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Tissue Transglutaminase Is a Multifunctional BH3-only Protein*

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Tissue transglutaminase (TG2) protein accumulates to high levels in cells during early stages of apoptosis both *in vivo* and *in vitro*. The analysis of the TG2 primary sequence showed the presence of an eight amino acid domain, sharing 70% identity with the Bcl-2 family BH3 domain. Cell-permeable peptides, mimicking the domain sequence, were able to induce Bax conformational change and translocation to mitochondria, mitochondrial depolarization, release of cytochrome *c*, and cell death. Moreover, we found that the TG2-BH3 peptides as well as TG2 itself were able to interact with the pro-apoptotic Bcl-2 family member Bax, but not with anti-apoptotic members Bcl-2 and Bcl-X_L. Mutants in the TG2-BH3 domain failed to sensitize cells toward apoptosis. In TG2-overexpressing cells about half of the protein is localized on the outer mitochondrial membrane where, upon cell death induction, it cross-links many protein substrates including Bax. TG2 is the first member of a new subgroup of multifunctional BH3-only proteins showing a large mass size (80 kDa) and enzymatic activity.

Tissue or type 2 transglutaminase (TG2,[1] EC 2.3.2.13) is a multifunctional enzyme belonging to the transglutaminase family (1). Its primary enzymatic activity resides in a Ca^{2+}-dependent reaction in which γ-carboxyamide groups of peptide-bound glutamine residues serve as acyl donors and primary amino groups of several compounds function as acceptor substrates (2). This reaction results in the post-translational modification of proteins by establishing ε(γ-glutamyl)lysine and *N*,*N*-bis(γ-glutamyl)polyamine isodipeptide linkages (3). Covalent TG2-dependent cross-linking leads to the polymerization of substrate proteins that can be dismantled only by the proteolytic degradation of the protein chains (4). While Ca^{2+} is a positive regulator of TG2 enzymatic activity, GTP is a negative one. It has been demonstrated that high intracellular GTP concentrations shift the enzyme activity from cross-linking to G-protein. At physiological intracellular GTP concentration, TG2 might act as the $G\alpha_h$ subunit of the GTP-binding protein (G_h) and form a ternary complex in association with the 50-kDa β subunit ($G\beta_h$) and the α_1-adrenergic receptor (5). Thus, the TG2/$G\alpha_h$ proves to be a multifunctional protein, which by binding GTP in a $G\alpha_h$GTP complex, can modulate receptor-stimulated phospholipase-C (PLC) activation. The binding of GTP to TG2/$G\alpha_h$ prevents the activation of the pro-apoptotic cross-linking activity (6–9). It has been proposed that the fine modulation of TG2 enzymatic activity exerted by GTP, Ca^{2+}, and nitric oxide allows cells to survive in the presence of high TG2 protein levels (10). In addition, recent studies suggest that TG2 in its G protein configuration could lead to prevention of cell death (11–13).

In addition to these two main enzymatic activities, it has been proposed very recently that TG2 might also function as a protein-disulfide isomerase (14) and as a kinase (15), depending on its subcellular localization.

Under physiological conditions, TG2 is a ubiquitous enzyme, although high expression levels could be detected only in particular cell subsets (*e.g.* endothelial, mesangial, and smooth muscle cells). However, during both physiological (*i.e.* mammary gland regression, interdigital web shaping) and pathological (*i.e.* HIV infection, hepatitis) onset of apoptosis, a large increase in the enzyme synthesis and cross-linking activity could be observed (16–18). During apoptosis, the drop of intracellular GTP concentration and the increase of intracellular Ca^{2+} levels are responsible for the activation of TG2 cross-linking activity, which results in the assembly of highly cross-linked intracellular protein polymers (4). This detergent-insoluble protein scaffold contributes to the stabilization of dying cells, before their clearance by phagocytosis. In addition to this "late" contribution to the apoptotic process, TG2 might act also as an upstream regulator of the mitochondrial pathway. In a previous report (19), we showed that overexpression of TG2 in neural cells results in a 4–5-fold more rapid accomplishment of the death program, as compared with their parental counterpart that expresses low levels of TG2. We observed that mitochondria of TG2-overexpressing cells were greatly modified with respect to both their ultrastructure and physiology. Mitochondria appeared often clustered in discrete cytoplasmic regions, with few cristae and extremely electron-dense matrix.

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The authors would like to dedicate this article to Prof. Francesco Autuori on the occasion of his 71st birthday.

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[1] The abbreviations used are: TG2, tissue transglutaminase; CsA, cyclosporin a; ΔΨ, mitochondrial membrane potential; JC-1, 5–5′,6–6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol carbocyanine iodide; MMP, mitochondrial membrane potential; PI, propidium iodide; STS, staurosporine; PLC, phospholipase C; HIV, human immunodeficiency virus; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; z-VAD, carbobenzoxy-valyl-alanyl-aspartyl; FCS, fetal calf serum; PBS, phosphate-buffered saline; MOPS, 4-morpholinepropanesulfonic acid; TMRM, tetramethylrhodamine ester; cyt *c*, cytochrome *c*; ELISA, enzyme-linked immunosorbent assay; ROS, reactive oxygen species; ANT, antennapedia.

These ultrastructural modifications are coupled with a constitutive hyperpolarization of the organelles, a decrease of intracellular GSH level and an increased reactive oxygen species (ROS) production. The TG2-dependent hyperpolarization of mitochondria preceded and was separated from the PTP opening and the release of cytochrome *c*, which took place only upon induction of apoptosis.

Prompted by these results, we investigated whether TG2 interaction/modification of proteins known to influence mitochondrial physiology and/or to control the mitochondrial apoptosis pathway might play a role in the observed commitment to death. To address these questions, we investigated the effect exerted by TG2 accumulation in mitochondria, focusing our attention to the possible interaction of TG2 with Bcl-2 family members. The choice of this class of protein was basically because of their well defined role in the regulation of the mitochondrial pathway of apoptosis (20, 21). It has been widely accepted that the pro-apoptotic factors Bax and Bak are important regulators of the mitochondrial permeability transition and that other Bcl-2 family members could modulate their action. In particular, the anti-apoptotic Bcl-2 and Bcl-X_L are able to inhibit Bax/Bak mitochondrial action, while the pro-apoptotic BH3-only subfamily are activators of Bax and Bak, also at the mitochondrial level (22, 23).

Our results demonstrate for the first time that TG2 possesses a well defined and functional BH3 domain. Through this domain the enzyme might behave as a BH3-only protein, able to interact, even in the absence of any apoptotic stimuli, with pro-apoptotic members Bax and Bak, but not with anti-apoptotic Bcl-2 and Bcl-X_L. Upon cell death induction, the interaction with Bax increased, and many mitochondrial proteins were post-translationally modified by TG2. It is worth noting that Bax acts as one of the major mitochondrial substrates, and its modification led to the formation of large molecular weight polymers.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—Human SK-n-BE(2) neuroblastoma cells and stably transfected TG2 derivatives (TGA) were grown as described (24). For all experiments, cells were seeded in tissue culture flasks, multiwell plates, and chamber slides (NUNC), and allowed to attach overnight before treatment. The seeding density varied according to the type of experiment. Expression vectors for TG2 wild type (TG2-wt), deleted of the BH3 domain (TG2-ΔBH3), with cysteine 277 mutated to serine and with leucine 204 mutated to aspartic acid (TG2-LE), were generated by PCR and cloning in the pcDNA4/HisMax vector (Invitrogen). Deletion of the TG2-BH3 domain was accomplished by substitution of the nucleotides corresponding to the amino acids ^{204}LKNAGRDCS^{212} with an EcoRI site via PCR. Mutations of the leucine 204 (CTG-GAG) and cysteine 277 (TGC-AGC) were also accomplished by PCR, with primers containing the mutations. Transient transfections of SK-n-BE(2) cells were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cytofluorimetric analysis of cell death induction by staurosporine (1 μM) was conducted 48 h after transfection.

Synthetic Peptides and Cell Treatment—Peptides bearing the BH3 sequence of interest fused to antennapedia (ANT) sequence, as to allow cell entry, were designed as follows: ANT: NH_2-RQIKIWFQNRRMKWKK-COOH; ANT-BH3 TG2: NH_2-RQIKIWFQNRRMKWKKDVNPKFLKNAGRDCSR-COOH; ANT-BH3 Bax: NH_2-RQIKIWFQNRRMKWKKKKLSECLKRIGDELDS-COOH; ANT-BH3 TG2 L-E; NH_2-RQIKIWFQNRRMKWKKDVNPKFEKNAGRDCSR-COOH. All peptides were synthesized, with or without biotin at the N terminus, by Sigma-Genosys, purified by HPLC and controlled by mass spectroscopy. Upon arrival peptides were dissolved in Me_2SO at 50 or 100 mM and stored in aliquots at −80 °C.

SK-n-BE(2) cells were treated with 50 μM ANT-BH3 peptides for 30 min in culture medium without FCS, then supplemented with FCS, and incubation was continued for the indicated time period.

Induction of Apoptosis by ANT-BH3 Peptides and Cell Viability Assessment—For cell viability assessment (CellTiter Proliferation Assay, Promega) 1–2 × 10^4 cells/well were seeded in 0.2 ml of complete culture medium in 96-well tissue culture plates. After 24 h, cells were washed with culture medium without FCS and treated with peptides (50 μM). At the indicated time, measurement of cell viability was determined following the manufacturer's protocol. For apoptosis evaluation and measurement of mitochondrial membrane potential (MMP) 2 × 10^6 cells were seeded in 25-cm^2 tissue culture flasks and after 24 h treated as above. Quantitative flow cytometry evaluation of apoptosis was performed by double staining, using the FITC-conjugated annexin V/propidium iodide (PI) apoptosis detection kit (Eppendorf) and evaluation of DNA fragmentation in ethanol-fixed cells using PI (Sigma). For cell death inhibition, cells were treated 15 min with 1 mM cyclosporin A (CsA, Sigma) before adding BH3 peptides.

Analysis of Mitochondrial Membrane Potential in Living Cells—Analysis of MMP in control and peptide-treated cells was conducted with the JC-1 probe. Briefly, cells were stained with 10 μM JC-1 (Molecular Probes) as previously described (25) and analyzed by flow cytometry. Tetramethylrhodamine ester (1 μM, TMRM, Molecular Probes) was also used to confirm the data obtained using JC-1.

Analysis of Cytochrome c Release in Living Cells and on Isolated Mitochondria—For cytochrome *c* release assays, 1 × 10^6 cells were seeded in 6-well plates and after 24 h were treated with 50 μM BH3 peptides as described above. At the indicated times, cells were harvested by trypsin treatment and collected by centrifugation. After washing three times in PBS, cells were suspended in 250 μl of Hypotonic Buffer (2 mM $MgCl_2$, 10 mM KCl, 10 mM Tris-HCl, pH 7.6) supplemented with complete protease inhibitor mixture (Roche Applied Science), and incubated on ice for 10 min. Cells were homogenized with a Teflon homogenizer with B-type pestle as previously reported (26) and, after addition of an equal volume of Mitochondria Buffer (400 mM sucrose, 10 mM TES, 400 μM EGTA, pH 7.2), centrifuged twice for 10 min at 900 × *g* at 4 °C. The supernatant was recovered and further centrifuged for 15 min at 17,000 × *g* at 4 °C. The pellet fraction was considered to be the mitochondria, and the supernatant was the cytosol. Release of cytochrome *c* was assessed by Western blot with monoclonal anti-cyt *c* antibody (65981A, BD PharMingen). Anti-COX subunit IV antibody (20E8-C12, Molecular Probes) was used as a control of mitochondrial isolation.

For peptide treatments, mitochondria were isolated with a further 10 min centrifugation at 10,000 × *g* at 4 °C of the 900 × *g* supernatant, obtained as above. The mitochondrial pellet was suspended in swelling buffer (SB: 0.1 M sucrose, 0.5 M sodium succinate, 50 mM EGTA, pH 7.4, 1 M H_3P0_4, 0.5 M MOPS, 2 mM rotenone), kept on ice, and used within 2 h from the preparation. Mitochondria (1 mg of protein/ml) were treated with 10 and 50 μM of different BH3 peptides in the presence or absence of 5 μg/ml cyclosporin a for 20 min at room temperature, followed by centrifugation (10,000 × *g*, 10 min, 4 °C). Proteins contained in the supernatants were then concentrated in 90% acetone (4 °C, 18 h; centrifugation at 17,000 × *g*, 20 min, 4 °C), and the precipitate was subjected to Western blot with anti-cytochrome *c* antibody.

Swelling Experiments—For swelling experiments mitochondria, isolated as described above, were suspended in 1.5 ml of SB (0.5 mg protein/ml). As a general rule, 4 min after starting to record, the reagents to be tested were added. Total recording time was 20 min. As a positive control we used 300 μM $CaCl_2$, which opens the protein transition pore causing high amplitude swelling, accompanied by a decrease of ΔΨ and an increase of outer membrane permeability. CsA (5 μg/ml) added before Ca^{2+} prevented ΔΨ loss. ΔΨ measurement was performed by cytofluorimetric analysis of TMRM (1 μM; Molecular Probes) incorporation. Low levels of TMRM incorporation (revealed by a decrease of red fluorescence) indicated a low ΔΨ. We tested the effect produced by different peptides (*e.g.* TG2-BH3, BAX-BH3, and TG2-BH3 L-E) on the ΔΨ either in the presence or absence of CsA. All samples were analyzed with a FACScan cytometer (Becton Dickinson) equipped with a 488 argon laser. To exclude debris, samples were gated based on light scattering properties in the side scattering (SSC) and forward scattering (FSC) modes. The red fluorescence emission of untreated mitochondria was considered as basal emission and recorded for 4 min; after this time reagents to be tested were added, and the effect was monitored for the following 16 min. Dot plots of red fluorescence emission as a function of the time, obtained in each condition, were statistically analyzed using CellQuest software to determine the percentage of mitochondria with depolarized membrane.

Cyt *c* presence in the supernatants of swelling reactions, performed in the absence of TMRM, was assessed with a commercial ELISA kit (R&D System). The cyt *c* release was quantified and expressed as pg/ml in all tested samples, including the positive control.

Co-precipitation between BH3 Domain Peptides and Bax—Mitochondria (0.5 mg) isolated from SK-n-BE(2) cells were treated with 5 and 10

μM biotinylated BH3 domain peptides for 20 min at room temperature, followed by centrifugation at 10,000 × *g* for 10 min at 4 °C to eliminate the supernatant. Pellets were lysed with CHAPS-IP buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 150 mM KCl, 1% CHAPS) for 30 min on ice and, after addition of 50 μl of prewashed streptavidin paramagnetic particles (MagneSphere, Promega), incubated for 2 h at 4 °C under gentle shaking. The beads were recovered and washed six times. Proteins attached to the beads were solubilized in sample buffer and after separation on 4–12% Nu-PAGE (Invitrogen) subjected to Western blot with anti-Bax antibody (N-20, Santa Cruz Biotechnology). For whole cell co-precipitation, cells were treated with 50 μM biotinylated peptides and, after lysis in CHAPS-IP buffer, peptides and interacting proteins were isolated as described above.

Identification of Bax as a TG2 Protein Substrate—TG2-overexpressing cells (TGA) were grown in the presence of 5 mM 5-(biotinamido)pentylamine (EZ-link, Pierce) and treated for 2 h with 1 μM staurosporine (Sigma) in the presence or absence of 100 μM z-VAD (Promega). Cells were harvested and lysed in CHAPS-IP buffer, and biotinylated TG2 substrates were separated on 4–12% Nu-PAGE (Invitrogen). After transfer to nitrocellulose, TG2 substrates were revealed with streptavidin-horseradish-conjugated peroxidase (Amersham Biosciences, 1:3000). Mitochondria from the same cells were isolated and, after lysis in CHAPS-IP buffer, biotinylated TG2 protein substrates were purified by streptavidin paramagnetic particles (MagneSphere, Promega) following the manufacturer's protocol. Purified proteins were separated on 4–12% Nu-PAGE gels and revealed by Western blot with anti-Bax N-20 antibody.

Immunoprecipitations—For immunoprecipitation experiments TG2-overexpressing cells (TGA), seeded in 175-cm^2 tissue culture flasks, were treated with 1 μM staurosporine for 2 h, harvested with trypsin, and washed twice in PBS. Whole cell extracts were obtained by lysis with CHAPS-IP buffer for 30 min on ice and clearing at 14,000 × *g* at 4 °C for 10 min. For each reaction 500 μg of total protein were preincubated with 50 μl of prewashed Dynabeads-protein G (Dynal) for 1 h at 4 °C, with gentle shacking. Immunoprecipitations were performed by adding 5 μg of anti-TG2 (CUB 7402, NeoMarkers), or anti-Bax (N-20, Santa Cruz Biotechnology and 6A7, BD PharMingen) or anti-Bak (G-23, Santa Cruz Biotechnology) antibodies, and 50 μl of prewashed Dynabeads-protein G. After 4 h or overnight incubation, beads were recovered and washed six times with CHAPS-IP buffer. Immunoprecipitated proteins were detached from beads by boiling in sample buffer and separated on 4–12% Nu-PAGE. After transfer to nitrocellulose, membranes were incubated with anti-TG2 (0.2 μg/ml), anti-Bax (N-20, 0.2 μg/ml; 6A7, 1 μg/ml), and anti-Bak (0.2 μg/ml) antibodies for 1 h and then appropriate secondary horseradish peroxidase-conjugated secondary antibodies were added for 1 h. The signals were detected with Supersignal (Pierce).

Immunofluorescence—For immunofluorescence experiments SK-n-BE(2) and TGA cells were seeded in 4-well chamber slides and treated or not with ANT-BH3 peptides, as described above. After washing with PBS, cells were fixed on paraformaldehyde (4% w/v) and picric acid (0.19% v/v) for 20 min at room temperature and washed three times in PBS. After permeabilization with PBS, 0.1% Triton X-100 for 10 min at room temperature and blocking in PBS, 0.1% Triton X-100, 10% FCS, primary antibody was added, and incubation was carried out for 1 h at room temperature. For indirect immunofluorescence anti-Bax (N-20, 2 μg/ml; 6A7, 1 μg/ml) and anti-Hsp60 (SPA-806, 1 μg/ml, StressGen) antibodies were used. Alexa Fluor-conjugated secondary antibodies (Molecular Probes) were used as suggested by the manufacturer. Direct labeling of anti-TG2 and anti-Bax (2D2, Santa Cruz Biotechnology) monoclonal antibodies with Alexa Fluor was performed with Zenon Mouse IgG Labeling Kit (Molecular Probes) following the manufacturer's instructions. Briefly, 6 μg of each monoclonal antibody were labeled with 30 μl of Zenon Mouse IgG labeling reagent for 5 min at room temperature, and then the reaction was blocked by addition of 30 μl of Zenon blocking reagent and further incubation for 5 min. The formed complex was added to permeabilized fixed cells, and incubation was carried out for 1 h in the dark. After washing three times with PBS, cells were subjected to a second fixation for 15 min at room temperature. Cells were washed three times with PBS and nuclei counterstained with Hoechst 33342 (Molecular Probes). The dried slides were mounted with Prolong Antifade Kit (Molecular Probes) and examined with a Nikon Eclipse TE200 epifluorescence microscope equipped with Coolsnap CCD camera. Images were assembled with Adobe Photoshop.

RESULTS

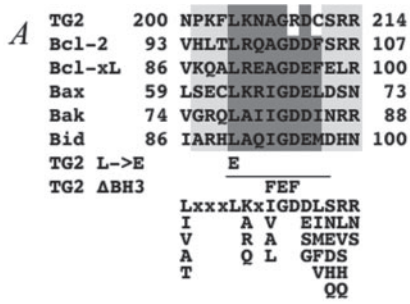
TG2 Has a Functional BH3 Domain—TG2 protein selectively accumulates to high levels in cells undergoing cell death by apoptosis both *in vivo* and *in vitro* (27, 28). In keeping with this, we previously showed that TG2-overexpressing cells (TGA) were primed toward apoptosis, and their mitochondria were greatly modified both at the ultrastructural and functional levels (19). On the basis of this evidence, we decided to further investigate the role exerted by TG2 at the mitochondrial level.

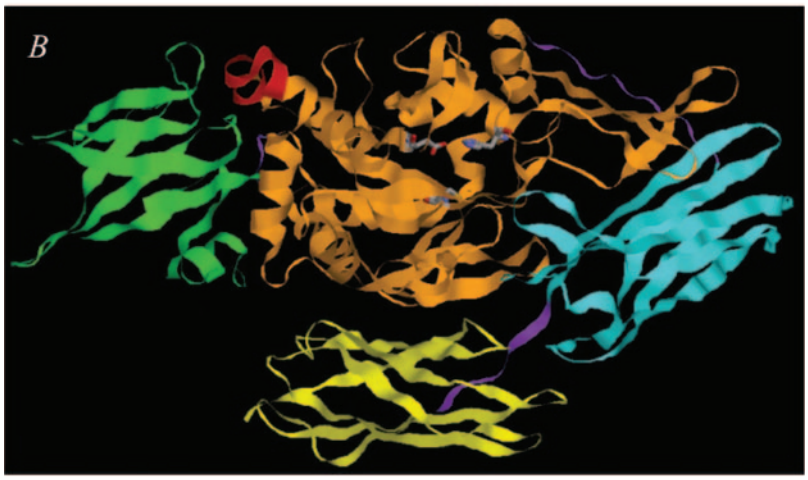
We first analyzed the primary structure of TG2, in search of sequences that might be involved in its subcellular localization or responsible for specific protein-protein interactions. This analysis led us to the identification of an eight amino acid region (amino acids 204–212 in the enzyme's catalytic core) that shares more than 70% identity with the Bcl-2 BH3 domain (Fig. 1*A*). This sequence showed the presence of the highly conserved leucine, at position 204, and only two different amino acids out of eight. At first glance, the substitution of the aspartic acid 209, which is highly conserved in all other proteins of the Bcl-2 family, with arginine might be considered as relevant, given the opposite charge of the two amino acids. On the other hand, it might not be as drastic with respect to steric hindrance and folding properties. The other substitution, phenylalanine 211 with cysteine, occurs in an amino acid that seems not to be relevant for the domain-mediated interaction, at least as described for the other Bcl-2 family members (29). In addition, by comparing the surrounding sequence to the 12 amino acid BH3 consensus found on Prosite (ca.expasy.org/prosite/), we found only one other difference consisting of a shift of the first amino acid of the domain.

Computer-assisted analysis of the published crystal structure of TG2 (8) revealed that the identified TG2 domain is structured into two α-helices, the conformation expected for the BH3 domain of Bcl-2 family proteins (30), and exposed to the interaction with solvent (represented in *red* in Fig. 1*B*). Analyses carried out to identify other putative BH domains in TG2 primary sequence do not lead to the identification of regions showing relevant homology to them.

In order to assess the relevancy and functionality of this identified domain, we generated two different TG2 mutants in which the BH3 domain had been either deleted (TG2 ΔBH3) or the leucine 204 mutated to glutamic acid (TG2 L-E), because this amino acid has been described to be critical for the homo-/heterodimerization of Bax (29). Wild-type and mutated TG2s were expressed in SK-n-BE(2), by transient transfection, and sensitization to cell death induction was addressed. When we treated transfected cells with 1 μM staurosporine we observed that, as expected, expression of wild-type TG2 sensitized cells toward death. On the other hand, when the two mutants were expressed, this sensitization to death completely disappeared (Fig. 1*C*). We also observed that, as described (13), TG2 bearing a mutation in the cysteine of the active site (C277S) was unable to sensitize cells toward cell death (data not shown). Taken together, these data suggest that the identified domain is a functional one and that TG2 sensitization to death relies on this BH3 domain, which proves to be necessary but not sufficient for priming cells toward apoptosis. In fact, the C277S mutant that retains the BH3 domain but lacks the transamidating activity indicates the TG2 enzymatic activity as necessary for the sensitization to death.

Effect of TG2-BH3 Peptide in Vivo—To further assess whether the identified domain behaves as previously characterized BH3 domains, we decided to use synthetic peptides bearing the domain sequence fused to the C terminus of the antennapedia sequence (ANT-BH3 peptides), as described by





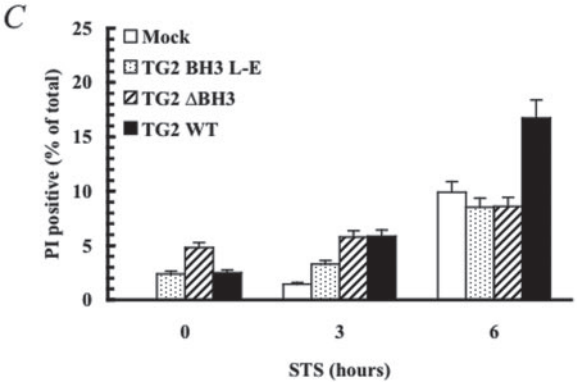


FIG. 1. **TG2 possesses a functional BH3 domain.** *A*, alignment of TG2 and Bcl-2 family BH3 domains. The conservation along the eight core amino acids is highlighted in *black*, while *gray* highlights the neighboring ones. Consensus sequence from Prosite. TG2 L-E and ΔBH3, indicates the introduced mutations. *B*, crystal structure of TG2 showing that the BH3 domain (amino acids 204–212, *red*) is located in the catalytic core of TG2 (*orange*) and structured in two inversely oriented α-helices that are exposed to the interaction with solvent. *Green*, *blue*, and *yellow* define the β-sandwich and the two β-barrel regions of the protein, respectively. The catalytic triad, Cys^{277}, His^{335}, and Asp^{358} is represented in *ball and stick*. *C*, time course analysis of cell death induction in SK-n-BE(2) cells transfected with wild type and mutated TG2, after treatment with 1 μM staurosporine. Mutation of highly conserved Leu^{204}, as well as complete deletion of the BH3 domain, abolished the TG2-dependent sensitization toward STS-induced apoptosis.

others (31). The antennapedia sequence delivers peptides through the plasma membrane, thus allowing the study of the intracellular effect of the domain of interest (32, 33). We generated also a set of peptides with biotinylation at the N terminus to allow their purification by streptavidin paramagnetic particles (see "Experimental Procedures"). As the experimental model, we used the neuroblastoma SK-n-BE(2) cell line, which has low levels of endogenous TG2 (see Fig. 5*C*). Cells were treated with 50 μM peptides bearing the ANT sequence alone, as a negative control, the ANT-BH3 Bax sequence, as positive control, and ANT-BH3 TG2 sequence. Time course analysis of cell viability, as assessed by MTS assay (Fig. 2*A*), showed that both the BH3 peptides induced cell death that reached about 80% of the treated cells after 6 h of incubation. We assessed also phosphatidylserine exposure and propidium iodide (PI) incorporation after peptide treatment. Fig. 2*B* shows a representative experiment in which cells were treated with different BH3 peptides for 6 h in the presence or absence of mitochondrial membrane permeability transition pore inhibitor CsA (34). It is interesting to note that CsA is able to inhibit both phosphatidylserine externalization and PI uptake in TG2-BH3 peptide-treated cells, while it had little or no effect on Bax-BH3 peptide-treated cells (Fig. 2*B*). In addition, TG2-BH3 peptides bearing the mutation of the leucine 204 (TG2 LE) did not induce phosphatidylserine externalization nor PI incorporation, suggesting that this amino acid was important for the functionality of the BH3 domain. These data were consistent with what we previously observed in TG2-overexpressing cells (TGA) (19). After staurosporine treatment, they rapidly die by apoptosis via the mitochondrial pathway, and this phenotype could be reverted by CsA. In that report, we hypothesized that the observed sensitivity of TGA cells toward apoptosis might be related to the hyperpolarized state of their mitochondria, which rapidly undergo MMP loss and cytochrome *c* release (19). To address whether this mitochondrial behavior might be related to the presence of the identified TG2-BH3 domain, we analyzed the mitochondrial status in SK-n-BE(2) prior to and after treatment with ANT-BH3 peptides. We did not detect any hyperpolarization of mitochondria (Fig. 2*C*). This result was probably caused by the fact that both TG2- and Bax-BH3 peptides were able to induce a very rapid decrease, after 1 h, of the mitochondrial membrane potential ($\Delta\psi$). In keeping with what we previously observed in TGA cells, pretreatment with CsA completely inhibited the mitochondrial depolarization in cells

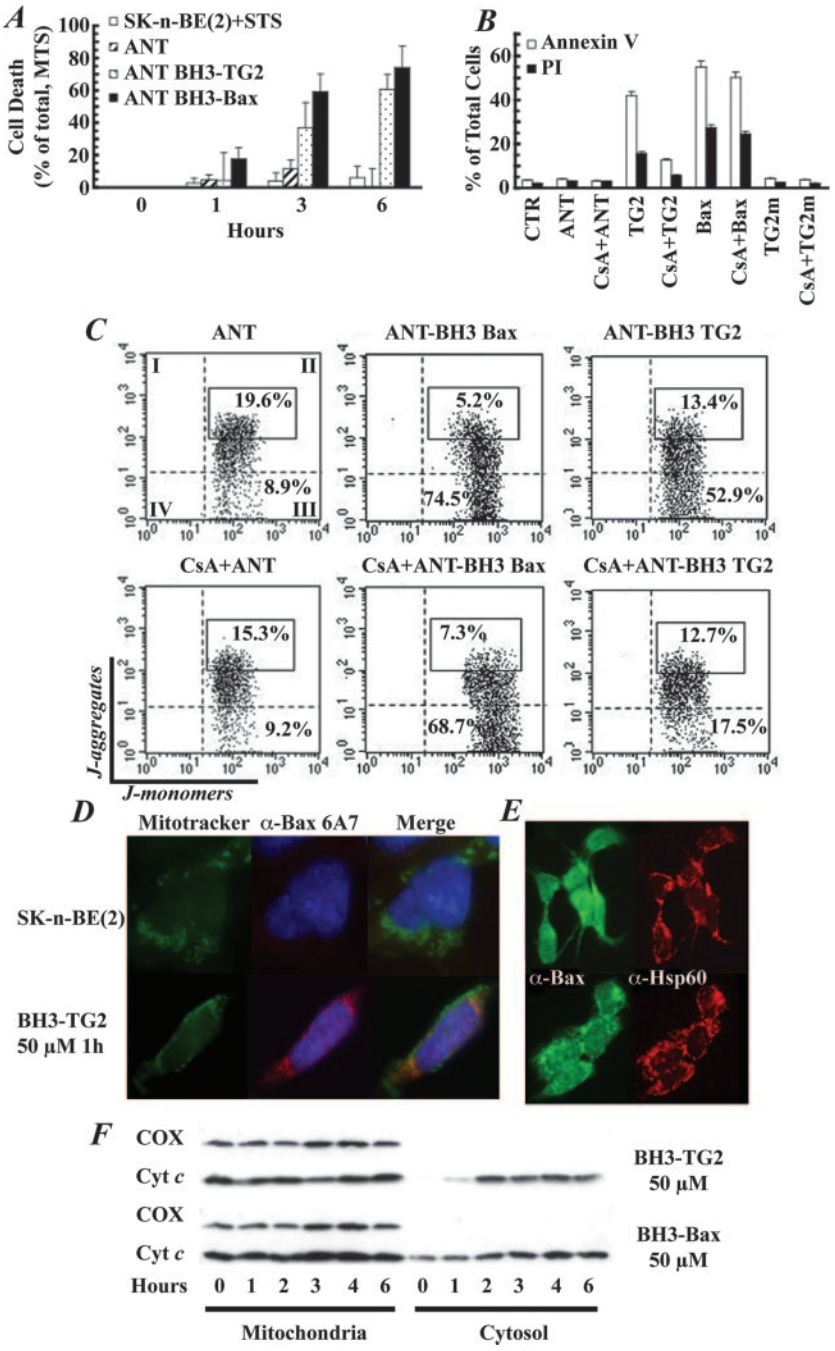


FIG. 2. **Cell permeable TG2-BH3 peptide induces cell death, mitochondrial depolarization, Bax conformational change, and translocation as well as cytochrome *c* release.** SK-n-BE(2) cells were treated for different time periods with 50 μM cell-permeable peptides bearing the internalization sequence alone (*ANT*, negative control), Bax BH3 (*ANT-BH3 Bax*, positive control), and TG2-BH3 (*ANT-BH3 TG2*) domains. *A*, time course analysis of cell viability (MTS assay) revealed that BH3 peptides induce progressive loss of cell functionality. 1 μM STS treatment was used as control. *B*, biparametric flow cytometry analysis of cells double-stained with annexinV-FITC/PI after 6 h of BH3 peptide treatment, in the presence or absence of CsA. CsA completely inhibited TG2-BH3-induced cell death, whereas it had no effect on Bax-BH3-induced cell death. The peptide-bearing TG2-BH3 mutated at Leu^{204} is ineffective in respect to cell death induction. *C*, biparametric flow cytometry analysis of MMP after staining with JC-1 in the presence or absence of CsA. Both Bax and TG2-BH3 peptides induce early loss of $\Delta\Psi$ (data presented refer to 1 h of treatment), and only the TG2-BH3 peptide is inhibited by CsA pretreatment. *D* and *E*, TG2-BH3 peptide induces Bax conformational change and translocation to mitochondria. SK-n-BE(2) cells were treated with 50 μM TG2-BH3 peptide for 1 h, fixed, and labeled with antibodies directed against Bax. *D*, staining with α-Bax 6A7 antibody, specific for Bax conformational change, and Mitotracker green for mitochondria. *E*, staining with α-Bax N-20 antibody, for overall Bax, and Hsp60 as mitochondrial marker. *F*, TG2-BH3 peptide induces cytochrome *c* release *in vivo*. SK-n-BE(2) cells were treated with 50 μM BH3 peptides for the indicated times, and cyt *c* release was assayed by Western blot. Cytochrome *c* oxidase (*COX*) was used as a control for mitochondria preparation. Both TG2- and Bax-BH3 peptides induce early cytochrome *c* release.

treated with the TG2-BH3 peptide, whereas it had a negligible effect on Bax-BH3-treated cells. The different sensitivity to CsA inhibition suggests that TG2-BH3 might induce mitochondrial membrane permeabilization by a mechanism different from that of Bax-BH3. The translocation of the pro-apoptotic Bcl-2 family member Bax to mitochondria has been described as a relevant step toward mitochondrial membrane permeabilization (20, 21), We investigated whether the TG2-BH3 peptide was able to induce the activation/translocation of Bax protein. In cells treated for 1 h with 50 μM TG2-BH3 peptide, we observed the Bax conformational change, detected by labeling with specific 6A7 monoclonal antibody (Fig. 2*D*), as well as translocation of Bax to mitochondria, deduced from the punctate staining visible after labeling overall of Bax with polyclonal N-20 antibody (Fig. 2*E*). To address whether this Bax translocation was related to mitochondrial membrane depolarization we analyzed the ability of TG2- and Bax-BH3 peptides to induce cyt *c* release. Fig. 2*F* shows that cyt *c* release is induced by both peptides, and it takes place at the same time of $\Delta\psi$ collapse. Interestingly, the TG2-BH3 peptide is as effective as Bax-BH3 peptide in inducing cyt *c* release *in vivo*. Data presented so far were consistent with the mitochondria-regulated cell death observed in TGA cells (19), which also showed an increase of the intracellular ROS. When we checked the ROS production levels, we did not observe any variation after treatment with either TG2- or Bax-BH3 peptides (data not shown), suggesting that this event might be a consequence of the sustained mitochondrial hyperpolarization observed in TGA cells.

Effect of TG2-BH3 Peptides on Isolated Mitochondria—Cytochrome *c* release from mitochondria is supposed to be achieved in different ways, implicating both the formation of specific channels or the rupture of the outer mitochondrial membrane (20, 21). In order to understand which model could account for

TG2-dependent action, we addressed the effect of TG2-BH3 peptide on isolated mitochondria. To this aim we investigated whether BH3 peptides were able to induce swelling of mitochondria from SK-n-BE(2) cells, and if CsA had any effect on it. Fig. 3*A* shows a representative profile of mitochondrial swelling induced by peptide treatment monitored by variations in TMRM fluorescence. TG2- and Bax-BH3 peptides are both able to induce mitochondrial swelling, and, again, CsA inhibition is effective only when the TG2-BH3 peptide is employed. This observation was in accordance with what we observed in living cells, and it was a further indication of a different mode of action for TG2-BH3 with respect to Bax. We also addressed the ability of both peptides to release cyt *c* from isolated SK-n-BE(2) mitochondria. Fig. 3*B* shows a representative Western blot of the supernatants obtained from mitochondria treated with 10 and 50 μM peptides, in the presence or absence of CsA 5 μg/ml. Calcium chloride and ANT peptide were used as positive and negative controls, respectively. These data indicate that both peptides were able to induce cytochrome *c* release in a concentration-dependent manner, and that, also in this case, CsA inhibition was effective only for the TG2-BH3 peptide. To confirm this finding we evaluated the amount of cytochrome *c* released after treatment with 50 μM peptide in the presence or absence of CsA with an ELISA assay. Fig. 3*C* shows that both peptides induce cyt *c* release to a similar extent, and CsA was effective only for TG2-BH3.

Because of the different efficacy of CsA to inhibit TG2- and Bax-BH3 peptide action, both *in vivo* (Fig. 2, *B* and *C*) and on isolated mitochondria (Fig. 3, *A–C*), and given the observed activation/translocation of Bax (Fig. 2, *D* and *E*), we chose to address the possibility that TG2-BH3 peptide was able to directly interact with Bax. To verify this hypothesis, we treated both SK-n-BE(2) cells and isolated mitochondria with N terminus biotinylated BH3 peptides (see "Experimental Procedures"), and, after purification by streptavidin paramagnetic particles, the interacting proteins were analyzed by Western blot. Fig. 3*D* shows that both peptides are able to co-precipitate Bax from isolated SK-n-BE(2) mitochondria, in a concentration-dependent manner, although no interaction was observed with Bcl-2 or Bcl-X_L (data not shown). Similar results were obtained when cells were treated with biotinylated peptides, and interacting proteins were analyzed (data not shown). These data suggest that the TG2-BH3 domain might be responsible for Bax-TG2 interaction and that this leads to an activation of Bax, resulting in its translocation to mitochondria and consequently causing cytochrome *c* release.

TG2 Interacts with Bax and Bak—On the basis of the above described data we hypothesized that TG2 might be able to modulate cytochrome *c* release by interacting with Bcl-2 family members and that this interaction might be modulated during cell death induction and progression. In order to address this hypothesis, we performed immunoprecipitations on TG2-overexpressing cells before and after cell death induction with 1 μM STS for 2 h. Whole cell extracts were prepared in CHAPS-IP buffer, to preserve native forms of Bax and Bak (35), and we looked for co-precipitations of TG2 with Bcl-2 family members using antibodies directed against TG2, Bax, and Bak. Fig. 4*A* shows that, even under steady state conditions, TG2 co-precipitates with Bax, and this interaction is enhanced after cell death induction. The interaction between Bax and TG2, as well as its increase upon cell death induction, was confirmed by immunoprecipitation with the anti-Bax N-20 antibody (Fig. 4*B*). Moreover, when we used as bait the anti-Bax 6A7 antibody, specific for Bax conformational change, we observed that TG2 co-precipitated with the Bax that has undergone the conformational change, and, also in this case, the interaction between the two proteins increased after apoptosis induction (Fig. 4*C*). These data indicate that TG2 interacts with Bax in living cells and that this interaction is enhanced upon induction of apoptosis. We also checked the ability of TG2 to interact with other members of the Bcl-2 family, and we observed co-precipitation with Bak (Fig. 4*A*); but in this case, there were no variations in the amount of protein associated before and after induction of cell death. Interestingly, no binding with the anti-apoptotic members Bcl-2 or Bcl-X_L had been detected (data not shown).

Bax Acts as a TG2 Substrate at the Mitochondrial Level—The TG2 main enzymatic activity relies on its ability to form cross-links between glutamines and lysines of proteins (2). The proposed mechanisms for Bax-mediated cyt *c* release involve both interaction with existent mitochondrial channels and/or aggregation of Bax itself, to form pores on the mitochondrial outer membrane (20, 21, 36). Given the observed interaction between TG2 and Bax, we verified whether Bax might act as a substrate for TG2 enzymatic activity. TG2-overexpressing cells were pre-incubated with the cell permeable TG2 synthetic substrate 5-(biotinamido)pentylamine (EZ-link; Ref. 37), treated with staurosporine 1 μM for 2 h, in the presence or absence of the caspase inhibitor z-VAD, and the TG2 protein substrates that incorporated EZ-link were revealed by Western blot with horseradish peroxidase-conjugated streptavidin. We observed (Fig. 5*A*) that under steady state conditions only a few proteins incorporated EZ-link (at least one of them was TG2 itself; data not shown). But when TG2 activation takes place, upon cell death induction with staurosporine, we observed the incorporation of EZ-link into many protein substrates, with masses ranging from 17 to 200 kDa. It is interesting to note that the inhibition of caspases results in a slightly different pattern of bands, suggesting that TG2 and caspases share some substrates (Fig. 5*A*, *lane 3*). To address the possibility that Bax might act as a TG2 substrate at the mitochondrial level, we purified mitochondria from cells treated as described above, and we purified the EZ-link-labeled proteins with streptavidin paramagnetic particles. Fig. 5*B* shows a Western blot of whole cell extract and purified mitochondrial TG2 substrates, probed with anti-Bax N-20 antibody. In the whole cell extract (Fig. 5*B*, *WCE*), the antibody recognized the monomeric form of Bax, at 20 kDa, as well as other bands that have an apparent mobility in the range between 50 and 150 kDa. In the lane corresponding to purified mitochondrial TG2 protein substrate (Fig. 5*B*, *MP*) the 20-kDa band is lacking, whereas the others are still present. The apparent molecular mass suggests that they might be Bax multimers, as already reported by other authors (38, 39). To confirm these results, we analyzed the status as well as the distribution between cytosol and mitochondria of Bax protein in SK-n-BE(2) and TG2-overexpressing cells. Fig. 5*C* shows that the monomeric form of Bax is equally distributed between cytosol and mitochondria. Whereas, when we looked at Bax multimers, they appeared to localize in mitochondria and to be more abundant in TGA cells, which also showed about half of the TG2 proteins in the mitochondria (*upper panel*). The presence of a strong signal at about 50 kDa in the cytosol fraction, as well as the presence of a doublet of bands at about 28 kDa in the mitochondrial fraction, might be caused by a nonspecific cross-reaction of the antibody. We obtained similar data for Bak, even if the presence of multimers was not so evident (data not shown). This evidence prompted us to analyze Bax status in TGA cells by immunofluorescence. Staining of Bax with the monoclonal α-Bax 2D2 antibody (Fig. 5*D*) results in diffuse staining, corresponding to the cytosolic monomeric form, as well as in punctate aggregated staining that might correspond to the multimeric forms observed by Western blot

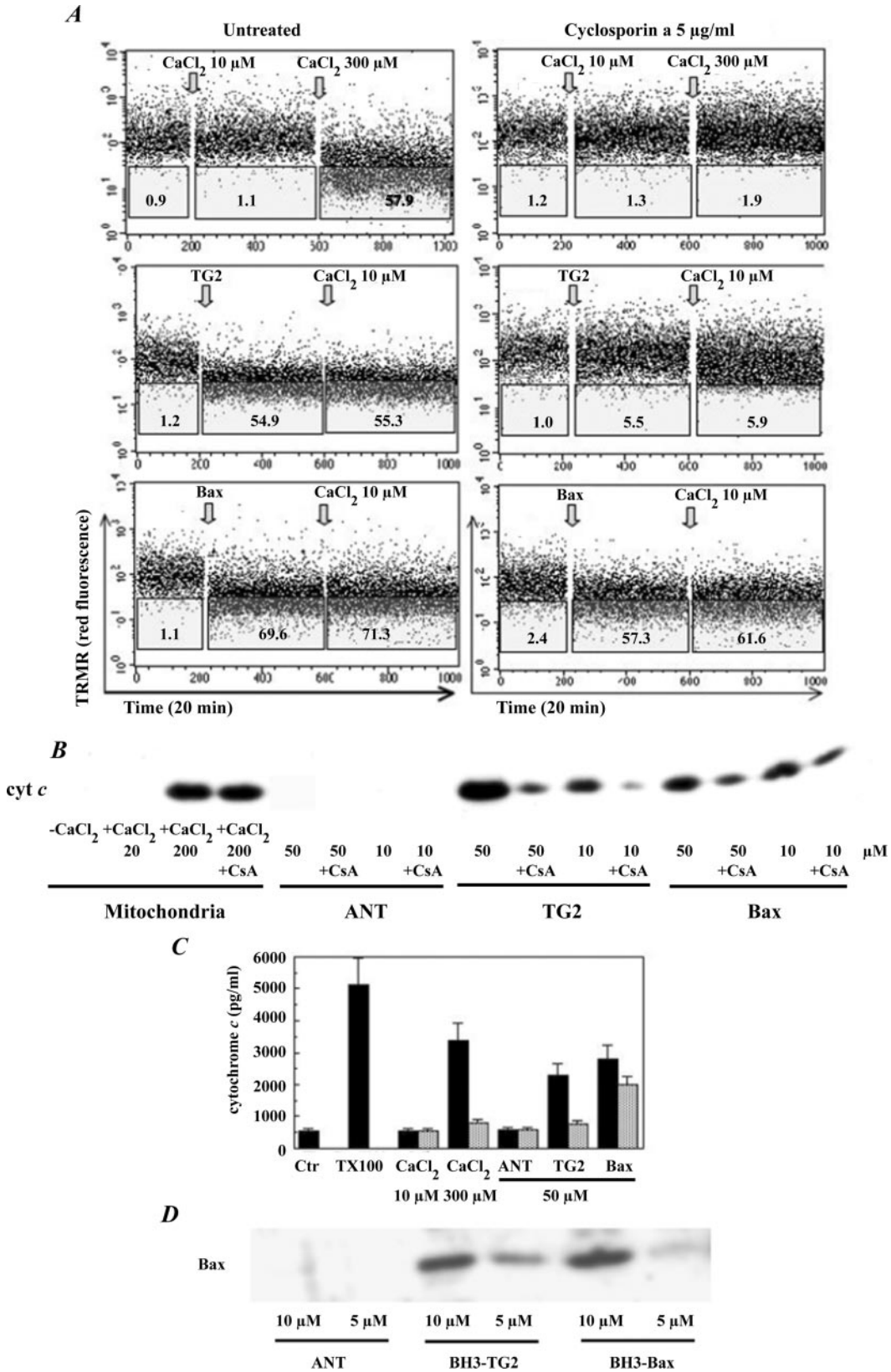


FIG. 3. **TG2-BH3 induces mitochondrial swelling and cytochrome *c* release on isolated mitochondria.** *A*, mitochondria, isolated from SK-n-BE(2) cells, were treated with BH3 peptides, and the MMP was monitored by variations in TMRM fluorescence as a function of the time. Both peptides induce a rapid loss of MMP, whose loss is inhibited by CsA (5 μg/ml) only in the case of the TG2-BH3 peptide. In the first row, a positive control of Ca^{2+}-induced MMP loss is shown. Numbers in the *boxed area* represent the percentage of mitochondria showing loss of MMP. *B* and *C*, TG2-BH3 peptide induces cytochrome *c* release from isolated mitochondria. Mitochondria isolated from SK-n-BE(2) cells were treated with 10 and 50 μM peptide for 30 min in the presence or absence of CsA. Cytochrome *c* release was monitored by Western blot (*B*) and ELISA (*C*). Calcium chloride and/or Triton X-100 (*TX100*) treatments were used as positive controls. The peptides were able to induce release of nearly all cytochrome *c* (*black bars*) but CsA inhibition (*gray bars*) is more effective for the TG2-BH3 peptide. *D*, BH3 peptides interact with Bax. Mitochondrial proteins interacting with biot-ANT BH3 peptides were isolated as described under "Experimental Procedures," and the presence of Bax was revealed by Western blot with α-Bax antibody. Both peptides are able to co-precipitate Bax in a concentration-dependent manner.

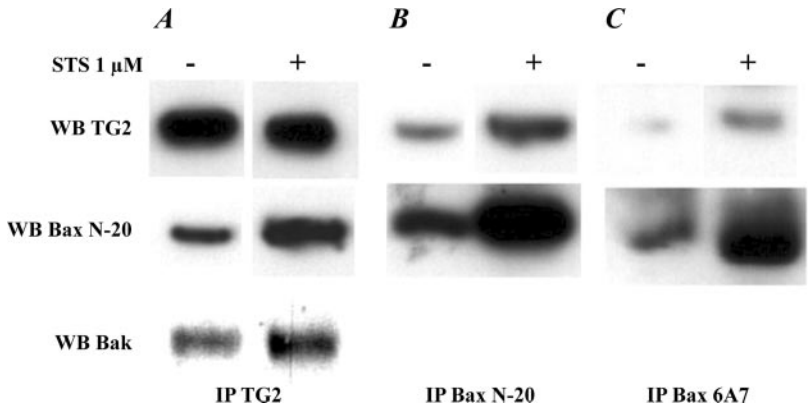


FIG. 4. **TG2 interacts with pro-apoptotic Bcl-2 family members Bax and Bak.** Western blot analysis of interaction between TG2, Bax, and Bak in TGA cells prior to and after apoptosis induction. Whole cell extracts, from cells treated with 1 μM STS for 2 h, were prepared in CHAPS-IP buffer and subjected to immunoprecipitations with antibodies directed against TG2 (Ab1) and Bax (N-20 and 6A7). *A* and *B*, TG2-Bax interaction is detectable in TG2-overexpressing cells even in the absence of any apoptotic stimulus and is increased after STS treatment. *C*, TG2 interacts with Bax that had undergone conformational change, and this interaction is increased after STS treatment. TG2-Bak interaction takes place even in the absence of any apoptotic stimulus, but it is not increased after STS treatment (*A*).

(Fig. 5*C*). Staining of Bax with the monoclonal α-Bax 6A7 antibody, which recognizes the conformational change of Bax, results in the same punctate staining observed with the α-Bax 2D2 antibody. These data are in accordance with what we observed in immunoprecipitation experiments (Fig. 4*C*), where, even under steady state conditions, the α-Bax 6A7 antibody co-precipitates Bax and TG2. Taken together this evidence confirms that there is an interaction between TG2 and Bax, but also indicates that TG2 might post-translationally modify Bax via the formation of SDS insoluble cross-links, which result in the appearance of stable Bax polymers at the mitochondrial level.

DISCUSSION

The intrinsic, or mitochondrial, pathway of apoptosis depends on the alteration of organelle membrane permeability, which leads to the release of small intermembrane-contained proteins; among them is cytochrome *c*. The regulation of this phenomenon has been proposed to rely on the balanced activity of pro- and anti-apoptotic members of the Bcl-2 family. The Bcl-2 family consists of about 20 proteins that share sequence and structure homology with Bcl-2 (30, 40, 41). There are four specific domains, termed Bcl-2 homology (BH1, BH2, BH3, BH4), that show high conservation among the family and are critical for determining cell survival *versus* apoptotic function of these proteins as well as their ability to interact with each other and with other regulatory proteins. The anti-apoptotic members of the family, Bcl-2 and Bcl-X_L, show the presence of all four BH domains; the pro-apoptotic, can be further subdivided into the multidomain, comprising Bax and Bak, which contain the BH1, BH2, BH3 domains, and the BH3-only proteins, which possess only the BH3 domain (30, 40, 41). The multidomain proteins Bax and Bak are able to homo- and heterodimerize, and this ability has been described as important for the modulation of the mitochondrial changes that take place after cell death induction (42–44). The expanding characterization of the biological significance of the various members of the BH3-only protein family revealed them as important upstream elements in the initiation and amplification steps of apoptosis. The interactions that these proteins establish with pro- and anti-apoptotic members of the Bcl-2 family, as well as with other proteins in various cellular compartments, are responsible for the integration of cell survival *versus* death signals and result in a fine-tuning of the apoptotic cascade. The regulation of their activity relies on both transcriptional (as their cellular levels increased significantly as a consequence of the induction of apoptosis) and post-translational regulation, *i.e.* phosphorylation or cleavage (45). In keeping with these characteristics, TG2 was among the first apoptosis-related genes demonstrated to be induced in cells programmed to die (27, 46–48), and its enzymatic activity is finely tuned in living cells (2). Moreover, the cross-linking activity of TG2 is responsible for the post-translational modification of various protein substrates involved in the apoptotic process. Studies from different laboratories have suggested that TG2 might have more than one function within the cascade of events leading to the establishment of the apoptotic phenotype (49, 50). We previously demonstrated that TG2 plays a role in the priming of mammalian cells toward apoptosis by seriously modifying mitochondrial physiology (19). In that report, we suggested that the observed mitochondrial alterations might be the consequence of a TG2-mediated modification of proteins involved in mitochondrial homeostasis. In this report we addressed this possibility by looking for possible TG2 protein partners and substrates. We discovered that TG2 possesses an eight amino acid region sharing more than 70% of identity with the BH3 domain of the Bcl-2 family. The only two different amino acids in this TG2 domain (Arg^{209} and Cys^{211}) do not seem to be relevant for its functionality. On the other hand the overall conserved leucine (Leu^{204}), described as important for homo- and heterodimerization of Bax, is present (29). In addition, the analysis of the crystal structure of TG2 showed this region as structured in an α-helix, as for all other BH3 domains, and exposed on the protein surface. Expression of mutated TG2, in which the domain was completely deleted or the leucine 204 mutated to aspartic acid, results in a complete abolishment of the sensitization to death observed when wild type TG2 was expressed. Cell permeable TG2-BH3 peptide induces, both *in vivo* and on isolated mitochondria, swelling, depolarization, and cytochrome *c* release at a rate comparable to that of the Bax-BH3 peptide. The only observed difference between the two peptides relies on the complete inhibition exerted by the permeability transition pore inhibitor CsA on the TG2-BH3 peptide. In addition, TG2-BH3 peptide induces an early conformational change, coupled with translocation to mitochondria, of Bax. Co-precipitation assays demonstrated its direct interaction with Bax. By immunoprecipitation assay, we confirmed that the TG2 protein itself is able to interact with pro-apoptotic members of the Bcl-2 family Bax and Bak, even in the absence of any apoptotic stimuli. As far as Bax is concerned the interaction with TG2 proves to be increased upon

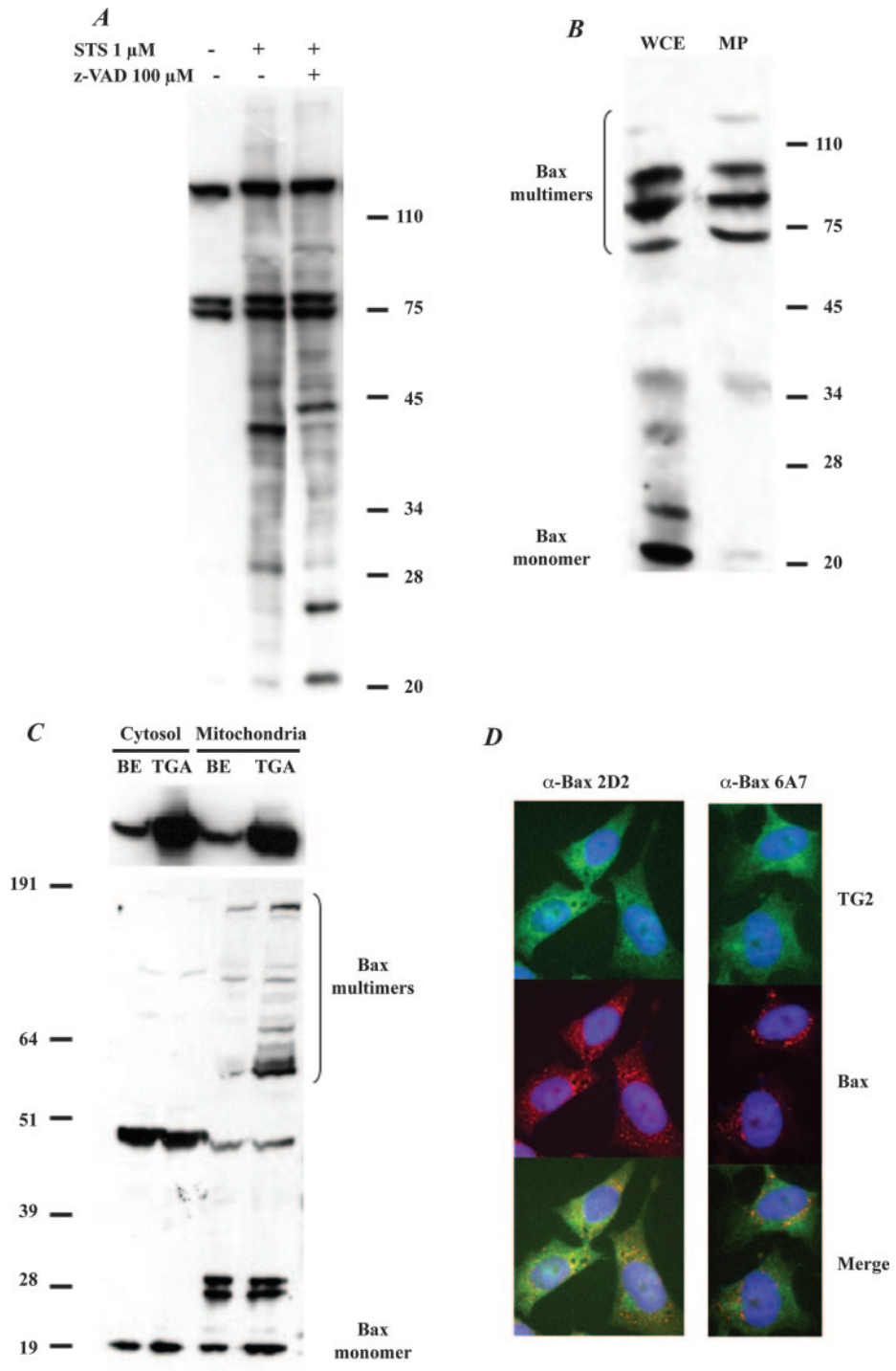


FIG. 5. **TG2-Bax interaction modifies Bax status in multiple ways.** *A*, identification of proteins that acts as substrate for TG2 cross-linking activity. EZ-Link-labeled TG2 protein substrates in the whole cell lysate of TGA cells prior to and after treatment with STS (1 μM, 2 h) in the presence or absence of caspase inhibitor z-VAD (100 μM) were identified by Western blot analysis with peroxidase-conjugated streptavidin. Few proteins incorporated the biotinylated substrate under steady state conditions, while more protein substrates appear after TG2 activation. Some of the proteins that are substrates for TG2 cross-linking activity are also caspase substrates, as indicated by the appearance of additional bands in the z-VAD-treated sample. *B*, identification of Bax as a TG2 cross-linking substrate at the mitochondrial level. Mitochondria of TGA cells, treated with 1 μM STS for 2 h in the presence of EZ-link, were purified, and then the biotinylated proteins were purified. After SDS-PAGE separation, Western blot with α-Bax antibody (N-20) was performed to reveal the presence of Bax. The monomeric form of Bax was detected only in whole cell extract (*WCE*), whereas bands corresponding to multimers of Bax, ranging between 50 and 200 kDa, were detected in both WCE and mitochondrial streptavidin-purified samples (*MP*). *C*, Bax is present in an aggregate form on the mitochondria of TGA cells. Mitochondrial and cytosolic proteins from SK-n-BE(2) and TGA cells were analyzed by Western blot with α-Bax antibody (N-20). In both cytosolic fractions Bax is present mainly as a monomer, whereas in the mitochondria the antibody detected bands corresponding to the multimeric form of Bax. The band migrating at an apparent molecular mass of 60 kDa proves to be enhanced in mitochondria in TGA cells. *D*, immunofluorescence analysis of Bax status in TG2-overexpressing cells. TGA cells were stained for TG2 (*green*) and Bax (*red*) by Alexa dye-conjugated monoclonal antibodies (see "Experimental Procedures"). For detection of overall Bax protein the α-Bax 2D2 antibody was used, whereas for conformationally changed Bax the α-Bax 6A7 antibody was used. The presence of aggregated and conformationally changed Bax is visible in untreated TGA cells.

cell death induction, whereas we did not observe any change in the ratio of Bak that associates with TG2. It is interesting to note that the conformational change that Bax undergoes after apoptosis induction has no effect on its ability to interact with TG2, but rather seems to be causative of the increased association of the two proteins. In addition, we found out that in TG2-overexpressing cells cytosolic Bax seemed to be mainly in the monomeric form, whereas in mitochondria it appeared also in a conformationally changed and aggregated form, similar to the one described by Youle and co-workers (51). From our data, it is not possible to state unequivocally whether the conformationally changed/aggregated Bax interacts with cytosolic rather than mitochondrial TG2 fraction or *vice versa*. Nevertheless, as the TG2-BH3 peptide is able to induce the Bax conformational change, we could speculate that the TG2-Bax interaction takes place in the cytosol and might promote the Bax conformational change/aggregation and translocation to mitochondria. It is worth noting that in TG2-overexpressing cells, about half of total TG2 protein is localized on the mitochondria, where after apoptosis induction it cross-links many protein substrates, among which is Bax itself. This TG2-dependent Bax cross-linking leads to the formation of Bax polymers with an apparent electrophoretic mobility that suggests they result from the oligomerization of multiple molecules of Bax. This kind of Bax aggregation is very similar to the one previously described (38, 39), and Bax multimerization has been proposed as the mechanism by which it might form pores on the outer mitochondrial membrane that lead to the release

of cytochrome *c*. In this view, TG2 cross-linking action might stabilize the aggregated Bax, resulting in a stably open pore on the outer mitochondrial membrane. We also observed interaction of TG2 with Bak, but this kind of interaction does not seem to change after induction of apoptosis, and we observed the presence of Bak multimers to a lesser extent than Bax multimers. The ability of TG2 to interact with the pro-apoptotic members of the Bcl-2 family Bax and Bak supported the biological significance of the domain in the whole protein context. The lack of interaction with the anti-apoptotic members of the Bcl-2 family (we never observed interaction of TG2 with Bcl-2 or Bcl-X_L) prompted us to hypothesize a model for TG2 regulation of Bax activation. Under physiological Ca^{2+} concentration, TG2 is inactive as a cross-linking enzyme. We might speculate that the interaction between TG2 and Bax in the cytosol is responsible for the further localization of both proteins in mitochondria, where they might be in close proximity. In this configuration TG2-Bax interaction might be anti-apoptotic, as it blocks Bax action on mitochondria and prevents cytochrome *c* release. After apoptosis induction, the early increase of calcium concentration at the mitochondrial level activates the cross-linking activity of TG2, which by polymerizing Bax stabilizes its pore-forming conformation. This action results in a more rapid release of cytochrome *c* compared with cells that do not have high TG2 levels. This hypothesis seems to be confirmed by the complete abolition of the priming effect toward apoptosis, observed in transfections of TG2 in which the BH3 domain was either deleted or mutated (L204E). In this view, TG2 seems to act both as a regulator of the apoptotic response upstream to mitochondria and as an accelerator of the process at the mitochondrial level. This evidence also suggests that TG2 behaves as a Bid-like BH3-only protein, as proposed by Letai *et al.* (39).

TG2 action at the mitochondrial level seems not to be limited to this stabilizing effect on Bax, because we previously observed that mitochondria of TG2-overexpressing cells were hyperpolarized, and there was an increase in ROS levels. In contrast with these observations, the TG2-BH3 peptide was not able to induce either hyperpolarization of mitochondria or increases in ROS levels. We have evidence that these effects are the consequence of a different and more complex action exerted by TG2 on some mitochondrial membrane proteins.[2]

In conclusion we found that TG2 might contribute to the control of the mitochondrial pathway of apoptosis by interacting with and post-translationally modifying pro-apoptotic members of the Bcl-2 family. The presence of a BH3 domain in TG2, which is of large mass size (80 kDa) and possesses different enzymatic activities (cross-linking, G-protein, PDI), suggests the existence of a new class of BH3-only proteins.

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