Unreliability of the Cytochrome c-Enhanced Green Fluorescent Fusion Protein as a Marker of Cytochrome c Release in Cells That Overexpress Bcl-2*

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A cytochrome c-enhanced green fluorescent protein chimera (cyt-c-EGFP) was used to monitor the release of cytochrome c from mitochondria in Bcl-2-negative and Bcl-2-positive MDA-MB-468 breast cancer cells. A comparison was made with the intracellular distribution of endogenous cytochrome c based on Western blotting of cell fractions and immunocytochemistry. The release of endogenous cytochrome c from mitochondria into the cytoplasm was detected in Bcl-2-negative cells treated with the kinase inhibitor staurosporine or the calcium-ATPase inhibitor thapsigargin. No release of endogenous cytochrome c was evident in Bcl-2-positive cells, consistent with earlier evidence that Bcl-2 overexpression inhibits cytochrome c release from mitochondria. Cyt-c-EGFP appeared to be localized to the mitochondria in Bcl-2-negative cells and to be released into the cytoplasm following treatment with either staurosporine or thapsigargin. However, in Bcl-2-positive cells the pattern of distribution of cytochrome c-EGFP was inconsistent with that of endogenous cytochrome c, due to accumulation of both cyt-c-EGFP and free EGFP in the cytoplasm of both treated and untreated cells. In summary, cyt-c-EGFP may be useful for monitoring cytochrome c release in living cells that do not express high levels of Bcl-2 but is an unreliable marker of cytochrome c release in cells that overexpress Bcl-2.

Apoptosis is induced by diverse signals and regulated positively or negatively by members of the Bcl-2 family. Recent studies indicate that mitochondrial dysfunction is a critical step in apoptosis and that mitochondria are a major site of action of both proapoptotic and antiapoptotic Bcl-2 family members (1–3). Many apoptotic signals induce the release of cytochrome c from its usual location between the inner and outer mitochondrial membranes into the cytoplasm, where it binds to the cytoplasmic protein Apaf-1, thereby activating caspases involved in the execution phase of apoptosis (4). Moreover, recent studies indicate that the proapoptotic proteins Bax and Bid trigger apoptosis by inducing cytochrome c release, while the antiapoptotic proteins Bcl-2 and Bcl-xL inhibit apoptosis by blocking cytochrome c release (5–8).

We have been investigating the role of calcium in apoptosis induction, using as a model system the induction of apoptosis by thapsigargin (TG),1 a selective inhibitor of the endoplasmic reticulum (ER)-associated calcium-ATPase (9, 10). The ER is the major intracellular reservoir of calcium ions in non-muscle cells (11). The TG-sensitive ER calcium-ATPase functions as a pump to maintain the high calcium concentration in the ER lumen required for a number of vital cellular functions including protein processing through the secretory pathway, translation, and cell division (12–14). Moreover, calcium is released from the ER in response to physiologic stimuli, producing pulses and waves of elevated cytoplasmic calcium concentration that regulate cellular processes as diverse as mitochondrial enzyme activity, transcription, and apoptosis (15). By inhibiting the ER-associated calcium-ATPase, TG allows calcium to efflux from the ER lumen into the cytoplasm, producing an elevation of cytoplasmic calcium concentration and depleting the ER lumen of calcium (16, 17). TG treatment induces apoptosis in a variety of cell types (18, 19), mediated by caspase activation and inhibited by Bcl-2 overexpression (20). Information gained from studies of TG-induced apoptosis should contribute to a better understanding of the physiological forms of apoptosis in which calcium release from the ER appears to play a role, including glucocorticosteroid-induced apoptosis (18, 21, 22) and apoptosis induced by growth factor withdrawal (23).

In the present study, we set out to determine if TG-induced apoptosis is mediated by cytochrome c release from mitochondria using the kinase inhibitor staurosporine (STS) as a positive control and to determine the effect of Bcl-2 on cytochrome c release following treatment with these agents. Until recently, cytochrome c release from mitochondria had been detected primarily by cell fractionation. Recently, a cytochrome c-green fluorescent protein chimera was reported as a method of monitoring cytochrome c release in living, unfractionated cells (24, 25). Therefore, a cytochrome c-enhanced green fluorescent protein chimera (cyt-c-EGFP) was used in the present study to monitor cytochrome c release in TG-treated cells. However, as reported here, the intracellular distribution of cyt-c-EGFP did

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1 The abbreviations used are: TG, thapsigargin; ER, endoplasmic reticulum; STS, staurosporine; cyt-c, cytochrome c; EGFP, enhanced green fluorescent protein; PBS, phosphate-buffered saline; TMRM, tetramethylrhodamine methyl ester.
not reflect that of endogenous cytochrome c in cells that over-express Bcl-2 and was therefore an unreliable marker of cytochrome c release under this condition.

MATERIALS AND METHODS

Cell Culture—The MDA-MB-468 breast cancer cell line was cultured in IMEM (Biofluids) with 10% heat-inactivated fetal calf serum (Atlas Biologicals) supplemented with penicillin, streptomycin, and l-glutamine (Life Technologies, Inc.).

Cell Treatment and Apoptosis Assays—Cells were plated on a 12-well plate at 100,000 per well 1 day before treatment. TG, purchased from Alexis (San Diego, CA), and STS, purchased from Sigma, were dissolved in Me2SO. Control cells were treated with an equal concentration of the Me2SO solvent (≤0.2% final concentration). The proportion of cells with apoptotic nuclear morphology was measured using acridine orange/ethidium bromide double staining on an epiﬂuorescence microscope.

Cell Fractionation—Cell fractionation was performed with the FluoroAce™ Apopain Assay kit (Bio-Rad) according to the manufacturer's protocol. Assays were performed in a 96-well plate (Falcon) and fluorescence was read with a Cytofluor Multwell Plate Reader Series 4000 (PerSeptive Biosystems, Framingham, MA). Purified apopain (Bio-Rad) was used as a positive control and Ac-DEVD-fluoromethyl ketone (Ac-DEVD-fmk) as a negative control. The reactions were allowed to proceed for 1 h at room temperature. During this time, fluorescence was read 4 times, and the linearity of the reactions was conﬁrmed with puriﬁed apopain.

Expression Vectors and Transfection Procedures—The pSFFVneo expression vector was provided by Gabriel Nunez, University of Michigan, and the human Bcl-2 cDNA subcloned into pSFFVneo was provided by Roger Miesfeld, University of Arizona. Transfections were performed with FuGene 6 (Roche Diagnostics). Transfected cells were selected with G418 (0.8 mg/mL) selection.

The cDNA encoding wild-type rat cytochrome c was subcloned into the EGFP-N1 expression vector to generate a C-terminal-linked cyt-c-EGFP fusion protein as previously described (24). MDA-MB-468 cells stably transfected with either the pSFFVneo vector or the pSFFVneo-Bcl-2 expression vector were secondarily transfected with the cyt-cEGFP plasmid (BD Pharmingen, 65891A; 1:1000); mammalian anti-cytochrome c (Santa Cruz, SC-8383; 1:100); and anti-EGFP (CLONTECH, 8362-1; 1:100).

Immunocytochemistry—Cells were incubated with Hoechst 33342 (5 µg/mL; Molecular Probes, Eugene, OR) for 30 min at 37 °C and subsequently ﬁxed with 4% paraformaldehyde for 10 min at room tempera- ture. Cells were then washed by two washes with PBS and incubated at room temperature in PBS/0.05% saponin, followed by a 10-min incubation in ice-cold methanol. After two washes with PBS, the cells were blocked with 5% goat serum in PBS for 30 min. Cells were incubated with mouse anti-rat cytochrome c antibody (BD Pharmingen, 65971A) at a 1:200 dilution for 1 h, washed twice with PBS, and then incubated with 1:100 goat anti-mouse Alexa488 (Molecular Probes) for 30 min. Both antibody incubations were performed in 5% goat serum in PBS. Cells were then washed with PBS. Cells were viewed on a Zeiss Axios- vert S100 epiﬂuorescence microscope with a 63×/1.4 NA objective and a 1.6× optical filter. Alexa 488 ﬂuorescence was visualized using an XF67 filter cube (Omega Optical) exc:485/nm; em:535. Hoechst 33342 ﬂuorescence was visualized using the XF67 filter cube exc:380/em: 470. Fluorescence images were obtained using a Hammamatsu Orca-100 digital camera. Images were processed using Simple PCI (Compix Inc., Imaging Systems, Cranberry Twp., PA).

Cytochrome c-EGFP Imaging—Control and Bcl-2-overexpressing MDA-MB-468 cells expressing cyt-c-EGFP chimeric protein were plated on glass bottom Petri dishes (no. 1.5, MatTek, Ashland, MA) the day before the experiment. Cells were then loaded immediately before imaging with a cell permeable nuclear dye, HOECHST 33342 (Molecular Probes) at 500 ng/mL for 20 min. Cells were imaged using a Zeiss Axiovert S-100 inverted epiﬂuorescence microscope with a 4×/oil/1.3 NA objective (Thornwood, NY) and an InCyt Im 1 single wavelength fluorescence imaging system (Intracellu- laring Imaging Inc., Cincinnati, OH). The imaging system consisted of a 300 watt xenon light source, a Sutter Instrument Lambda 105 filter wheel for excitation ﬁlters (480 nm for EGFP and 380 nm for HO- ECHS 33342), a liquid light cable leading the excitation light to the specimen, and a triple ﬁlter cube (XF67, Omega Optical, Brattleboro, VT) for emission wavelength selection (4’‚6-diamidino-2-phenylindole/ﬂuorescein isothiocyanate/Texas Red). Images were collected with a 12 bit charge-coupled device camera. Images were pseudocolored using Adobe Photoshop imaging software (Adobe Systems Inc., San Jose, CA). To investigate the localization of cyt-c-EGFP, cells were incubated with 150 nM tetramethylrhodamine methylster (TMRM, Molecular Probes) in culture medium for 15 min at 37 °C. Cells were then placed on the microscope stage and incubated in medium containing 30 nM TMRM during the experimental period. Confocal images of cyt-c-EGFP were collected using a 488 nm excitation light from an argon/krypton laser, a 560 nm beam splitter, and a 500–550 nm band pass barrier filter using a Zeiss 410 laser scanning confocal microscope and a 63×/1.4 NA oil immersion planapochromat objective (Thornwood, NY). The images of TMRM ﬂuorescence were collected using a 568 nm excitation light from the argon/krypton laser, a 560 nm beam splitter, and a 590 nm long pass barrier filter. For measurements of cyt-c-EGFP and TMRM ﬂuo- rescence, laser power was attenuated with neutral density ﬁlters by at least 90 and 99%, respectively, to avoid photodamage caused by laser illumination.

RESULTS

The MDA-MB-468 breast cancer cell line was stably transfected with the empty pSFFVneo vector or the pSFFVneo-Bcl-2 vector. Bcl-2 was barely detected by Western blotting in untransfected cells and cells transfected with the empty vector but was expressed at high levels in two clones transfected with pSFFVneo-Bcl-2 (Fig. 1A). We reported previously that MDA- MB-468 cells undergo apoptosis when treated with 100 nM TG (20). The induction of apoptosis by TG is a slow process that evolves over 48–72 h, and is inhibited by Bcl-2 overexpression (Fig. 1B). In contrast, the induction of apoptosis by STS was determined within a few hours of STS addition, but was also inhibited by Bcl-2 (Fig. 2A). The difference in kinetics of apoptosis induction by TG and STS was confirmed by measuring caspase-3 activation (Fig. 3). In TG-treated cells, caspase-3 was not activated until 48 h post-treatment, whereas in STS-treated cells, caspase-3 activation was detected within 6 h of treatment. Bcl-2 overexpression inhibited caspase-3 activation in both TG- and STS-treated cells.
To investigate the effect of STS and TG treatment on the intracellular distribution of cytochrome c using live cell fluorescence microscopy, cells that stably overexpress Bcl-2 or empty vector transfected cells were secondary transfected with the cyt-c-EGFP plasmid. EGFP-positive cells were sorted and collected by flow cytometry. Expression of cyt-c-EGFP in vector control and Bcl-2 expressing cells was documented by Western blotting (Fig. 4A). Cyt-c-EGFP displayed a punctate pattern of fluorescence that matches that of the mitochondrial marker TMR M (Fig. 4B). The same pattern was observed in MDA-MB-468 cells coexpressing Bcl-2 and cyt-c-EGFP (not shown).

The pattern of distribution of cyt-c-EGFP in cells was observed by fluorescence microscopy following treatment with STS or TG (Fig. 5). Within 4 h of adding 1 μM STS, the intracellular localization of cyt-c-EGFP changed from a punctate mitochondrial pattern to a diffuse cytoplasmic pattern (Fig. 5A). The change in cyt-c-EGFP distribution induced by STS was accompanied by the development of nuclear morphological changes typical of apoptosis, detected by Hoechst staining (Fig. 5B). Bcl-2 overexpression inhibited the staurosporine-induced change in cyt-c-EGFP distribution (Fig. 5C) and also inhibited nuclear apoptotic changes (Fig. 5D). These findings suggest that STS induces release of cyt-c-EGFP from mitochondria and that cyt-c-EGFP release is inhibited by Bcl-2 overexpression.

TG treatment for 48 h also changed the intracellular distribution of cyt-c-EGFP from a punctate mitochondrial pattern to a diffuse cytoplasmic pattern (Fig. 5E). Over this time period, the intracellular distribution of cyt-c-EGFP did not appear to change in untreated cells. As with STS treatment, the change in cyt-c-EGFP distribution induced by TG was observed in cells undergoing apoptosis, detected by changes in nuclear morphology (Fig. 5F). Bcl-2 overexpression inhibited TG-induced apoptosis (Fig. 5H). However, Bcl-2 overexpression did not prevent the appearance of a diffuse cytoplasmic pattern of cyt-c-EGFP distribution following TG treatment (Fig. 5G).

The preceding observations suggest that Bcl-2 may act downstream of cytochrome c release to inhibit apoptosis in TG-treated cells. However, this conclusion is based on the assumption that the intracellular distribution of cyt-c-EGFP corresponds to that of endogenous cytochrome c. Therefore, the effect of STS and TG on the intracellular distribution of endogenous cytochrome c was assessed by two independent methods. First, cells were fractionated according to a standard procedure to separate mitochondria from cytoplasm, and cytochrome c in the cytosolic fraction was assessed by Western blotting. Release of endogenous cytochrome c into the cytosolic fraction was evident following both STS and

**Fig. 1.** Bcl-2 overexpression in MDA-MB-468 cells inhibits TG-induced apoptosis. A, wild-type, empty vector, and two Bcl-2-overexpressing clones (10 and 22) were assayed for Bcl-2 expression level by Western blotting. Equal amounts of protein were added to each lane. Panel B, Bcl-2 prevents TG-induced apoptosis. Wild-type and Bcl-2-overexpressing cells were treated with 100 nM TG or vehicle, and the percentage of apoptotic cells at the time intervals shown was measured by fluorescence microscopy based on the presence or absence of typical nuclear morphological changes in acridine orange/ethidium bromide-stained cells. Symbols represent the mean ± S.D. of three independent experiments.

**Fig. 2.** Inhibition of STS-induced apoptosis by Bcl-2. Empty vector control and Bcl-2-overexpressing MDA-MB-468 cells were treated with 1 μM STS, and apoptosis was assessed by the morphology of acridine orange-stained nuclei. Symbols represent the mean of at least three separate experiments.

**Fig. 3.** Bcl-2 overexpression inhibits STS- and TG-induced caspase activation. Empty vector or Bcl-2-overexpressing MDA-MB-468 cells were treated with 100 nM TG for the indicated times, and samples were collected for determination of caspase-3 activity. Fold induction from control group activity is shown (n = 3; mean ± S.E.). Cells were treated with 1 μM staurosporine for 6 h prior to caspase-3 activity measurement. Fold induction from control group activity is shown (n = 2; mean ± S.E.). Statistical comparisons between different treatment groups were performed using the enzyme activity data. The asterisk denotes a statistically significant difference (p < 0.05) versus control group according to the Student’s t test for independent samples.
Fig. 4. Cyt-c-EGFP expression in MDA-MB-468 cells. A, MDA-MB-468 cells expressing empty vector or Bcl-2 as described in the legend to Fig. 2 were stably transfected with a construct encoding cyt-c-EGFP. The level of cyt-c-EGFP by Western blotting is shown. B, MDA-MB-468 neo-cells stably expressing cyt-c-EGFP were incubated with 150 nM TMRM, and images were collected by confocal microscopy. Cyt-c-EGFP displayed a punctate pattern of fluorescence that matches that of TMRM. The same pattern was observed in MDA-MB-468 cells coexpressing Bcl-2 and cyt-c-EGFP (not shown).

TG treatment, and in each case was inhibited by Bcl-2 overexpression (Fig. 6). These findings were confirmed by immunocytochemistry in which a monoclonal antibody to cytochrome c detected endogenous cytochrome c as a punctate pattern characteristic of mitochondrial localization (Fig. 7). The location of cytochrome c in mitochondria was confirmed by co-localization with the mitochondrial-specific dye Mitotracker. In neo-control cells, treatment with either STS or TG induced release of endogenous cytochrome c into the cytoplasm, producing diffuse fluorescence and eliminating the punctate mitochondrial pattern (Fig. 7, panels A and B). Moreover, Bcl-2 overexpression inhibited release of endogenous cytochrome c into the cytoplasm (Fig. 7, C and G). In summary, two independent assays of endogenous cytochrome c indicate that Bcl-2 inhibits cytochrome c release from mitochondria in cells treated with either STS or TG, whereas cyt-c-EGFP gave the misleading result that Bcl-2 did not inhibit cytochrome c release following TG treatment.

To investigate this discrepancy, cells that expressed cyt-c-EGFP were analyzed by cell fractionation and Western blotting to compare the intracellular distribution of cyt-c-EGFP with that of endogenous cytochrome c (Fig. 8, A and B). A polyclonal antibody that recognizes both endogenous cytochrome c and cyt-c-EGFP fusion protein was employed. Both STS (Fig. 8A) and TG (Fig. 8B) induced endogenous cytochrome c release in neo-control cells, and this release was inhibited by Bcl-2 overexpression. In neo-control cells treated with either STS or TG, release of cyt-c-EGFP fusion protein into the cytoplasmic fraction was observed, fully consistent with the pattern of endogenous cytochrome c. However, the Western blotting pattern was distinctly different for endogenous cytochrome c versus cyt-c-EGFP in Bcl-2-overexpressing cells in which the cyt-c-EGFP fusion protein was detected in the cytoplasmic fraction of untreated cells (Fig. 8). Both cytoplasmic and mitochondrial fractions are shown for comparison in Fig. 8B. The ratio of cytosolic cyt-c-EGFP to mitochondrial cyt-c-EGFP was much higher than the corresponding ratio for endogenous cytochrome c. These findings suggest that cyt-c-EGFP is incompletely taken up by mitochondria of Bcl-2-positive cells or is released from mitochondria upon cell disruption and fractionation.

To further investigate the cyt-c-EGFP expressing cells, Western blots of cytosolic fractions were reprobed with antibody to EGFP (Fig. 8C). Consistent with data obtained using cytochrome c antibody, the cyt-c-EGFP appeared to be redistributed into the cytoplasm following STS or TG treatment in neo-control cells, whereas in Bcl-2-overexpressing cells cyt-c-EGFP was detected in cytosolic fractions from both treated and untreated cells. Furthermore, an unexpected finding was the presence of free EGFP in cytosolic fractions.

In summary, the use of cyt-c-EGFP as a marker of cytochrome c release in cells that overexpress Bcl-2 was complicated by the inappropriate accumulation of both free EGFP and cyt-c-EGFP in the cytosol. Note that the level of cyt-c-EGFP did not appear to increase following TG treatment in Bcl-2-positive cells (Fig. 8B). But examination of the same cells by fluorescence microscopy (Fig. 5G) suggested that cyt-c-EGFP was released from mitochondria of Bcl-2-positive cells following TG treatment. Based on the findings described in Fig. 8C, this misleading result was due to increased accumulation of free EGFP in the cytoplasm of Bcl-2-positive cells following TG treatment.

DISCUSSION

Evidence that Bcl-2/Bcl-xL inhibit cytochrome c release from mitochondria originated with studies in which STS, etoposide, and ultraviolet radiation were employed as apoptosis inducers (27, 28) and has subsequently been confirmed in studies using not only STS (7), but also anti-Fas (5, 29, 30), tumor necrosis factor (8, 29), the viral protein E1A (6), and the p53 tumor suppressor gene (31). Also, Bcl-2 and Bcl-xL have been reported to inhibit Bax-induced release of cytochrome c both in vitro from isolated mitochondria (32–34) and in vivo (7, 35).

However, there are at least two reports indicating Bcl-2/Bcl-xL may work downstream of cytochrome c release to inhibit caspase-3 activation and apoptosis. In one case, cytochrome c release was induced by exogenous expression of Bax (36), while in the other case cytochrome c release was induced by a porphyrin-derived photosensitizer (37). In each of these situations, caspase-3 activation and cell death were inhibited by Bcl-2 or Bcl-xL. Thus, Bcl-2 and Bcl-xL appear to act downstream of cytochrome c release to inhibit apoptosis in certain situations. Several additional findings suggest that the ability of Bcl-2 and Bcl-xL to inhibit cell death is not due solely to inhibition of cytochrome c release from mitochondria. First, Bcl-xL inhibits cell death induction in cytochrome c-insensitive MCF7 cells (38). Second, Bcl-2 inhibits apoptosis induction by microinjected cytochrome c (39). Third, Bcl-2 inhibits caspase-3 activation induced by the addition of cytochrome c to cell extracts (40).

Therefore, the goal of the present study was to determine whether or not cytochrome c release occurs during TG-induced apoptosis, and, if so, the effect of Bcl-2 on cytochrome c release. To this end, we used three complementary assays of cytochrome c release. Two of the assays were dependent upon detection of endogenous cytochrome c release, either by immunohistochemistry or by cell fractionation and Western blotting. The third assay used cyt-c-EGFP as a marker of cytochrome c release. Both assays of endogenous cytochrome c release indicated that TG-induced apoptosis is associated with cytochrome c release, although apoptosis induction and release of cytochrome c was delayed by about 48 h following TG addition to cell cultures. This was in striking contrast to the rapid induction of apoptosis and release of endogenous cytochrome c detected within 4 h of adding STS to cells. Nevertheless, Bcl-2...
overexpression inhibited apoptosis as well as endogenous cytochrome c release in both TG- and STS-treated cells.

Unexpectedly, the behavior of cyt-c/H18528EGFP was not fully consistent with the behavior of endogenous cytochrome c in Bcl-2-overexpressing cells, raising concern about the validity of cyt-c/H18528EGFP as a marker of cytochrome c release. The discordance between endogenous cytochrome c and cyt-c/H18528EGFP might not have been recognized if only cells lacking Bcl-2 (i.e., untransfected or neo-transfected MDA-MB-468 cells) were used in experiments. Indeed, release of cyt-c/H18528EGFP from mitochondria into the cytoplasm was detected by both fluorescence microscopy and cell fractionation/Western blotting in both STS- and TG-treated Bcl-2-negative MDA-MB-468 cells, in accordance with the behavior of endogenous cytochrome c under the same treatment conditions. Thus, the discordance between the intracellular distribution of cyt-c/H18528EGFP and endogenous cytochrome c was mainly in cells that overexpress Bcl-2, where both cyt-c/H18528EGFP and free EGFP were found to accumulate in the cytoplasm of untreated cells.

Other laboratories have reported the use of cyt-c/EGFP fusion protein as a marker of endogenous cytochrome c release (24, 25). But cells that overexpress Bcl-2 were not employed in those studies. Furthermore, in the case of Bcl-2-negative cells in the present study, there was complete concordance between the intracellular distribution of endogenous cytochrome c and cyt-c/EGFP in cells treated with STS or TG. Hence, cyt-c/EGFP...
indeed appears to be a reliable marker of cytochrome c release from mitochondria in cells where Bcl-2 is not overexpressed. Why then might Bcl-2 overexpression lead to a discrepancy between the intracellular localization of cyt-c-EGFP compared with endogenous cytochrome c and also to the accumulation of free EGFP in the cytoplasm? Although the answer to this question is not known with certainty, there are at least two potential explanations. One possibility is that the presence of Bcl-2 on the outer mitochondrial membrane interferes with transport of cyt-c-EGFP fusion protein into the inner mitochondrial membrane space. As a consequence, some of the cyt-c-EGFP fusion protein might be loosely associated with the outer mitochondrial membrane and therefore readily released into the cytosol following cell disruption and fractionation. A second possibility is that Bcl-2, by inhibiting caspase activation and apoptosis induction, allows cells to accumulate high levels of cyt-c-EGFP in the cytoplasm. Cytoplasmic accumulation of cyt-c-EGFP might be toxic to the control (neo-transfected) cells; hence, the cyt-c-EGFP-positive control cell population may be selected for low or absent cytoplasmic cyt-c-EGFP accumulation. Indeed, this may have been the case in the present study since Bcl-2-positive cells had considerably higher basal level of cytosolic cyt-c-EGFP than Bcl-2-negative cells. Another vexing problem with the use of cyt-c-EGFP as a marker of cytochrome c release in Bcl-2-overexpressing cells was the accumulation of free EGFP in the cytoplasm. This was also observed in Bcl-2-negative cells when treated with TG. Most likely, cyt-c-EGFP that accumulates in the cytoplasm of Bcl-2-positive cells is cleaved by an unknown protease to produce free EGFP. Moreover, perhaps elevated cytosolic calcium following TG treatment increases the turnover and degradation of cyt-c-EGFP, producing free EGFP.

In summary, both STS and TG induce release of endogenous cytochrome c from mitochondria, and Bcl-2 overexpression inhibits this release. Unexpectedly, a dichotomy between the distribution of endogenous cytochrome c and cyt-c-EGFP fusion protein was revealed, requiring caution in the use cyt-c-EGFP fusion protein as a marker of endogenous cytochrome c release, particularly in cells that overexpress Bcl-2.

Acknowledgments—We thank Nancy Wang and Edmunds Reineiks for guidance in fluorescence microscopy and Manjunatha Bhat for helpful discussions. We also thank Anna-Lisa Niemenson for assistance in confocal microscopy and for providing the Living Colors Antibody.

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