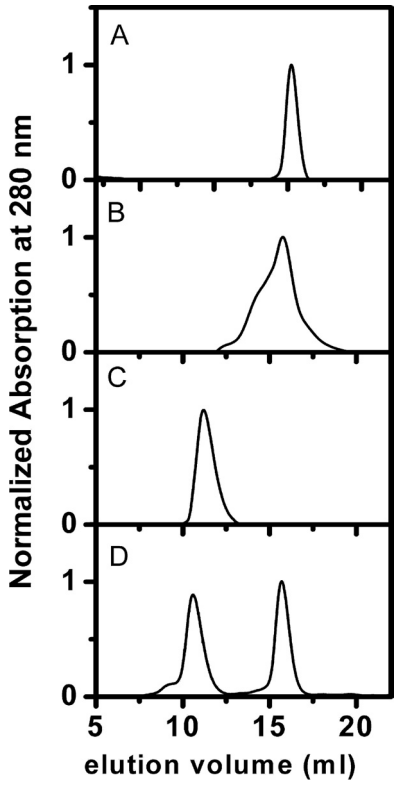

FIGURE 3. **Analytical gel filtration of BAXΔC(G40C) incubated with indicated detergents.** BAXΔC(G40C) was incubated with 2% (w/v) of indicated detergent for 1 h at 4 °C. BAXΔC(G40C) incubated with detergent was passed through a Superdex-200 column equilibrated with 10 mM HEPES/KOH, pH 7.0, 300 mM KCl 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 detergents. *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).

BAXΔC(G40C) eluted as a monomer with a molecular mass of slightly less than 25 kDa (Fig. 3*A*), whereas in the presence of 2% (w/v) *n*-octyl glucoside, protein eluted at a molecular mass slightly below 440 kDa (Fig. 3*C*). 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. 3*B*). These results are consistent with previously reported analytical gel filtration of BAXΔC (7) and show that mutant BAXΔC(G40C) interacts

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. 4*A*) (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. 4*A*), 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. 4*B*). Comparison of the maximum carboxyfluorescein release values for both proteins after 90 min presents two instructive observations (Fig. 4*C*). 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.

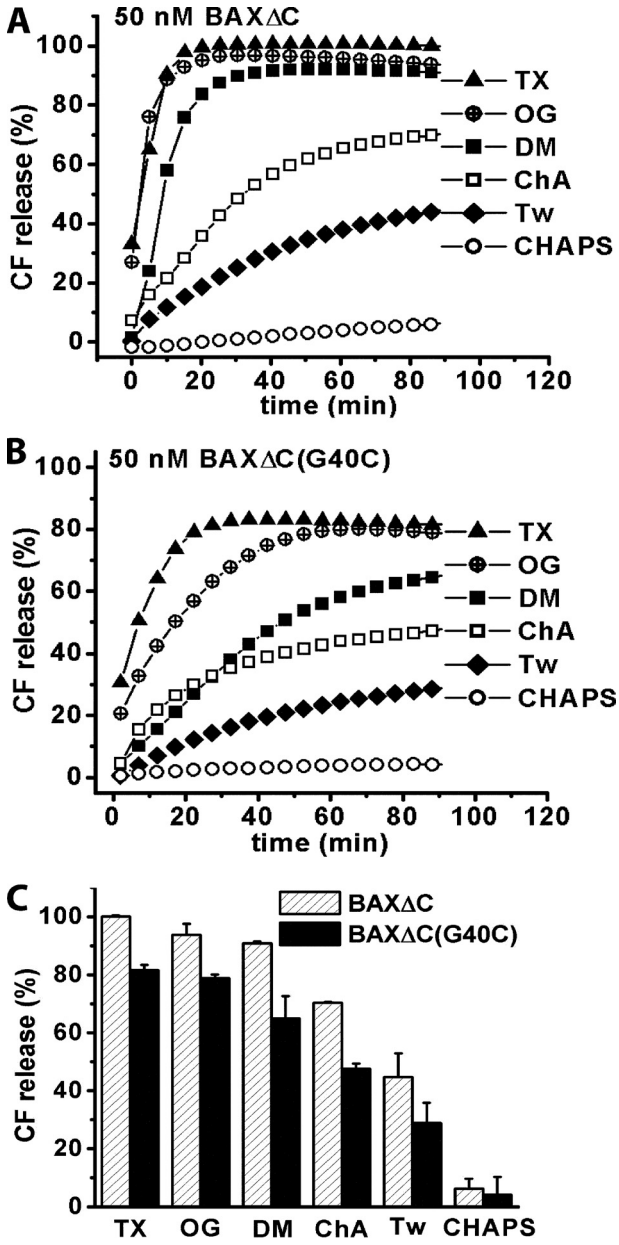

FIGURE 4. **Release of CF from liposomes by the detergent-activated BAXΔC and BAXΔC(G40C).** *A,* monomeric inactive BAXΔC was incubated for 1 h at 4 °C with 2% (w/v) *n*-octyl glucoside (*OG*), 0.6% (w/v) dodecyl maltoside (*DM*), 0.076% (w/v) Triton X-100 (*TX*), 0.038% (w/v) Tween 20 (*Tw*), 2% (w/v) CHAPS, or 2% (w/v) cholic acid (*ChA*). During this incubation the concentration of each detergent was above the CMC, whereas protein concentration was 70 μM. Before addition to liposomes protein was diluted to 50 nM. Total lipid mass in each assay was 175 μg. Liposomes composition was DOPC: DOPA 70:30 mol %. Final detergent concentration in the release assays was below the CMC for each detergent. Releases were normalized to carboxyfluorescein release by 20% Triton X-100 and corrected for basal carboxyfluorescein release by each detergent. Each release curve represents an average of at least three independent experiments. *B,* release of carboxyfluorescein from liposomes by detergent-activated BAXΔC(G40C). Protein was treated the same way as BAXΔC in *A*. *C,* single point comparison of the carboxyfluorescein release values at 90 min for BAXΔC and BAXΔC(G40C) activated with indicated detergents. Representative *error bars* are shown for each release curve.

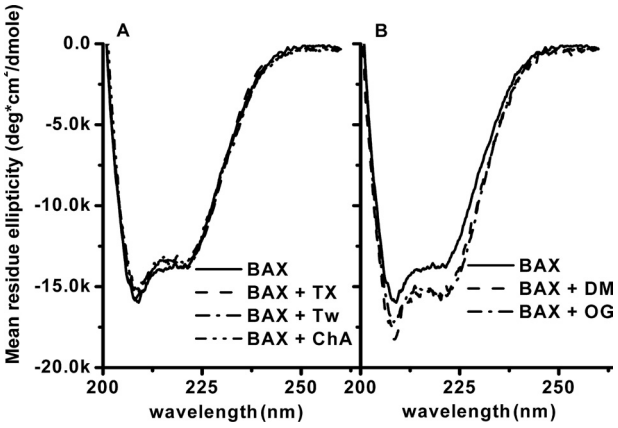

FIGURE 5. **CD spectroscopy of BAXΔC in the presence of detergent micelles in 10 mM HEPES, pH 7. 0, 100 mM KCl.** *A,* comparison of the CD spectra for BAXΔC alone and in the presence of either 0.08% Triton X-100 (*TX*), 0.04% Tween 20 (*Tw*), or 2% cholic acid (*ChA*). *B,* comparison of the CD spectra for BAXΔC alone and in the presence of either 2% *n*-octyl glucoside (*OG*) or 0.6% dodecyl maltoside (*DM*).

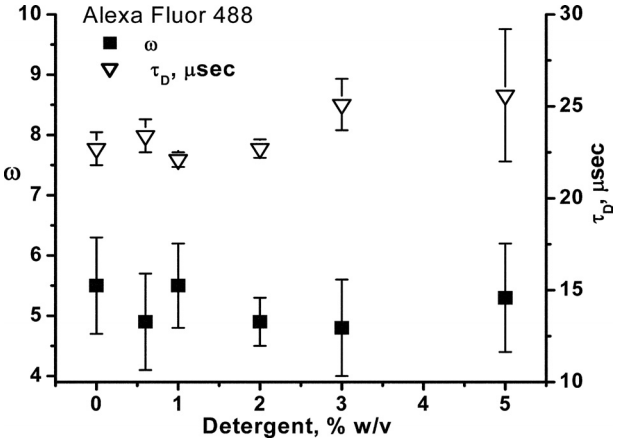

FIGURE 6. **Effect of detergent on the shape and size of the FCS detection volume.** Measurement of the diffusion time (τ_D) of AlexaFluor 488 and the structure parameter of the detection volume (ω) at various detergent concentrations. *n*-Octyl glucoside was used as a detergent of choice, whereas buffer composition was 10 mM HEPES, pH 7.2, 100 mM KCl. *Error bars* were calculated based on nine 10-s measurements. For some data points *error bars* are smaller than the symbol.

Commonly AlexaFluor 488 dye is used for calibration of the FCS detection volume (30, 31). During this calibration procedure the diffusion time (τ_D) of the dye molecules and the structure parameter of the FCS detection volume (ω) were obtained by fitting the measured autocorrelation curve using Equation 1. Using such analysis for AlexaFluor 488 diffusing freely in solution in the absence of detergent, we get the following values: τ_D 22.7 ± 0.9 μs, ω = 5.5 ± 0.8. To determine whether the presence of detergent has an effect on our FCS measurements, we determined τ_D and ω values for AlexaFluor 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).

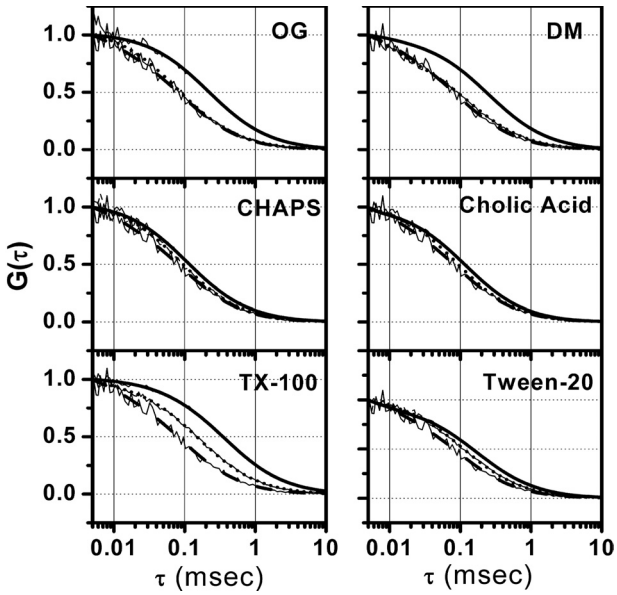

FIGURE 7. **Normalized FCS autocorrelation curves of fluor-BAXΔC incubated with various detergents, as indicated (*OG*, *n*-octyl glucoside; *DM*, dodecyl maltoside; *TX-100*, Triton X-100).** For each autocorrelation experiment, protein was incubated in 10 mM HEPES/KOH, pH 7.2, 100 mM KCl, with 2% (w/v) of the indicated detergent at 4 °C for 1 h. In each *graph* the normalized autocorrelation curve for fluor-BAXΔC in the absence of detergent (*dashed line*), normalized autocorrelation curve for fluor-BAXΔC incubated with 2% (w/v) of the indicated detergent (*solid line*), and normalized autocorrelation curve for fluor-BAXΔC after detergent was diluted to the concentration below its CMC (*dotted line*). Raw data for each autocorrelation curve are shown with *thin jagged line*. See Table 1 for the numerical results of the analysis of these autocorrelation curves.

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, τ_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 concentration 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		
	τ_D	Molecular mass	Detergent concentration, (w/v)	τ_D	Molecular mass
	μs	*kDa*	*%*	*μs*	*kDa*
No detergent	91 ± 4		0	91 ± 4	
n-Octyl glucoside	223 ± 5	280 ± 12	0.003	100 ± 5	25 ± 1
Triton X-100	366 ± 8	1236 ± 223	0.003	158 ± 4	99 ± 2
Dodecyl maltoside	262 ± 21	453 ± 31	0.003	90 ± 4	18 ± 1
CHAPS	115 ± 4	38 ± 1	0.004	87 ± 3	17 ± 1
Cholic acid	124 ± 5	48 ± 1	0.003	86 ± 2	17 ± 1
Tween 20	161 ± 12	105 ± 2	0.003	112 ± 3	35 ± 1

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 for 25 μM BAX	Fluorescence brightness for 3 μM BAX	Detergent concentration (w/v)	Fluorescence brightness
	kHz	*kHz*	*%*	*kHz*
No detergent			0	5.5 ± 0.1
n-Octyl glucoside	7.4 ± 0.2	7.2 ± 0.3	0.003	4.7 ± 0.2
Triton X-100	10.5 ± 0.1	10.4 ± 0.1	0.003	7.1 ± 0.1
Dodecyl maltoside	8.5 ± 0.1	10 ± 0.1	0.003	5.0 ± 0.1
CHAPS	5.9 ± 0.1	5.8 ± 0.1	0.004	5.6 ± 0.1
Cholic acid	3.49 ± 0.06		0.003	4.71 ± 0.09
Tween 20	4.70 ± 0.24		0.003	5.41 ± 0.06

ciation of the protein-detergent micelles as well as of the detergent micelles in solution. Each measurement of fluor-BAXΔC size after detergent dilution was done at least 1 h after dilution to allow dissociation of the detergent micelles bound to the protein. In all cases the diffusion time of fluor-BAXΔC decreased significantly upon detergent dilution (Table 1 and Fig. 7). For all detergents, except Triton X-100, the diffusion time decreased close to that of fluor-BAXΔC monomer. These observations indicate that upon detergent dilution the molecular weight of the protein-detergent micelle complexes is reduced, and for most of the detergents this molecular weight is reduced to that of a fluor-BAXΔC monomer. In addition, it should be mentioned that *n*-octyl glucoside-treated fluor-BAXΔC used in the FCS dilution experiments was used with similar dilution as in the cytochrome *c* release experiments (Fig. 1*C*).

Fluorescence Intensity Distribution Analysis of Fluor-BAXΔC in Detergent Micelles—Analysis of the distribution of the photon counts in the FCS data sets was used to determine the number of fluor-BAXΔC molecules per protein-detergent micelle formed with micelles of all tested detergents. This analysis used the FIDA algorithm developed by Kask *et al.* (18). Using FIDA we determined fluorescence brightness of BAX molecules before, during, and after interaction with detergent micelles. 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. 8*A* (*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

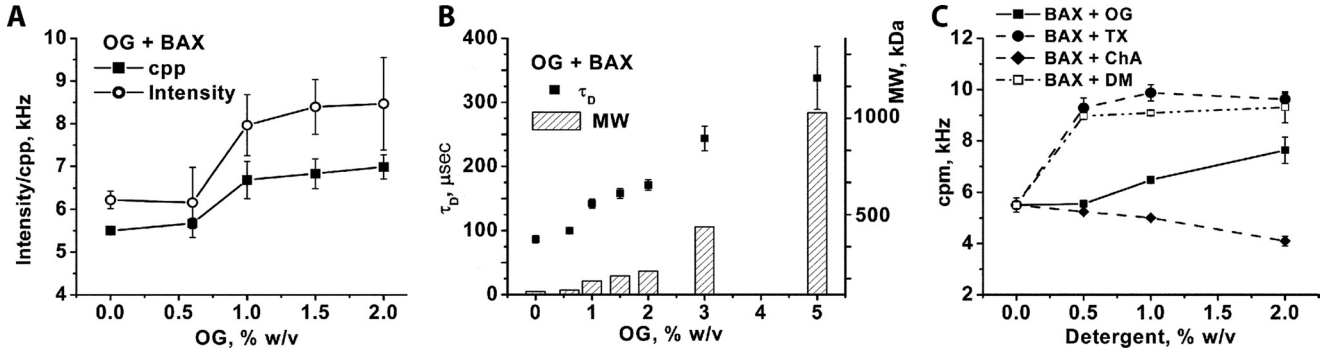

FIGURE 8. **Enhancement of the fluor-BAXΔC fluorescence intensity upon interaction with detergents.** *A,* constant concentration (3 μM) of fluor-BAXΔC was incubated with increasing concentrations of *n*-octyl glucoside (*OG*) for 1 h at 4 °C. The fluorescence intensity of the protein samples incubated with detergent was measured using ConFocor 3 (Zeiss, Germany). For each measurement the protein incubated with detergent was diluted to a final concentration of 24 nM in EB buffer containing the same detergent concentration as in the protein sample. All measurements were done at 22 °C. cpp values were determined using the single-component fit of the FCS autocorrelation curves. *B,* increase in the apparent molecular weight (*MW*) of BAXΔC upon interaction with micelles of *n*-octyl glucoside. Molecular weight values were calculated using Equation 6. *C,* change in the cpp value of fluor-BAXΔC with increasing detergent concentration. Samples were prepared, and measurements were done similar to the Fig. 6*A*. Detergent abbreviations are similar to Fig. 5.

resulting FCS autocorrelation curves also increased (Fig. 8*A, 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. 8*C*). 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. 8*B*).

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).

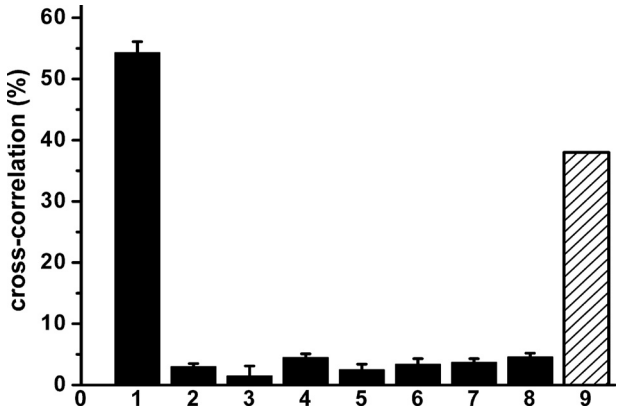

FIGURE 9. **Results of the FCCS experiments with fluor-BAXΔC (50% labeled) and Bodipy 630/650 maleimide-BAXΔC (80% labeled) in the presence of the indicated detergents.** *Lane 1,* cross-correlation standard (double-stranded RNA with AlexaFluor 488, Cy5 labels); *lane 2,* no detergent present; *lane 3,* +2% *n*-octyl glucoside; *lane* 4, +2% Triton X-100; *lane 5,* + 2% CHAPS; *lane 6,* + 2% cholic acid; *lane 7,* + 2% Tween 20; *lane 8,* + 2% dodecyl maltoside; *lane 9,* maximum expected cross-correlation value for a formation of protein dimer from a mix of 50 and 80% labeled protein. Experiments were done in 10 mM HEPES, pH 7.2, 100 mM KCl buffer at 22 °C. All detergent concentrations are given in % of w/v.

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 BAXΔC protein are shown not to interfere with the function of the full-length 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 BAXΔC(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-BAXΔC.

To assess the functional capability of fluor-BAXΔC, we tested its ability to promote cytochrome *c* release from isolated mitochondria as compared with that of BAXΔC. Because our engineered BAXΔC 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-BAXΔC releases 71 ± 15% of the mitochondrial cytochrome *c* when activated with *n*-octyl glucoside, whereas BAXΔC releases 100 ± 30% (Fig. 1). Based on this result we conclude that mutation and fluorophore labeling of BAXΔC 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 BAXΔC 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. 4*C*). Finally, we compared the integration of BAXΔC and its mutant into lipid membranes using SPR. We have recently developed methods to quantitatively study binding and integration of 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 BAXΔC, BAXΔC(G40C) was fully functional and integrated into lipid membranes as well as BAXΔC (Fig. 2). Taken together these studies indicate that BAXΔC(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.

Fluor-BAXΔC Forms High Molecular Weight Protein-Detergent Micelles with Most Activating Detergents—BAXΔC has been shown to form high molecular weight complexes with *n*-octyl glucoside but not with CHAPS (7). To show that our mutated BAX, BAXΔC(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, BAXΔC(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 BAXΔC (7) indicating that mutations introduced into BAXΔC 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-BAXΔC in the presence of *n*-octyl glucoside and CHAPS using FCS. In the FCS experiments we measured the diffusion time, τ_D, of fluor-BAXΔC molecules in the presence of 2% (w/v) of these detergents. Then by using Equation 6, we calculated the molecular weight of the fluor-BAXΔC protein-detergent micelle complexes (Table 1). The molecular weights of BAXΔC protein-detergent micelle complexes determined by FCS and analytical gel filtration were fairly similar, further demonstrating that mutagenesis and fluorescent labeling of BAXΔC 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 BAXΔC 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-BAXΔC 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-BAXΔC does not form high molecular weight complexes. Fluor-BAXΔC also did not form high molecular weight complexes with CHAPS, a zwitterionic detergent known for its inability to activate BAXΔC. In addition, all of the tested activating detergents did not induce significant secondary structure changes in the BAXΔC protein (Fig. 5).

For the studies of BAXΔC pore formation in lipid membranes, it is desirable that detergent is removed after BAXΔC 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 BAXΔC after interaction with detergent micelles. To determine the latter, excess detergent was removed from fluor-BAXΔC by dilution below the CMC. After removal of detergent micelles, the molecular weight of fluor-BAXΔC decreased to that of the fluor-BAXΔC monomer for most of the detergents (Table 1). The results for fluor-BAXΔC treated with Triton X-100 were an exception. Upon Triton X-100 dilution the molecular weight of fluor-BAXΔC was five times larger than that of fluor-BAXΔC 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-BAXΔC upon detergent dilution, and the second explanation is possible formation of fluor-BAXΔC homo-oligomers. To differentiate between these two explanations and to determine the stoichiometry of fluor-BAXΔC before, during, and after interactions with detergent micelles we performed FIDA.

BAXΔC Is Present as a Monomer in Protein-Detergent Micelles—To further investigate the stoichiometry of the fluor-BAXΔC-detergent micelles, we performed FIDA on the FCS data (Table 2). The fluorescence brightness of the individual protein-detergent micelles varied ~90% as a function of the detergent used, but all of them contained one 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 BAXΔC 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 millimolars 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 glycophorin 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 FCCS 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 characteristics that may concern *in vivo* activation of BAX. First, because BAX remains active after we effectively remove the detergent substrate upon which it has activated, there must be two stable conformations of monomeric solution BAX, one active and the other inactive. Because this activation requires a large template and is distinct from the oligomer-competent conformation, which BAX assumes in membranes, we can consider the possibility that BAX is activated as a soluble protein and then integrates into the membrane. Second, the existence of a soluble active form of BAX suggests that this process can be reversed, which is a potential therapeutic approach.

Conclusion—In this work we studied in detail the process of BAXΔC activation by nonionic detergents. Based on the results of our study we conclude that BAXΔC is a monomer before, during, and after interaction with detergent micelles. In this study we used fluorescently labeled analogue of BAXΔC in combination with two single-molecule sensitivity techniques (FCS and FIDA). Because the determination of the oligomeric state of proteins in detergent micelles is important for structural and functional studies of integral membrane proteins (35, 36), we are hopeful that the method presented here can be used for other proteins.

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