

Brief Definitive Report**A Caspase-activated Factor (CAF) Induces Mitochondrial Membrane Depolarization and Cytochrome c Release by a Nonproteolytic Mechanism**

By Margino Steemans, Vera Goossens, Marc Van de Craen, Franky Van Herreweghe, Katia Vancompernelle, Kurt De Vos, Peter Vandenabeele, and Johan Grooten

From the Department of Molecular Biology, Flanders Interuniversity Institute for Biotechnology and University of Gent, B-9000 Gent, Belgium

Summary

It is well established that apoptosis is accompanied by activation of procaspases and by mitochondrial changes, such as decrease in mitochondrial transmembrane potential ($\Delta\Psi_m$) and release of cytochrome c. We analyzed the causal relationship between activated caspases and these mitochondrial phenomena. Purified recombinant caspase-1, -11, -3, -6, -7, and -8 were incubated with mitochondria in the presence or absence of additional cellular components, after which $\Delta\Psi_m$ was determined. At lower caspase concentrations, only caspase-8 was able to activate a cytosolic factor, termed caspase-activated factor (CAF), which resulted in decrease in $\Delta\Psi_m$ and release of cytochrome c. Both CAF-mediated activities could not be blocked by protease inhibitors, including oligopeptide caspase inhibitors. CAF-induced cytochrome c release, but not decrease of $\Delta\Psi_m$, was blocked in mitochondria from cells overexpressing Bcl-2. CAF is apparently involved in decrease of $\Delta\Psi_m$ and release of cytochrome c, whereas Bcl-2 only prevents the latter. Hence, CAF may form the link between death domain receptor-dependent activation of procaspase-8 and the mitochondrial events studied.

Key words: caspase • cytochrome c • mitochondria

A family of cysteine proteases, termed caspases, plays a central part in both induction and execution of apoptosis mediated by Fas and the p55 TNF receptor. Caspases are expressed as inactive zymogens and become activated by autoproteolytic processing (1) or cleavage by other caspases (2) at specific aspartic acid residues. After activation, caspases cleave their substrates, e.g., caspase zymogens, as well as cytosolic, nuclear, and cytoskeletal proteins (3). For several caspases, participation in cell death, especially in Fas-induced apoptosis, has been established (4). After aggregation of Fas and subsequent formation of the death-inducing signaling complex (DISC), recruitment of procaspase-8 to DISC is followed by autoproteolytic activation of this caspase (5, 6). Depending on the amount of active caspase-8 generated at the receptor complex, two types of Fas-induced apoptosis have been described (7). In type I cells, recruitment of high amounts of procaspase-8 results in direct activation of executioner caspases accompanied by loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) and release of cytochrome c from the mitochondria. In type II cells, where low amounts of procaspase-8 are re-

cruited to DISC, mitochondrial membrane depolarization (MMD) and release of cytochrome c precede activation of caspase-3. This release of cytochrome c may initiate the execution phase (8) by Apaf-1-mediated processing of procaspase-9 to active caspase-9, resulting in activation of caspase-3 (9, 10). Accordingly, inhibition of MMD and cytochrome c release by overexpression of the antiapoptotic protein Bcl-2 blocks apoptosis in type II but not in type I cells (7).

In addition, other triggers of apoptosis, such as overexpression of the proapoptotic protein Bax (11), accumulation of the secondary messenger ceramide (12), treatment with etoposide or staurosporine (13, 14), and exposure to ionizing radiation (14, 15), are dependent on or accompanied by MMD and/or release of cytochrome c from the mitochondria. Recently, activation of a cytochrome c efflux-inducing factor (CIF) during staurosporine-induced apoptosis has been demonstrated (16). However, the mechanism by which death receptors induce MMD and the release of cytochrome c remains elusive. In particular, the relative contribution, if any, of the executioner caspases-3, -6, and -7,

but also of the initiation caspases-1, -11, and -8, to the generation of these mitochondrial phenomena remains an intriguing question.

In this paper, we analyzed the efficacy of purified recombinant caspase-1, -3, -6, -7, -8, and -11 to induce MMD in isolated mitochondria in the presence or absence of additional cellular components. We identified an intermediary protein, termed caspase-activated factor (CAF), which generates MMD and cytochrome c release by a nonproteolytic mechanism. Accordingly, CAF may constitute a crucial link between the caspase cascade and these mitochondrial events.

Materials and Methods

Cells. Murine T cell hybridomas PC60R55R75, PC60R55P75-Neo^r, and PC60R55P75-Bcl-2 (17) were cultured in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM l-glutamine, 5×10^{-5} M 2-ME, and 1 mM sodium pyruvate.

Reagents and Caspases. Carbonyl cyanide *m*-chlorophenylhydrazone (mCICCP) and atractyloside were purchased from Sigma Chemical Co. (St. Louis, MO). Rhodamine 123 (R123; Molecular Probes, Inc., Eugene, OR) was prepared as a 10 mM stock solution in DMSO and was used at 0.1 μ M. The caspase peptide inhibitors benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone (zDEVD-fmk) and benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone (zVAD-fmk; both from Enzyme Systems Products, Dublin, CA) as well as acetyl-Tyr-Val-Ala-Asp-chloromethylketone (Ac-YVAD-cmk; Calbiochem-Novabiochem International, San Diego, CA) were prepared as 100 mM stock solutions in DMSO and were used at 10 μ M. Purified recombinant murine caspases were prepared as described (18).

Preparation of Cytosol Extract. Cytosol was prepared by centrifugation (100,000 *g*; 30 min) of PC60 cells (0.5×10^9 cells/ml in cell-free system buffer) permeabilized with 0.07% digitonin. Cell-free system buffer comprised 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2 mM MgCl₂, 2.5 mM PO₄H₂K, 0.5 mM EGTA, 0.5 mM sodium pyruvate, 0.5 mM l-glutamine, and 10 mM Hepes-NaOH (pH 7.4), supplemented with 100 μ M chymostatin, 28 μ M E-64, 1 μ M leupeptin, 1 μ M pepstatin, and 0.3 μ M aprotinin.

Preparation of Organelles. 10×10^6 PC60 cells were permeabilized with 0.01% digitonin in 2 ml of isolation buffer, pH 7.4, comprising 220 mM mannitol, 68 mM sucrose, 2.5 mM PO₄H₂K, 1 mM EGTA, 10 mM Hepes-NaOH, and 0.5% BSA. Mitochondria were isolated by differential centrifugation. After homogenization, cell debris and supernatant were removed by centrifugation (20 s at 10,000 *g* and 10 min at 15,000 *g*, respectively) to pellet down the mitochondria. The latter were resuspended in isolation buffer and stored on ice for up to 1 h. All steps were carried out at 4°C.

Determination of MMD. Serial caspase dilutions were obtained in cell-free system buffer, supplemented with 5 mM dithiothreitol and 2 mM ADP. 10^5 permeabilized PC60 cells or isolated mitochondria from 10^5 PC60 cells were incubated with caspases for 30 min at 37°C. MMD was detected by flow-cytometric analysis of R123 accumulation (FACScalibur[®]; Becton Dickinson, Mountain View, CA [19]). R123 was excited with a 488-nm argon ion laser; fluorescence emission was measured at 525 nm.

Determination of Cytochrome c Release. 10^6 digitonin-permeabilized PC60 cells or isolated mitochondria from 10^6 PC60 cells were incubated in cell-free system buffer, with or without caspase-8 or in caspase-8-activated or untreated cytosol extract, both supplemented with 5 mM dithiothreitol and 2 mM ADP at 37°C for 30 min. Opened cells or mitochondria were pelleted by centrifugation (15,000 *g* for 5 min). Supernatant was analyzed by 15% SDS-PAGE, using mAb to cytochrome c (PharMingen, San Diego, CA) and ECL-based detection (Nycomed Amersham plc, Little Chalfont, Bucks, UK).

Fluorogenic Substrate Assay for Caspase Activity. Caspase-3-like activity was determined by incubation of cytosol extracts (containing 25 μ g total protein) with 50 μ M of the fluorogenic substrate acetyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-aminomethylcoumarin (Ac-DEVD-amc; Peptide Institute, Osaka, Japan) in 200 μ l cell-free system buffer. The release of fluorescent 7-amino-4-methylcoumarin was measured for 20 min at 2-min intervals by spectrofluorometry (CytoFluor 2300; PerSeptive Biosystems, Cambridge, MA) at 480 nm, using an excitation wavelength of 360 nm.

Results and Discussion

Caspase-8 Induces MMD in Cooperation with an Additional Cellular Factor. It was recently reported that caspase-1, -2, -3, -4, and -6 induce MMD in intact mitochondria (20). However, to obtain the MMD-inducing effect, high amounts of caspase activity were used. To verify whether additional cellular components promote the MMD-inducing activity at low caspase concentrations, we quantified the MMD-inducing capacity of serial dilutions from murine caspase-1, -11, -3, -6, -7, and -8 on isolated PC60 mitochondria and on mitochondria in permeabilized PC60 cells. Cells had been permeabilized with digitonin, a mild detergent which, at low concentration, dissolves plasma membranes without harming mitochondrial membranes (21). This open cell system allows the introduction of membrane-impermeable proteins while preserving the integrity of mitochondria and maintaining the presence of cytosolic components. MMD was determined by flow-cytometric analysis of $\Delta\Psi_m$ using the mitochondria-specific and potential-sensitive dye, R123 (19). Induction of MMD by uncoupling of the respiratory chain with the protonophore mCICCP or by atractyloside, an inhibitory ligand of the adenine nucleotide translocator which induces MMD by opening the permeability transition pores (22), resulted in a 10-fold drop in R123 fluorescence intensity both in isolated mitochondria and opened cell mitochondria (Fig. 1, *inset*). This shows that the two types of mitochondria display similar functionality and that both in vitro systems are suited for MMD analysis.

The critical concentration needed to induce MMD was determined for each caspase. In isolated PC60 mitochondria (Fig. 1), caspase-6 and -8 were highly inefficient and generated MMD at a concentration of 60 μ g/ml only. Caspase-1 and -3 were slightly more efficient, exhibiting a critical concentration of 30 μ g/ml. Caspase-11 and -7 scored best, with critical concentrations of 15 and 8 μ g/ml, respectively. These results are in agreement with previous reports demonstrating that caspases induce MMD in intact

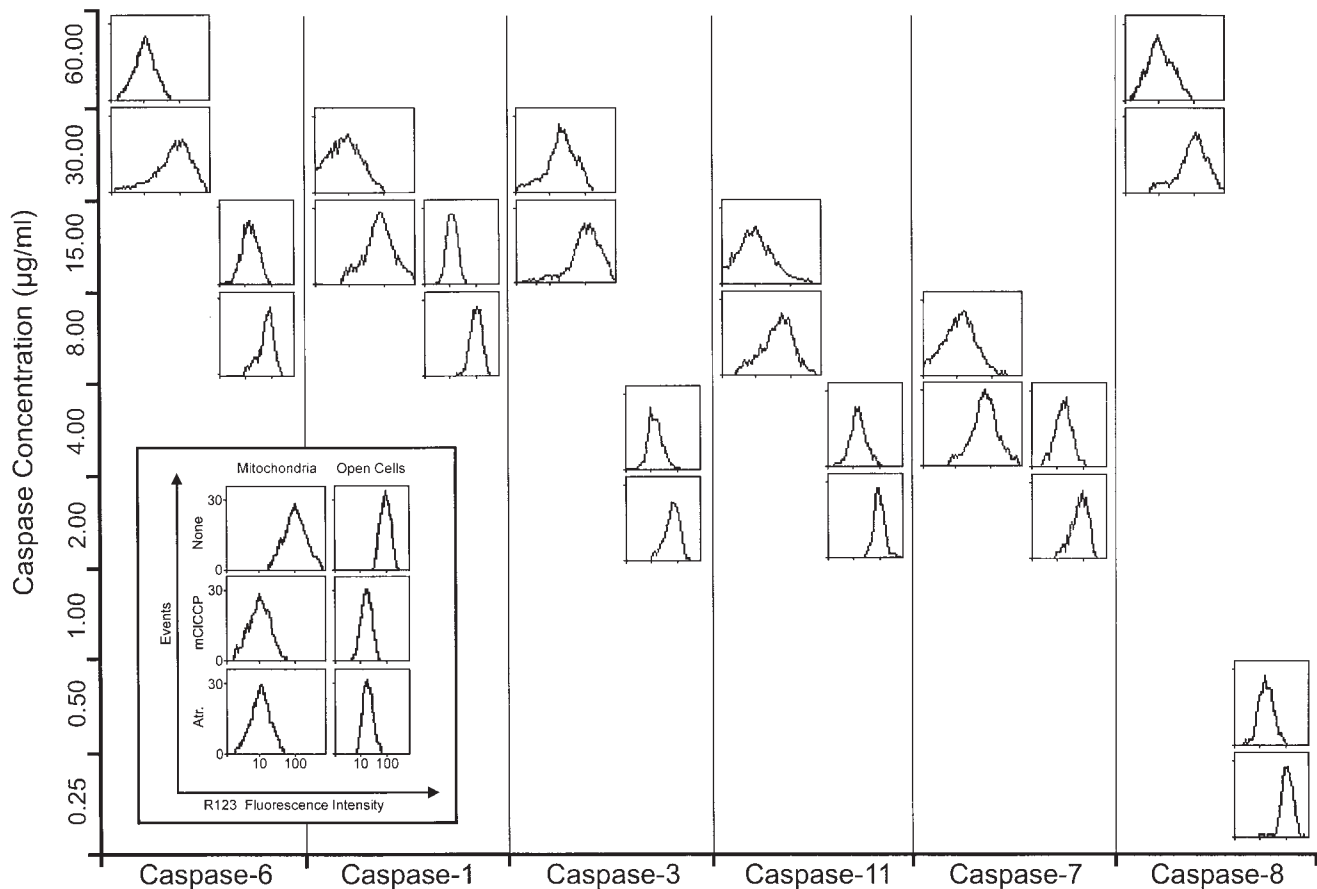


Figure 1. Critical caspase concentration for induction of MMD in isolated mitochondria and opened cell mitochondria. Serial dilutions of six purified recombinant caspases were analyzed for their ability to induce MMD in isolated PC60 mitochondria (*left panels of each column*) and digitonin-permeabilized PC60 cells (*right panels of each column*) after 30-min incubation at 37°C. $\Delta\Psi_m$ was measured using 0.1 μM R123 and flow cytometry. For each caspase, only the R123 fluorescence histograms from mitochondria treated with a caspase concentration just above and below the critical concentration to induce MMD are shown. Caspases are arranged according to their critical concentration for MMD induction in opened cell mitochondria and isolated mitochondria. *Inset*, analysis of MMD due to uncoupling of the respiratory chain by 50 μM mCICCP and to induction of permeability transition by 5 mM atractyloside after 30-min incubation at 37°C.

mitochondria (20), and further substantiate the low efficacy at which caspases, especially caspase-6 and -8, generate this response.

In contrast to the low efficacy in isolated mitochondria, all caspases showed a higher MMD-inducing activity in opened cell mitochondria (Fig. 1), the critical concentration decreasing twofold for caspase-1 and -7, fourfold for caspase-11 and -6, or eightfold for caspase-3. However, caspase-8 exhibited the most drastic shift, evolving from nearly no activity on isolated mitochondria to the most efficient activity in opened cells, with a critical concentration of 0.5 $\mu\text{g}/\text{ml}$. These results indicate that the caspases assayed induce, in intact cells, MMD mainly or exclusively (caspase-8) by cooperation with an intermediate cellular component(s), rather than by a direct mechanism. Since caspase-8 is the most effective caspase for inducing MMD in permeabilized cells and also the most apical caspase activated in the Fas-induced signal transduction pathway to cell death (5, 6), such cooperative activity may combine recruitment to DISC of procaspase-8 and occurrence of MMD.

Caspase-8 Activates a Cytosolic Protein that Induces MMD in Isolated Mitochondria by a Nonproteolytic Mechanism. To reconstitute the cytosolic signaling cascade to the mitochondria, cytosol extracts of digitonin-permeabilized PC60 cells were applied to isolated PC60 mitochondria. Without additional treatment, these cytosol extracts had no effect on $\Delta\Psi_m$ (Fig. 2). However, the addition of caspase-8 enabled the induction of MMD. The minimal caspase-8 concentration required to activate the cytosol extract was 0.5 $\mu\text{g}/\text{ml}$, at which concentration caspase-8 cannot induce MMD in isolated mitochondria. This substantiates the necessity of a cooperative cytosolic factor in order to obtain induction of MMD by caspase-8 in isolated mitochondria. The new factor is a protein, since the caspase-8-activated extract retained its MMD-inducing activity after dialysis, but became inactive after heat treatment (data not shown).

We also verified whether caspase-8 or secondary activated caspases in the cytosol extract are responsible for inducing MMD or, alternatively, whether MMD is induced by a secondary noncaspase effector molecule. To that end,

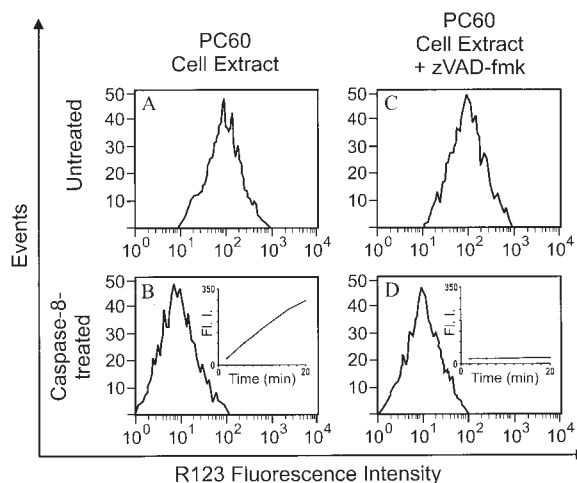


Figure 2. Caspase-8 induces MMD in isolated mitochondria by a cytosolic noncaspase intermediate. R123 fluorescence profiles of isolated PC60 mitochondria after 30-min incubation at 37°C with cell extract prepared from digitonin-permeabilized PC60 cells pretreated for 20 min with caspase-8 control buffer (A and E) and 1 μ g/ml caspase-8 (B and D). Respective cell extracts were postincubated with 10 μ M zVAD-fmk for 15 min (C and D). Caspase activity in the corresponding cytosol extracts was determined by accumulation of 7-amino-4-methylcoumarin fluorescence (F.I.), generated as a result of proteolytic modification of the fluorogenic caspase-3 substrate Ac-DEVD-amc (inset).

all caspase activity in the caspase-8-treated cytosol extract was blocked with 10 μ M of the broad-spectrum caspase inhibitor zVAD-fmk. Although this resulted in complete loss of caspase activity (Fig. 2, inset), the MMD-inducing activity of the treated extract remained unaffected (Fig. 2). In addition, Ac-YVAD-cmk and zDEVD-fmk, which at 10 μ M are specific inhibitors of the caspase-1 and -3 subfamilies, respectively, did not block the MMD-inducing activity in the caspase-8-activated extract (data not shown). In the cytosol extract of permeabilized PC60 cells, caspase-8 apparently activates an MMD-inducing activity that does not depend on caspase activity. This suggests the existence of an

additional effector protein, which induces MMD in isolated mitochondria. Because the protein is activated by caspase treatment, we termed it CAF.

Treatment of caspase-8 with zVAD-fmk rendered caspase-8 unable to generate CAF (data not shown). Thus, generation of CAF requires caspase-8 proteolytic activity, so CAF may be part of a proteolytic cascade. Accordingly, we verified whether the MMD-inducing activity of CAF itself is exerted by a proteolytic or nonproteolytic mechanism. However, protease inhibitors specific for different protease families did not affect the ability of caspase-8-treated cytosol extracts to induce MMD in isolated mitochondria (Table 1). These results show the existence of a caspase-activated protein, CAF, which possibly connects procaspase-8 recruitment and activation by death receptor aggregation with the occurrence of MMD.

CAF Induces Cytochrome c Release from Isolated Mitochondria. The major proapoptotic activity of mitochondria is the release of cytochrome c from the mitochondrial intermembrane space (11–15), resulting in activation of downstream caspase-3 (9, 10). We show that caspase-8-activated and zVAD-fmk-treated cytosol extract (CAF) induces cytochrome c release from isolated PC60 mitochondria (Fig. 3). Untreated cytosol extracts or caspase-8 by itself were unable to induce cytochrome c release. This indicates that CAF, by releasing cytochrome c from the mitochondria, may contribute to activation of caspase-3 and to execution of the apoptotic program.

Bcl-2 Prevents CAF-induced Cytochrome c Release, But Not MMD in Vitro. It was shown in various models of apoptosis that overexpression of Bcl-2 prevents MMD and cytochrome c release (7, 13, 14). Using permeabilized PC60 cells overexpressing Bcl-2 as a result of gene transfection (17), we analyzed the effect of Bcl-2 on CAF-induced MMD and cytochrome c release. Mitochondria in opened neomycin-resistant control cells showed cytochrome c release after treatment with caspase-8-activated and zVAD-fmk-treated cytosol extract as well as MMD, confirming that CAF entered the permeabilized cells. Addition of CAF

Table 1. Protease Inhibitors Do Not Block CAF-induced MMD

Inhibitor	Specificity	Inhibition of CAF
Antipain (74 μ M)	Papain, trypsin, cathepsin B	None
Aprotinin (0.3 μ M)	Serine proteases	None
Bestatin (130 μ M)	Metalloamino peptidases	None
Chymostatin (100 μ M)	α -, β -, γ -, δ -Chymotrypsin	None
E-64 (28 μ M)	Cysteine proteases	None
Leupeptin (1 μ M)	Serine and cysteine proteases	None
Pepstatin (1 μ M)	Aspartate proteases	None
Phosphoramidon (0.6 mM)	Metalloproteases	None

Caspase-8-activated PC60 cell extracts were treated with different protease inhibitors for 30 min at 20°C. CAF activity was determined on the basis of CAF-induced MMD in isolated PC60 mitochondria. MMD was measured by flow-cytometric $\Delta\Psi_m$ analysis.

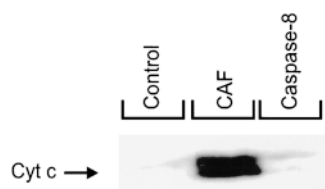


Figure 3. CAF induces release of cytochrome c from isolated mitochondria. Isolated PC60 mitochondria were incubated for 30 min at 37°C in the presence of control cell extract, caspase-8-activated zVAD-fmk-treated cell extract (CAF), or caspase-8 (1 µg/ml) alone. The presence of cytochrome c in the supernatant was determined by Western blot analysis.

to opened Bcl-2-overexpressing PC60 cells resulted in release of only marginal amounts of cytochrome c, whereas MMD was induced to the same extent as in neomycin-resistant control cells (Fig. 4). This shows that Bcl-2 primarily blocks the CAF-induced release of cytochrome c, which is the mitochondrial event crucial for execution of apoptosis, while CAF-induced MMD remains unaffected.

Conclusion. We provide compelling evidence for the existence of a cytosolic protein, CAF, which is activated by caspase-8 after proteolytic processing and which induces MMD as well as proapoptotic mitochondrial function of cytochrome c release by a nonproteolytic mechanism. Since caspase-8 by itself only induces MMD at extremely high (>30 µg/ml), hence nonphysiological concentrations, CAF is a possible component of the death receptor-induced pathway to the mitochondria. Furthermore, CAF-induced cytochrome c release was blocked by overexpression of Bcl-2, which meets the inhibitory activity of Bcl-2 on cytochrome c release and on MMD observed in several models of apoptosis (7, 12–14). However, in contrast to previous observations, overexpressed Bcl-2 did not prevent CAF-induced MMD in opened cell mitochondria. This might indicate that Bcl-2 prevents, by its effect on the mitochondrial volume homeostasis (23), the rupture of the

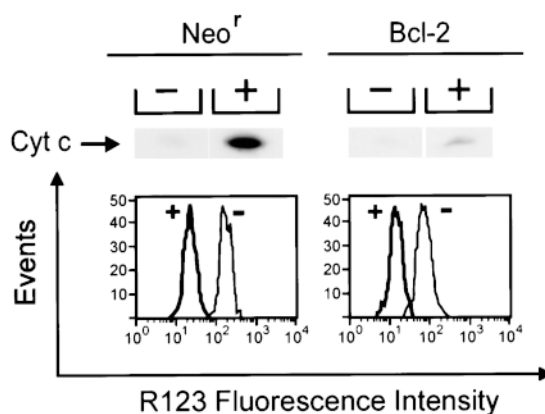


Figure 4. Overexpression of Bcl-2 prevents CAF-induced release of cytochrome c, but not induction of MMD. Digitonin-permeabilized Bcl-2 cells and neomycin-resistant PC60 cells were incubated for 1 h at 37°C in the presence of control cell extract (-) or caspase-8-activated zVAD-fmk-treated cell extract (+). Cells were analyzed for release of cytochrome c by Western blotting and induction of MMD by flow-cytometric determination of $\Delta\Psi_m$.

mitochondrial outer membrane and thus the release of cytochrome c, while its effect on the presumed opening of the permeability transition pore and the resulting MMD (22) remains limited. An alternative mechanism derives from the observation that cytochrome c release precedes MMD (13–15) or occurs in the absence of MMD (24). As a consequence and despite the fact that both CAF-induced mitochondrial events occurred almost simultaneously (data not shown), cytochrome c release and MMD may represent two independent outcomes of the same CAF-induced event differentially regulated by Bcl-2.

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Address correspondence to Johan Grooten, Department of Molecular Biology, Flanders Interuniversity Institute for Biotechnology and University of Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium. Phone: 32-9-264-53-10; Fax: 32-9-264-53-48; E-mail: johan.grooten@dmb.rug.ac.be

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