Mitochondrial control of cell death induced by hyperosmotic stress

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Abstract HeLa and HCT116 cells respond differentially to sorbitol, an osmolyte able to induce hypertonic stress. In these models, sorbitol promoted the phenotypic manifestations of early apoptosis followed by complete loss of viability in a time-, dose-, and cell type-specific fashion, by eliciting distinct yet partially overlapping molecular pathways. In HCT116 but not in HeLa cells, sorbitol caused the mitochondrial release of the caspase-independent death effector AIF, whereas in both cell lines cytochrome c was retained in mitochondria. Despite cytochrome c retention, HeLa cells exhibited the progressive activation of caspase-3, presumably due to the prior activation of caspase-8. Accordingly, caspase inhibition prevented sorbitol-induced killing in HeLa, but only partially in HCT116 cells. Both the knock-out of Bax in HCT116 cells and the knock-down of Bax in A549 cells by RNA interference reduced the AIF release and/or the mitochondrial alterations. While the knock-down of Bcl-2/Bcl-X_L sensitized to sorbitol-induced killing, over-expression of a Bcl-2 variant that specifically localizes to mitochondria (but not of the wild-type nor of a endoplasmic reticulum-targeted form) strongly inhibited sorbitol effects. Thus, hyperosmotic stress kills cells by triggering different molecular pathways, which converge at mitochondria where pro- and anti-apoptotic members of the Bcl-2 family exert their control.

Keywords Apoptosis · Bax · Bcl-2 · Hyperosmotic stress · Mitochondria · Sorbitol

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Abbreviations

$\Delta\Psi_m$	mitochondrial transmembrane potential
AIF	apoptosis-inducing factor
Cyt c	cytochrome c
$DiOC_6(3)$	3,3′dihexiloxalocarbocyanine iodide
FACS	fluorescence-activated cell sorter
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde phosphate dehydrogenase
HE	hydroethidine
HSP-60	heat shock protein of 60 kDa
IMS	mitochondrial intermembrane space
PI	propidium iodide
PS	phosphatidylserine
ROS	reactive oxygen species
siRNA	small interfering RNA
VDAC1	voltage-dependent anion channel 1

WT   wild type
Z-VAD-fmk *N*-benzyloxycarbonyl-Val-Ala-
           Asp.fluoromethylketone

Introduction

The best characterized and the most prominent pathways leading to apoptosis are called the extrinsic and intrinsic pathways. In the extrinsic pathway (also known as "death receptor pathway"), apoptosis is triggered by the ligand-induced activation of death receptors at the cell surface. Such death receptors include the tumor necrosis factor (TNF) receptor-1, CD95/Fas (the receptor of CD95L/FasL), as well as the TNF-related apoptosis inducing ligand (TRAIL) receptors-1 and -2 [1]. Upon binding of their ligands, death receptors recruit the adapter molecule FADD (Fas-associating death domain-containing protein) within the death-inducing signaling complex (DISC). Oligomerized FADD binds initiator caspases-8 and -10, in turn causing their dimerization and activation [2]. In the intrinsic pathway (also called "mitochondrial pathway"), apoptosis results from an intracellular cascade of events in which mitochondrial permeabilization plays a crucial role [3, 4]. The two pathways are interconnected at multiple levels, but the best characterized link is represented by the BH3-only protein Bid, which can be proteolitically activated by caspase-8 [2]. In its truncated active form (tBid), Bid translocates at mitochondria where it promotes membrane permeabilization, for instance by activating other pro-apoptotic members of the Bcl-2 family (*e.g.* Bax, Bak) [5].

Both routes to apoptotic death can be divided at least in three distinct phases: initiation, integration/decision and execution/degradation [6]. The initiation phase is highly heterogeneous and depends on the nature of the death-inducing signal, be it an extrinsic one (the ligation of a death receptor) or an intrinsic one (which may affect any cellular organelle including the nucleus, the endoplasmic reticulum (ER), lysosomes or mitochondria). The integration/decision phase involves the near-to-simultaneous activation of caspases and mitochondrial death effectors in a complex molecular interplay. During this phase the "decision to die" is taken and the "point-of-no-return" is trespassed. The execution/degradation phase, which is essentially a post-mortem process, is common to distinct types of apoptosis, meaning that the morphological and biochemical alterations that accompany late-stage apoptosis are independent of the initiating stimulus. Both the extrinsic and the intrinsic routes to apoptosis ultimately lead to cell shrinkage, chromatin condensation, nuclear fragmentation (which is frequently accompanied by internucleosomal DNA fragmentation), blebbing and phosphatidylserine exposure on the surface of the plasma membrane [7].

Most cell death in vertebrates proceeds via the intrinsic or mitochondrial pathway of apoptosis [8]. Here, the executioner caspases (including caspase-3) are cleaved and activated by the initiator caspase-9, which is activated by multimerization on the adapter molecule apoptosis-protease activating factor 1 (APAF-1) within a multiprotein complex called "apoptosome". APAF-1 pre-exists in the cytosol as a monomer, and its activation depends on the presence of cytochrome *c* (Cyt *c*) and ATP/dATP [9]. The release of Cyt *c*, which normally resides only in the mitochondrial intermembrane space (IMS) where it functions as an electron shuttle in the respiratory chain, is rate-limiting for the generation of the apoptosome [10]. Hence, mitochondrial membrane permeabilization (MMP) is the critical event responsible for caspase activation in the intrinsic pathway. MMP can even commit a cell to die when caspases are not activated. This "caspase-independent death" [11, 12] can occur because of an irreversible loss of mitochondrial function (often accompanied by an intense production of reactive oxygen species, *i.e.* ROS) as well as because of the mitochondrial release of caspase-independent death effectors including apoptosis-inducing factor (AIF) [13], endonuclease G (EndoG), and others [11, 12].

Hyperosmotic stress is one particular condition that can lead to cell death. For instance, hyperosmotic stress play an important role in the pathology of the ischemic heart muscle, where the rapid mobilization of osmolytes occurring upon ischemia promotes a sudden increase in local osmolarity [14]. Interestingly, in cultured cardiomyocytes, the activation of the NF-*κ*B pathway has been reported to mediate caspase-9 activation and eventually cell death as a consequence of sorbitol-induced hypertonic stress [15]. Hyperosmotic stress reportedly elicit numerous signal transduction pathways, and several papers insist on the fact that different osmolytes share the ability to trigger the extrinsic pathway of apoptosis, by activation of the CD95/FAS death receptor system [16, 17], the TNF-*α* death receptor system [18] or perhaps by interrupting trophic signals delivered by growth factor receptors [19]. Other physiological responses that are modulated by osmolarity and that may induce apoptosis include the acidification of endosomal compartments [20] as well as the degradation of cyclin-dependent kinases [21]. However, the implication of mitochondria in osmolyte-induced apoptosis has not yet been addressed in detail, in the mammalian system.

Here, we addressed the question to what extent mitochondria might contribute to apoptosis induction by hyperosmotic stress, as mimicked by sorbitol administration to cultured human tumor cells. Our results point that hypertonic stress is able to induce characteristic mitochondrial alterations involved in caspase-dependent and caspase-independent cell death, including ROS generation, in a cell type-specific fashion. Moreover, we demonstrate that members of the Bcl-2

family control osmolyte-induced apoptosis at the mitochondrial level.

Materials and methods

Cell lines, culture and treatments

Derivatives of the HCT116 cell line (parental and Bax$^{-/-}$) were a generous gift of Dr. B. Vogelstein [22] and routinely maintained in McCoy's 5A medium supplemented with 10% heat-inactivated fetal calf serum (FCS). HeLa cells were grown in DMEM (glucose 4,5 g/L) containing L-glutamine and 110 mg/L sodium pyruvate, supplemented with 10% FCS and 10 mM HEPES buffer. A549 were grown in F12-K medium containing L-glutamine, supplemented with 10% FCS, 100 units/ml penicillin G sodium and 100 μg/ml streptomycin sulfate. Rat-1 fibroblasts stably transfected with an empty control vector (CMV) or with a plasmid encoding Bcl-2, either in wild-type configuration (WT) or fused to peptides which allow the targeting to mitochondria (Acta) or to endoplasmic reticulum (Cb5) [23], were kindly provided by Dr. D. Andrews, and cultured in DMEM (glucose 4,5 g/L) containing L-glutamine and 110 mg/L sodium pyruvate, supplemented with 10% FCS, 10 mM HEPES buffer, 100 units/ml penicillin G sodium and 100 μg/ml streptomycin sulfate. All media and supplements for cell culture were purchased from Gibco-Invitrogen (Carlsbad, USA).

30–100 $\times$ 10^3 cells were seeded in 12-well plates and grown for 24 h prior to treatment with D-sorbitol (Sigma-Aldrich, St. Louis, USA) at concentrations varying from 0 to 300 mM (HeLa and HCT116 cells) of from 0 to 1 M (A549 cells) in the presence or absence of the pan-caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp.fluoromethylketone (Z-VAD-fmk, purchased from Bachem, Bubendorf, Switzerland) at the concentration of 50 μM or of the p53 inhibitor cyclic pifithrin-α (Sigma-Aldrich) at the concentration of 30 μM. Sorbitol treatments lasted up to a maximum of 48 h. As positive control for caspase-3 activation, cells were treated with 2 μM staurosporine (Sigma-Aldrich) for 6 h. All experiments were repeated at least two times with similar results.

Transfections and siRNAs

HeLa and HCT116 cells were transiently transfected with an empty control vector (CMV) or with a plasmid encoding Bcl-2, either in wild-type configuration (WT) or fused to peptides which allow the targeting to mitochondria (Acta) or to endoplasmic reticulum (Cb5) [23]. Liposome-mediated transfection was performed in 24-well plates by using Lipofectamine™ 2000 Transfection Reagent (Invitrogen), according to the manufacturer instructions. After 24 h, cells were subjected to sorbitol treatment prior to cytofluorometric determination of apoptosis-associated alterations.

The down-regulation of Bak and Bax was performed with siRNAs (Hs_BAK1_5 and Hs_BAX_10 HP Validated siRNAs, respectively) purchased from Qiagen (Hilden, Germany). Knock-down of Bcl-2, Bcl-X$_L$, p53 and the voltage-dependent anion channel 1 (VDAC1) was performed with siRNAs purchased from Sigma-Proligo (The Woodlands, USA). Bcl-2 sense 5′-GCUGCACCUGACGCCCUUCTT-3′, antisense 5′-GAAGGGCGUCAGGUGCAGCTT-3′ [24]; Bcl-X$_L$ sense 5′-CAGGGACAGCAUAUCAGAGTT-3′, antisense 5′-CUCUGAUAUGCUGUCCCUGTT-3′ [24]; p53 sense 5′-GACUCCAGUGGUAAUCUACTT-3′, antisense 5′-GUAGAUUACCACUGGAGUCTT-3′ [25]; VDAC1 sense 5′-GUACGGCCUGACGUUUACATT-3′, antisense 5′-UGUAAACGUCAGGCCGUACTT-3′. As a control, an siRNA with an "unrelated", irrelevant sequence was used ("UNR" sense 5′-GCCGGUAUGCCGGUUAAGUTT-3′, antisense 5′-ACUUAACCGGCAUACCGGCTT-3′). A549 cells were reverse-transfected at the time of plating by means of the HiPerFect transfection reagent (Qiagen), as follows. Transfection complexes were prepared in 100 μl serum-free, antibiotic-free F12-K by mixing 8 μl of HiPerFect transfection reagent with 40 pmol of siRNA and let stand at room temperature for 20–30 min, to allow for efficient complex formation. Transfection complexes were then mixed with 700 μl of complete medium (as detailed above) in which 30–40 $\times$ 10^3 cells had been previously resuspended. Finally, the resulting suspension was seeded as a whole in a single well of a 12-well plate. To allow for complete or near-to-complete protein down-regulation, transfected cells were cultured for 48 h prior to sorbitol treatment.
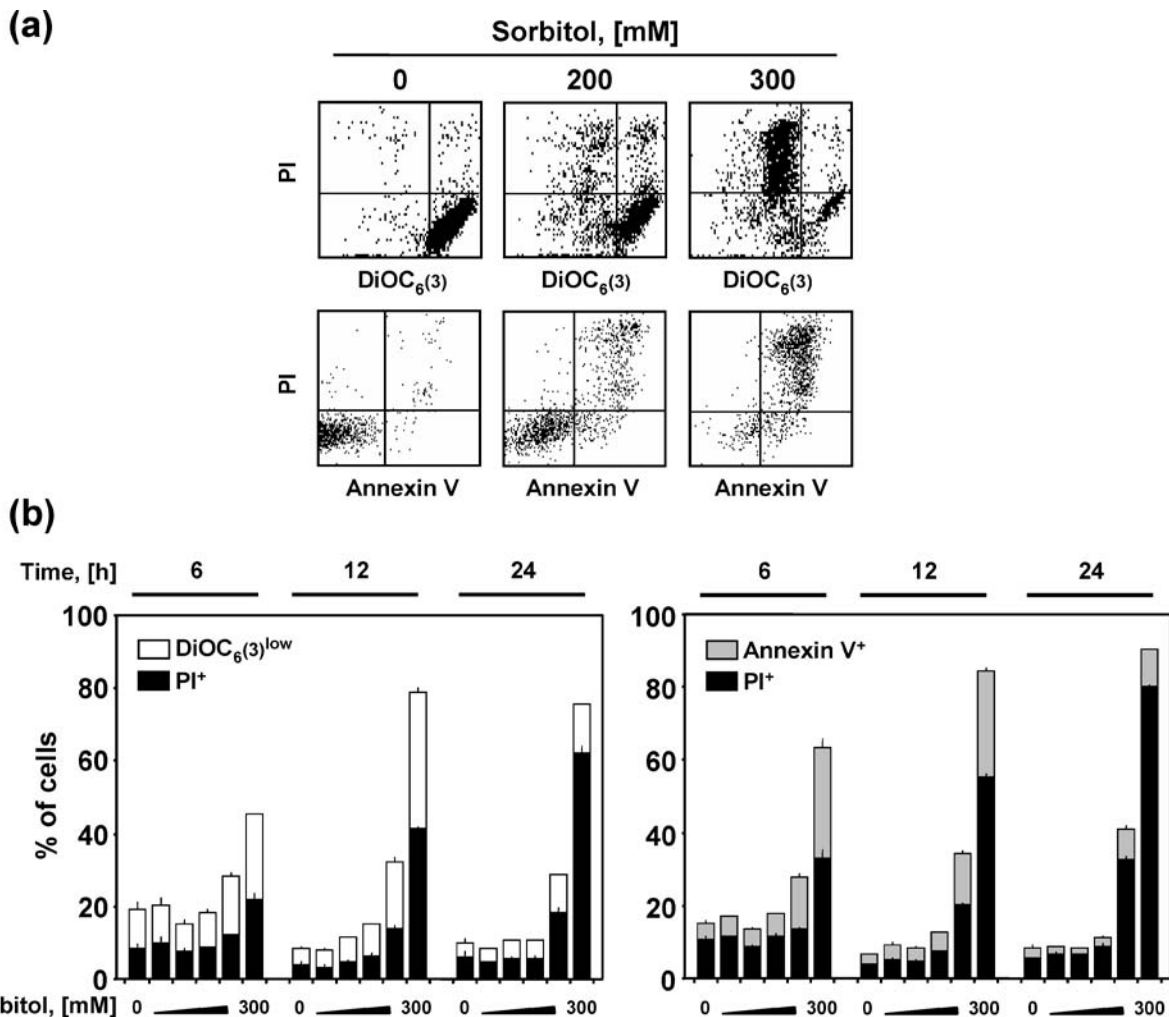
Cytofluorometric analysis and immunofluorescence

According to previously reported protocols [26], cells were labeled with the following fluorochromes to determine apoptosis-associated changes: propidium iodide (PI, 1 μg/ml, Sigma-Aldrich) for viability, 3,3′dihexiloxalocarbocyanine iodide (DiOC$_6$(3), 40 nM, Molecular Probes-Invitrogen) for $\Delta\Psi_m$ dissipation, annexin V-FITC (Molecular Probes-Invitrogen) for assessment of phosphatidylserine (PS) exposure. Hydroethidine (HE, 4 μM, Molecular Probes-Invitrogen) was employed to determine superoxide anion generation, as previously described [27]. In summary, HE is a reduced non-fluorescent precursor that, in the presence of ROS (and more precisely of superoxide anion species), is readily oxidized intracellularly into the fluorescent compound ethidium bromide, which binds to DNA and can be detected by virtue of its red fluorescence. Cytofluorometric analyses were performed by means of a FACS Scan (Becton Dickinson, San Jose, USA).

Data were statistically evaluated using Cell Quest software (Becton Dickinson).

Cells were fixed with paraformaldehyde (4% w/v) and subsequently stained with antibodies for the detection of AIF (for HCT116 cells: rabbit polyclonal IgG anti-AIF internal domain, cat. n° AB16501; Chemicon International, Temecula, USA; for HeLa cells: goat polyclonal IgG AIF (D-20), cat. n° SC-9416; Santa Cruz Biotechnology, Santa Cruz, USA), Bcl-2 (mouse monoclonal IgG Bcl-2 (100), cat.

n° SC-509; Santa Cruz Biotechnology), cytochrome c (rabbit polyclonal IgG cytochrome c (H-104), cat. n° SC-7179; Santa Cruz Biotechnology, Santa Cruz, USA) or HSP-60 (mouse monoclonal anti-heat shock protein 60 (clone LK1), cat n° H 4149, Sigma-Aldrich). Nuclei were labeled with 10 μg/ml Hoechst 33342 (Molecular Probes-Invitrogen). According to the specific double staining, primary antibodies were revealed either with anti-mouse, anti-rabbit or anti-goat IgG conjugated to either Alexa 488 (green) or

Fig. 1 *Hyperosmotic stress-induced apoptosis in HeLa cells.* HeLa cells were treated for up to 24 h with 0 (control), 25, 50, 100, 200 or 300 mM sorbitol, then submitted to cytofluorometric analysis of mitochondrial membrane potential (DiOC$_6$(3) staining), PS exposure (Annexin V staining) and viability (PI staining). (a) Representative dot plots of HeLa cells treated for 24 h with 0, 200 and 300 mM sorbitol. X-axis, DiOC$_6$(3) (upper panels) and Annexin V staining (lower panels); Y-axis, PI staining. (b) Time- and dose-dependent response of HeLa cells to sorbitol. White columns depict the percentage of cells that have a low mitochondrial membrane potential but are still viable (DiOC$_6$(3)^{low}). Black columns report the percentage of cells that have lost the intactness of plasma membrane (PI$^+$). Light grey columns indicate the percentage of cells that have exposed PS on the outer leaflet of the plasma membrane (Annexin V$^+$). Data are mean of triplicate experiments $\pm$ SEM. (c) ROS generation. HeLa cells were treated with 300 mM sorbitol

for the indicated time, then analyzed at FACS for the detection of ROS. Dark grey columns report the percentage of cells characterized by an intense production of superoxide anion (HE$^+$). Data are mean of duplicate experiments $\pm$ SEM. (d) Caspase activation upon sorbitol treatment. HeLa cells were treated with 300 mM sorbitol or with 2 μM staurosporine (STS, positive control for caspase-3 activation). After the indicated time, the cells lysates were run on a mono-dimensional SDS-PAGE, and analyzed by immunoblotting with antibodies specific for caspase-3, caspase-8 and GAPDH (loading control). Caspase-3 activation coincides with a reduction in the intensity of the pro-caspase band (35 kDa) paralleled by an increase of the bands corresponding to the active fragments (19 and 17 kDa). Caspase-8 activation results in the appearance of a band corresponding to the p10 active fragment. For additional details please refer to the section "Materials and methods"
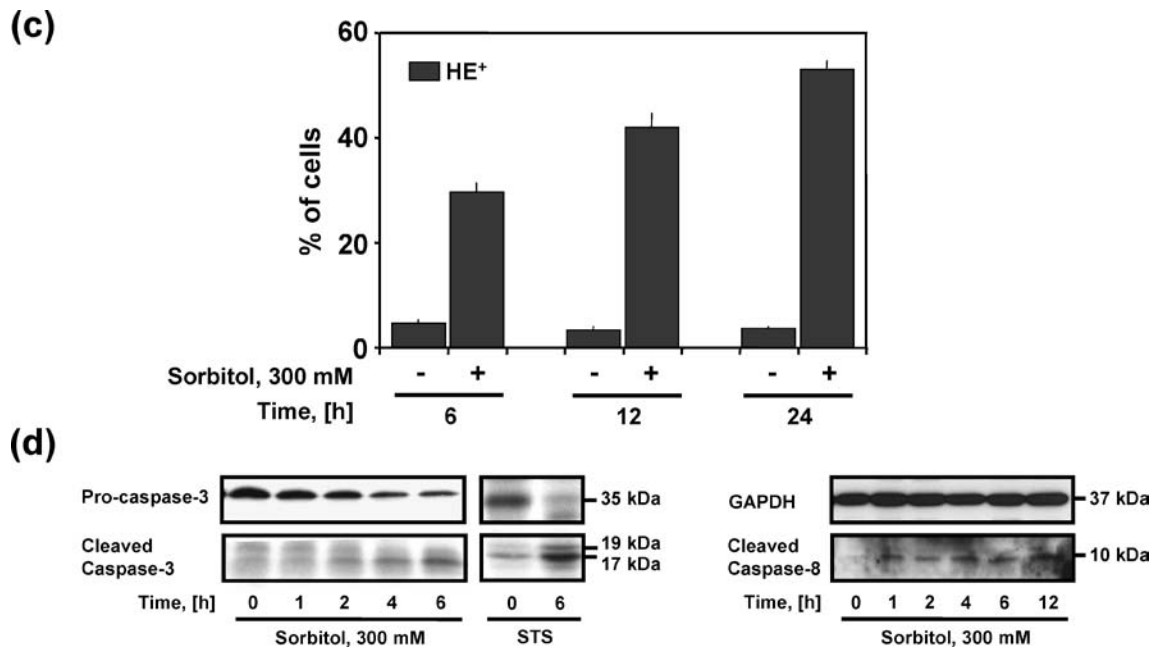
Fig. 1 (*Continued*).

Alexa 568 (red) from Molecular Probes-Invitrogen. Fluorescence microscopy assessments were performed with a Leica IRE2 microscope equipped with a Leica DC300F camera.

Analysis of protein expression

Protein samples of HeLa, HCT116 or A549 cells were prepared in lysis buffer, following standard established protocols. Aliquots of the extracted proteins (approx. 50 μg/lane) were separated according to molecular weight on a mono-dimensional 12% SDS-PAGE then subjected to immunoblots using antibodies specific for active caspase-8 (mouse monoclonal IgG anti cleaved caspase-8 (Asp384) (11G10), cat n° 9748; Cell Signaling Technology, Danvers, USA) Bak (rabbit polyclonal serum Bak, cat. n°, 556396; PharMingen, San Diego, USA), Bax (rabbit polyclonal IgG Bax (N-20), cat. n° SC-493; Santa Cruz Biotechnology), Bcl-2 (mouse monoclonal IgG Bcl-2 (100), cat. n° SC-509; Santa Cruz Biotechnology), Bcl-X (rabbit polyclonal IgG Bcl-X (Ab-1) (201-216), cat n° PC67; Calbiochem, San Diego, USA), GAPDH (mouse monoclonal IgG anti-glyceraldehyde-3-phosphate dehydrogenase (6C5), cat n° MAB274; Chemicon International, Temecula, USA), p53 (mouse monoclonal IgG p53 (DO-1), cat n° SC-126; Santa Cruz Biotechnology), total caspase-3 (rabbit polyclonal anti caspase-3 (Asp175), cat n° 9662; Cell Signaling Technology) or VDAC1 (rabbit polyclonal IgG anti-porin (Ab-5), cat n° PC548; Calbiochem). Membranes were then incubated with secondary goat anti-mouse (cat n° 1010-05) or anti-rabbit (cat. n° 4010-05) IgG conjugated to horseradish peroxidase (SouthernBiotech, Birmingham, USA) prior to revelation by means of ECL Detection Kit (Amersham Pharmacia, Pittsburgh, USA) and Hyperfilm x-ray films (Amersham Pharmacia).

Results and discussion

Cell-type specific response to hyperosmotic stress

Human tumor cells, including the HCT116 colon carcinoma and the HeLa cervical carcinoma cell lines, manifest the classical features of apoptosis with different sensitivity, when treated with high concentrations of the osmolyte D-sorbitol. Thus, HeLa cells exhibited rapid and massive apoptosis-associated alterations, including the dissipation of the mitochondrial transmembrane potential ($\Delta \Psi_m$) (Fig. 1(a, b)), the exposure of phosphatidylserine (PS) on the outer leaflet of the plasma membrane (Fig. 1(a, b)) and an increased generation of reactive-oxygen species (ROS) (Fig. 1(c)), before losing viability. In addition, HeLa cells manifested the proteolytic maturation of caspase-3, as indicated by the progressive degradation of the inactive pro-caspase-3 in parallel with the accumulation of a ~17 kDa fragment corresponding to the heavy chain of active caspase-3 (Fig. 1(d)). Caspase-3 activation was preceded by the activation of caspase-8, which reportedly occurs within the extrinsic pathway of apoptosis [2], witnessed by the early accumulation of the light chain (~10 kDa) of the active caspase-8.

As compared to HeLa cells, HCT116 cells were more resistant to sorbitol treatment, as indicated by two color staining with the $\Delta\Psi_m$-sensitive dye DiOC$_6$(3) and the vital dye propidium iodide (PI) as well as by reduced PS exposure (Fig. 2(a, b)). This correlated with a lower percentage of HCT116 cells, as compared to HeLa, that overproduced ROS after exposure to sorbitol (Fig. 2(c)). Moreover, HCT116 cells did not manifest any sign of caspase activation upon treatment with sorbitol (Fig. 2(d)), pointing to cell type-specific differences in the lethal process. Nonetheless, the mitochondrial changes induced by sorbitol occurred in a concentration and time-dependent fashion, in both HeLa and HCT116 cells, though to different extents (Figs. 1(b) and 2(b)).

To gain insights into the mechanism of $\Delta\Psi_m$ dissipation, cells were exposed to 300 mM sorbitol in the presence of the p53 inhibitor cyclic pifithrin-α or the pan-caspase inhibitor Z-VAD-fmk. As assessed by FACS analysis of $\Delta\Psi_m$, PS exposure and viability, pifithrin-α failed to protect HeLa (Fig. 3(a)) and HCT116 cells (Fig. 3(b)) from sorbitol-induced cell death, and rather sensitized HCT116 cells to the lethal effects of hypertonic stress (Fig. 3(b)). Z-VAD-fmk was able to prevent the $\Delta\Psi_m$ dissipation, PS exposure and loss of
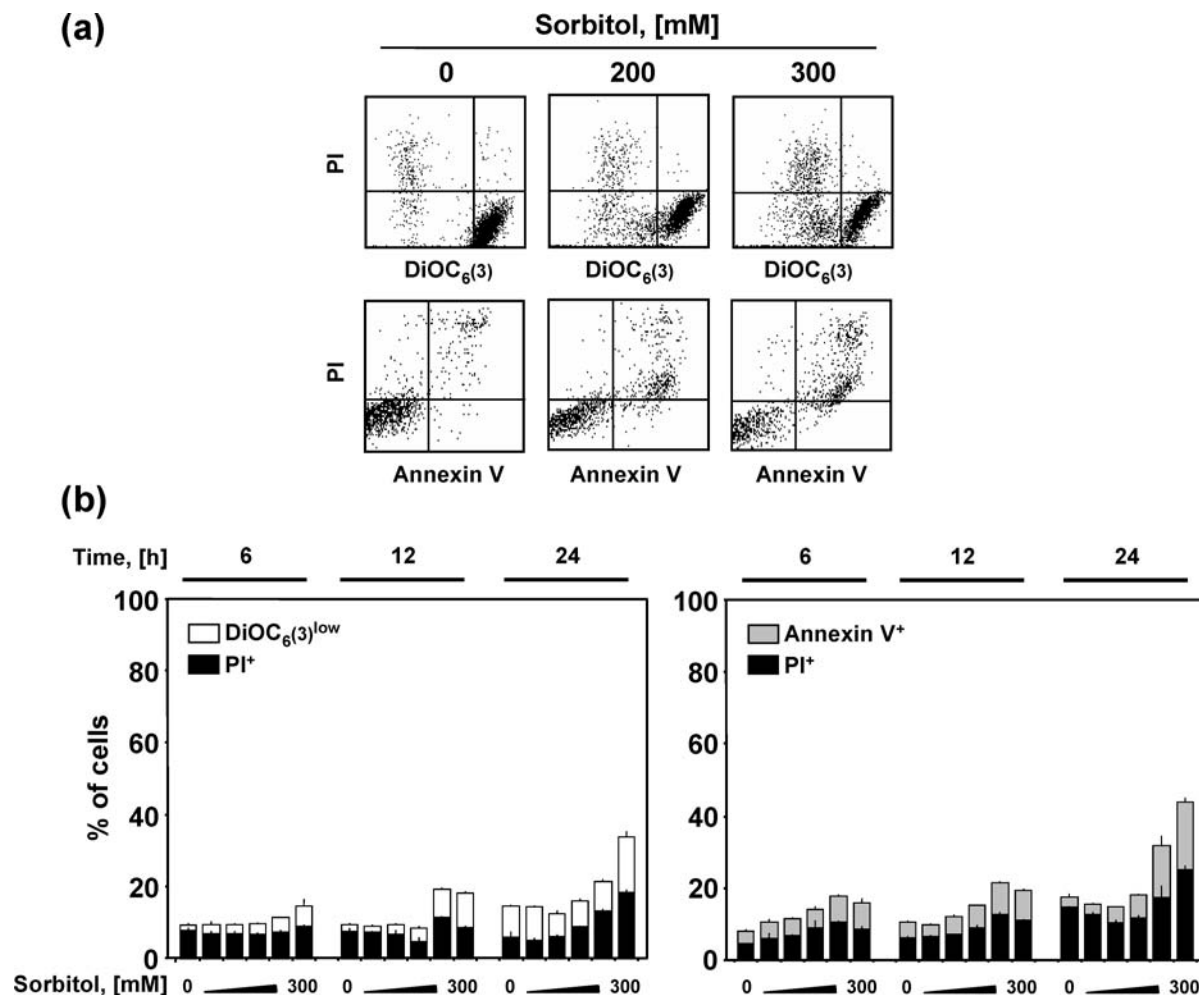
Fig. 2 *Hyperosmotic stress-induced apoptosis in HCT116 cells.* WT HCT116 cells were treated with 0 (control), 25, 50, 100, 200 or 300 mM sorbitol for up to 24 h, then subjected to cytofluorometric measurement of mitochondrial membrane potential (DiOC$_6$(3) staining), PS exposure (Annexin V staining) and viability (PI staining). (a) Typical dot plots of HCT116 cells treated with 0, 200 and 300 mM sorbitol for 24 h. X-axis, DiOC$_6$(3) (upper panels) and Annexin V staining (lower panels); Y-axis, PI staining. (b) Time- and dose-dependent response of WT HCT116 cells to sorbitol. White columns illustrate the percentage of cells which have dissipated the mitochondrial membrane potential but are still viable (DiOC$_6$(3)^{low}). The percentage of cells with disrupted plasma membrane (PI$^+$) is reported by black columns. Light grey columns represent the percentage of cells with PS exposed on the outer leaflet of the plasma membrane (Annexin V$^+$). Data are mean of triplicate experiments $\pm$ SEM. (c) ROS generation. WT HCT116 cells were treated for the indicated time with 300 mM sorbitol, stained with HE, and then subjected to cytofluorometric analysis for the detection of ROS. Dark grey columns illustrate the percentage of cells which overproduced superoxide anion (HE$^+$). Data are mean of duplicate experiments $\pm$ SEM. (d) Caspase activation upon sorbitol treatment. WT HCT116 cells were treated with 300 mM sorbitol or with 2 μM staurosporine (STS, positive control for caspase-3 activation) for the indicated time, followed by protein purification, SDS-PAGE and immunoblot detection of caspase-3, caspase-8 and GAPDH (loading control). For more detailed information please see also "Materials and methods" *(Continued on next page)*
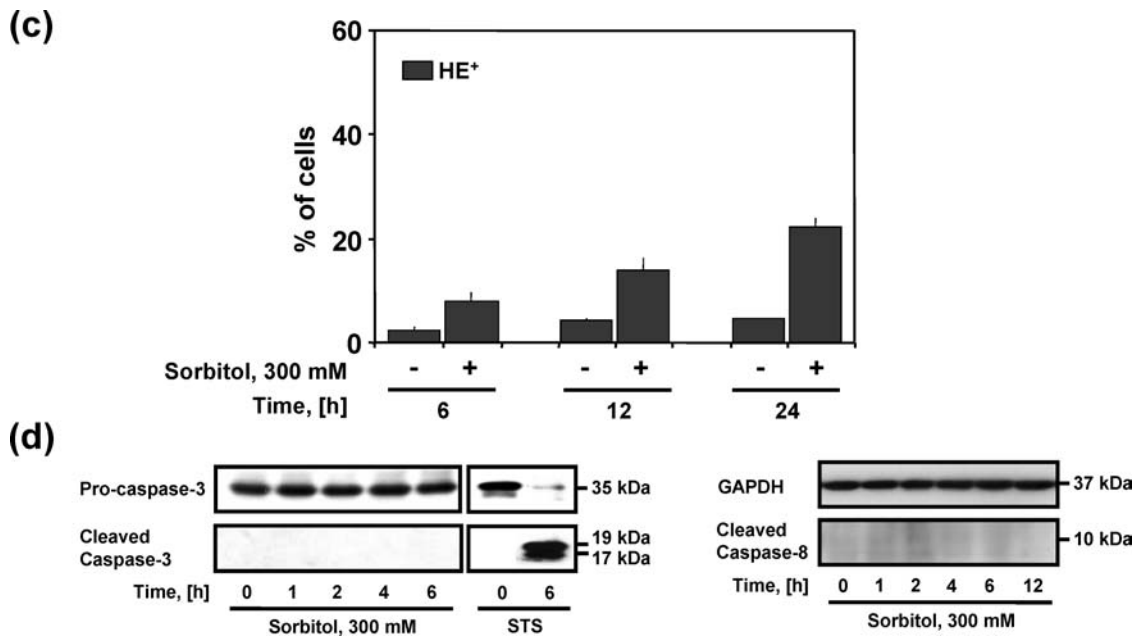
Fig. 2 (*Continued*).

viability in HeLa cells (Fig. 3(a)), but was only partially effective (especially concerning the loss of viability, as assessed by PI positivity) in HCT116 cells (Fig. 3(b)). This correlated with caspase-3 and caspase-8 activation in HeLa but not in HCT116 cells, and may be linked to the differential sensitivity demonstrated by these cell lines to hyperosmotic stress. Taken altogether, these results indicate that p53 is not required for the mitochondrial changes induced by hyperosmolarity and that the involvement of caspases is cell-type specific.

As an additional internal control, we took advantage of HCT116 cells that were either sufficient for Bax (wild type, WT) or lacked Bax expression as a result of homologous recombination (Bax$^{-/-}$). Of note, in the same conditions in which cyclic pifithrin-α and Z-VAD-fmk failed to exert cytoprotective effects, the removal of Bax did reduce (though partially) the sorbitol-triggered $\Delta\Psi_m$ dissipation (Fig. 3(c)), meaning that the $\Delta\Psi_m$ loss can be modulated by proteins such as Bax that control the mitochondrial phase of apoptosis. Altogether, these data indicate that caspases are activated by hyperosmotic stress in a cell type-specific fashion, thus supporting the notion that caspase activation is not an universal prerequisite for the $\Delta\Psi_m$ loss that defines the "point-of-no-return" of the lethal process [7].

AIF and Cyt *c* subcellular localization upon hyperosmotic stress

Since Bax was (at least partially) required for sorbitol-induced $\Delta\Psi_m$ loss, we suspected that specific mitochondrial alterations might accompany the cell death triggered by hy-

perosmotic stress. Thus, we performed immunofluorescence studies on cells that had been treated with sorbitol, to determine the subcellular localization of two proteins that under physiological conditions reside in the mitochondrial inter-membrane space (IMS), namely AIF and Cyt *c*. As shown in Fig. 4(a), WT HCT116 cells manifested a clear relocation of AIF from a cytoplasmic punctuate distribution (which closely resembles that of the mitochondrial marker protein HSP-60) in controls, to a more diffuse pattern all over the cell (including the nucleus) after sorbitol administration. In contrast, no similar mitochondrio-nuclear translocation of AIF was observed when Bax$^{-/-}$ HCT116 cells were treated with sorbitol (Fig. 4(a)). In sharp contrast with AIF, Cyt *c* remained in a clearly mitochondrial localization, co-staining with HSP-60, both in WT and Bax$^{-/-}$ HCT116 cells treated with sorbitol (Fig. 4(b)). These results pointed to a differential release mechanism for AIF and Cyt *c*. Indeed, it has been previously reported that AIF can exit mitochondria before Cyt *c*, for example when the latter is retained by electrostatic interactions with cardiolipin, a lipid of the inner mitochondrial membrane [28].

In an attempt to confirm these results in another cell line, we were surprised to find that in HeLa cells sorbitol failed to induce the release of both AIF (Fig. 5(a)) and Cyt *c* (Fig. 5(b)). Interestingly, the mitochondrial retention of Cyt *c* (Fig. 4(b)) correlated with the failure of HCT116 cells to activate caspase-3 (Fig. 2(d)), while the same did not hold true in HeLa cells, where the progressive activation of caspase-3 (Fig. 1(d)) occured independently from the liberation of Cyt *c* (Fig. 5(b)). In HeLa cells, the proteolytic maturation of caspase-3 may occur following the activation of caspase-8
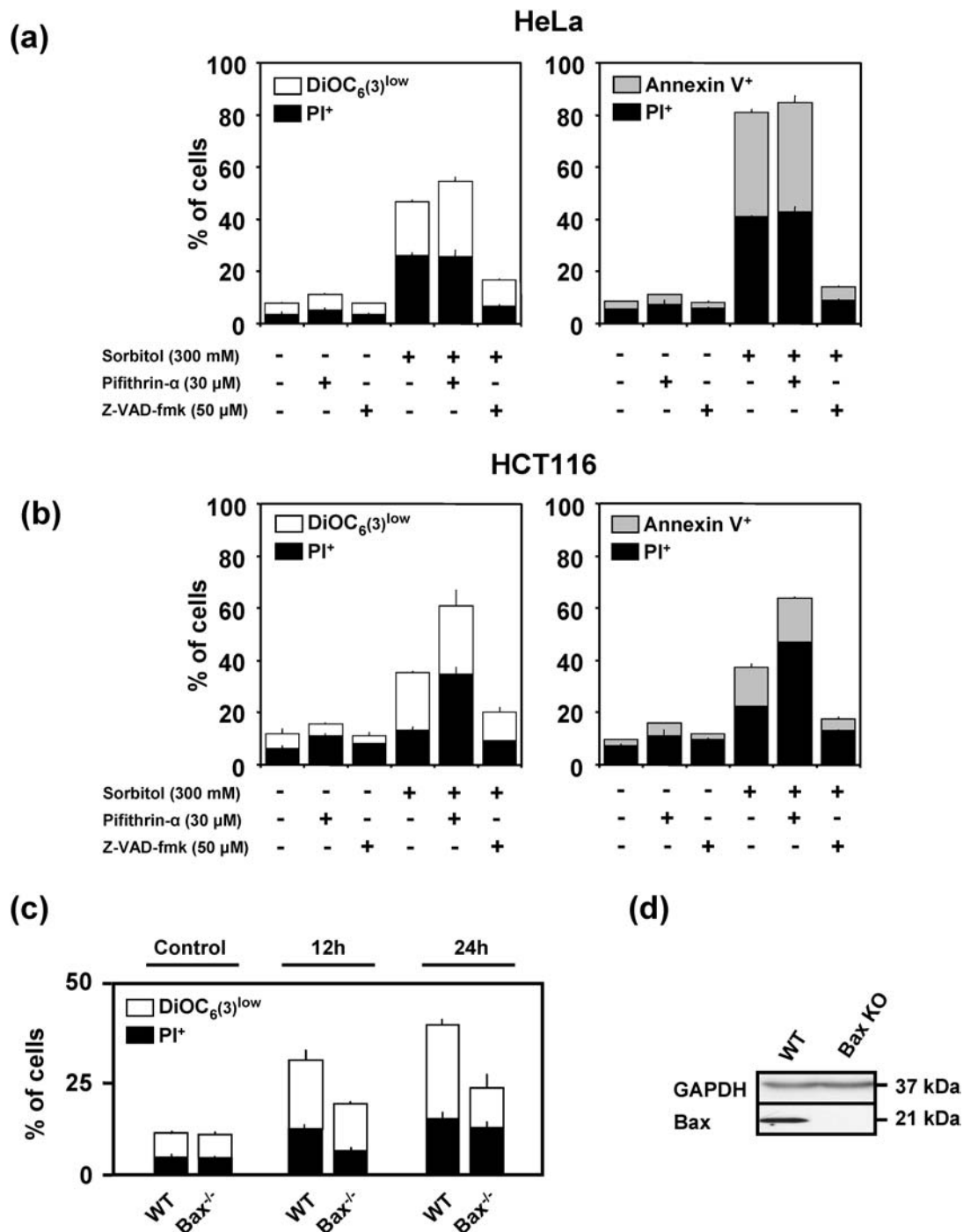
Fig. 3 *Roles of caspases, p53 and Bax in hyperosmotic stress-induced apoptosis*. HeLa (a) and WT HCT116 (b) cells were treated for 12 (a) or 24 (b) h with 0 (control) or 300 mM sorbitol, in the presence or not of the pan-caspase inhibitor Z-VAD-fmk or of the p53 inhibitor cyclic pifithrin-α, then analyzed at FACS for mitochondrial membrane potential (DiOC$_6$(3) staining), PS exposure (Annexin V staining) and viability (PI staining). (c) WT and Bax$^{-/-}$ HCT116 cells were treated for 12 or 24 h with 300 mM sorbitol then submitted to cytofluorometric analysis of mitochondrial membrane potential (DiOC$_6$(3) staining) and viability (PI staining). (a–c) White columns report the percentage of cells that have a low mitochondrial membrane potential but are still viable (DiOC$_6$(3)^{low}). Black columns specify the percentage of cells which have lost the integrity of plasma membrane (PI$^+$). (a, b) Light grey columns depict the percentage of cells which have exposed PS on the outer leaflet of the plasma membrane (Annexin V$^+$). Data are mean of triplicate experiments $\pm$ SEM. (d) To check for down-regulation, total proteins were purified from WT and Bax$^{-/-}$ HCT116 cells, run on mono-dimensional SDS-PAGE, then subjected to immunoblot analysis with antibodies against Bax and GAPDH (loading control). For further details please refer to the section "Materials and methods"
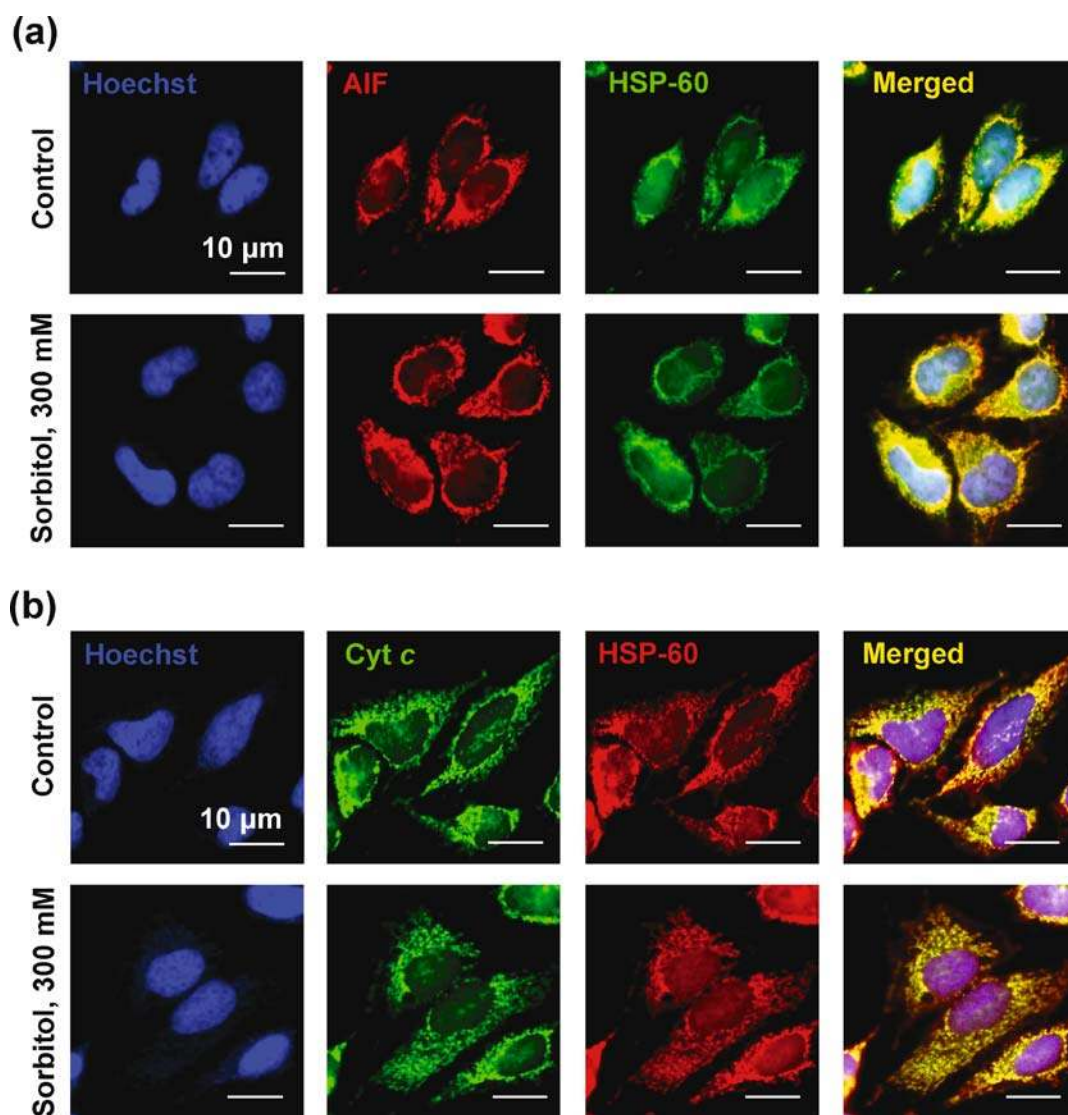
Fig. 4 *Subcellular localization of apoptosis-inducing factor (AIF) and cytochrome c (Cyt c) in HCT116 cells undergoing hyperosmotic stress-induced apoptosis*. (a, b) WT and Bax$^{-/-}$ HCT116 cells were treated or not with 300 mM sorbitol for 2 h, followed by immunofluorescence staining with antibodies specific for AIF (a) or Cyt c (b) and the 60 kDa heat-shock protein (HSP-60, which stains mitochondria). Diffuse AIF staining indicates that AIF is released from mitochondria upon sorbitol treatment in WT, but not in Bax$^{-/-}$, HCT116 cells (a). At the same time point, Cyt c is retained in mitochondria of sorbitol-treated HCT116 cells (WT and Bax$^{-/-}$) (b). White bars represent picture scale (10 μm)
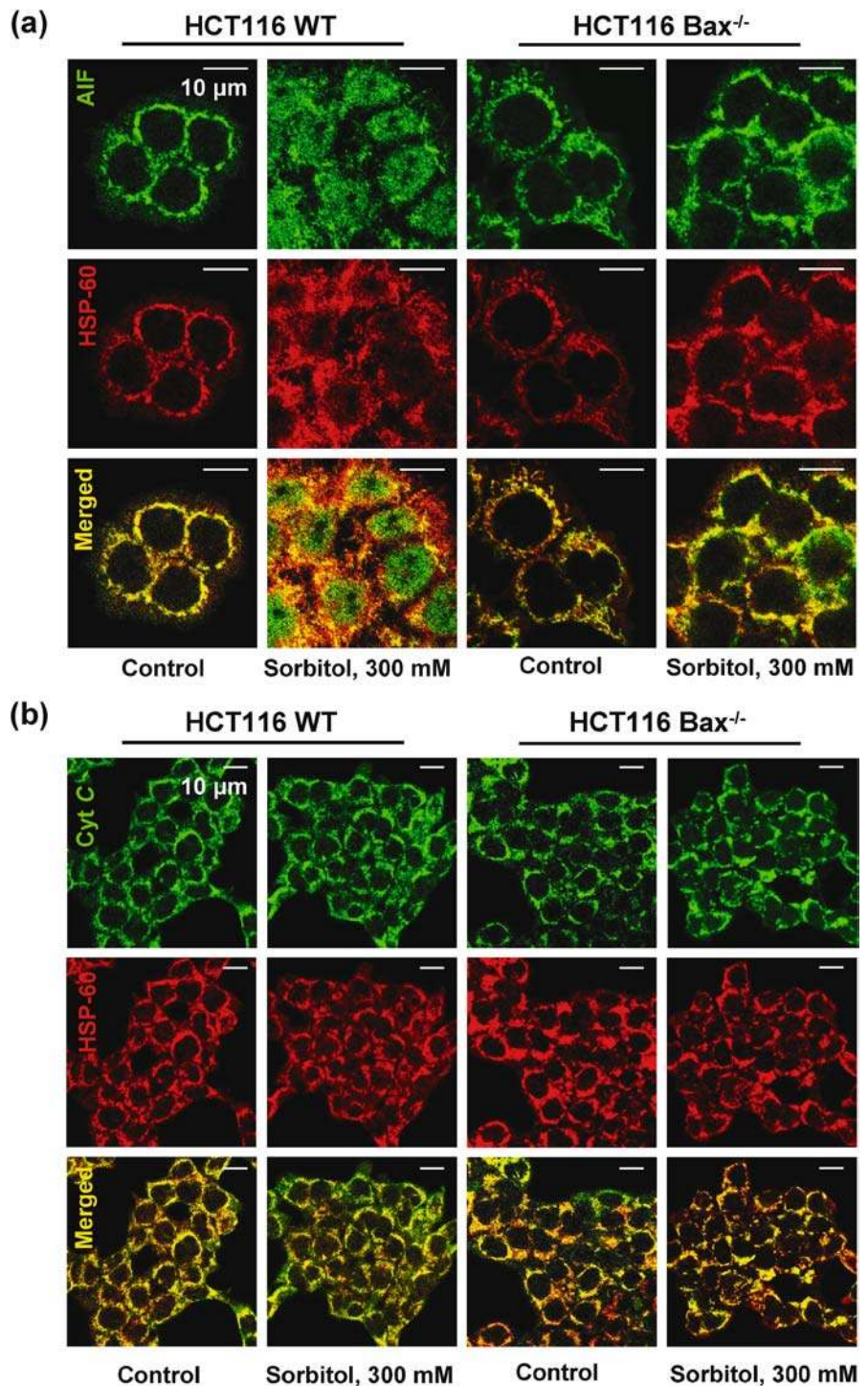
(Fig. 1(d)). In this context, it has previously been described that different osmolytes elicit the extrinsic pathway of apoptosis [16–18], thus leading to the activation of caspase-8 [2], which reportedly is able to cleave and activate pro-caspase-3 in a direct fashion [29]. In summary, hitherto unexplained cell type-specific differences must be taken into account when studying stress-elicited mitochondrial alterations. As

a possibility, hyperosmotic stress may trigger distinct proapoptotic mechanism in different cell types, either by acting on mitochondria to promote AIF release (as in HCT116 cells) or by activating the caspase cascade of the extrinsic pathway (as in HeLa cells). The involvement of mitochondria, be it direct or not, appears to be the unifying element of alterations induced by hypertonic stress.

Fig. 5 *Apoptosis-inducing factor (AIF) and cytochrome c (Cyt c) retention in HeLa cells undergoing hyperosmotic stress-induced apoptosis*. (a, b) HeLa cells were treated with 0 (control) or 300 mM sorbitol for 2 h, then stained with Hoechst 33342 and antibodies specific for AIF (a) or Cyt *c* (b) and the 60 kDa heat-shock protein (HSP-60, which co-localizes with mitochondria). Mitochondria of HeLa cells retained both AIF (a) and Cyt *c* (b) after 2 h of treatment with sorbitol. The same holds true for treatments up to 6 h (data not shown). White bars represent picture scale (10 μm).

## Bcl-2 family proteins control cell death induced by hyperosmotic stress

The data shown above indicate that osmotic stress is able to induce specific changes in the mitochondrial function that are regulated, at least partially, by pro-apoptotic Bcl-2 proteins such as Bax. We therefore took advantage of a non-small cell lung cancer model, A549 cells, that we are optimizing for use in high-throughput screening assays. A549 cells underwent sorbitol-induced cell death similarly to HeLa or HCT116 cells, yet were even more resistant to hypertonic stress (Fig. 6(a)). In this experimental system, the siRNA-mediated knock-down of several members of the Bcl-2 family (Fig. 6(b)), significantly influenced sorbitol-

induced killing. For instance, the knock-down of Bax (but not that of Bak) diminished sorbitol-induced killing, while the down-regulation of Bcl-2 or Bcl-X$_L$ enhanced cell death induced by osmotic shock (Fig. 6(c)). Notably, the knock-down of either p53 and of the voltage-dependent anion channel 1 (VDAC1, a major component of the mitochondrial permeability transition pore complex) [30, 31] had negligible effects on sorbitol-induced apoptosis (Fig. 6(c)). This correlates with the lack of cytoprotection conferred by cyclic pifithrin-α to HeLa and HCT116 cells treated with sorbitol (Fig. 3(a, b)).

To confirm the role of Bcl-2 in the pro-apoptotic cascades elicited by hyperosmotic stress, we transiently transfected HeLa and HCT116 cells with plasmids encoding either WT

Bcl-2 or Bcl-2 variants that selectively localize to mitochondria (Bcl-2 Acta) or to the endoplasmic reticulum (Bcl-2 Cb5) [23]. In HeLa, Bcl-2 Acta was able to reduce all the apoptosis-associated alterations induced by sorbitol, including $\Delta\Psi_m$ dissipation, PS exposure and loss of viability (Fig. 7(a)). In the same cells, WT Bcl-2 was effective only against the loss of $\Delta\Psi_m$, but did not decrease PS exposure nor PI positivity, and Bcl-2 Cb5 had no effects on sorbitol-induced

alterations (Fig. 7(a)). In HCT116 cells, WT Bcl-2 and Bcl-2 Cb5 were totally ineffective and only the Bcl-2 isoform targeted to mitochondria (Acta) provided some level of cytoprotection against hypertonic stress-induced apoptosis, by partially preventing $\Delta\Psi_m$ dissipation and loss of viability, but not PS exposure (Fig. 7(b)). Again, these data point to the existence of cell type-specific responses to hyperosmotic stress.
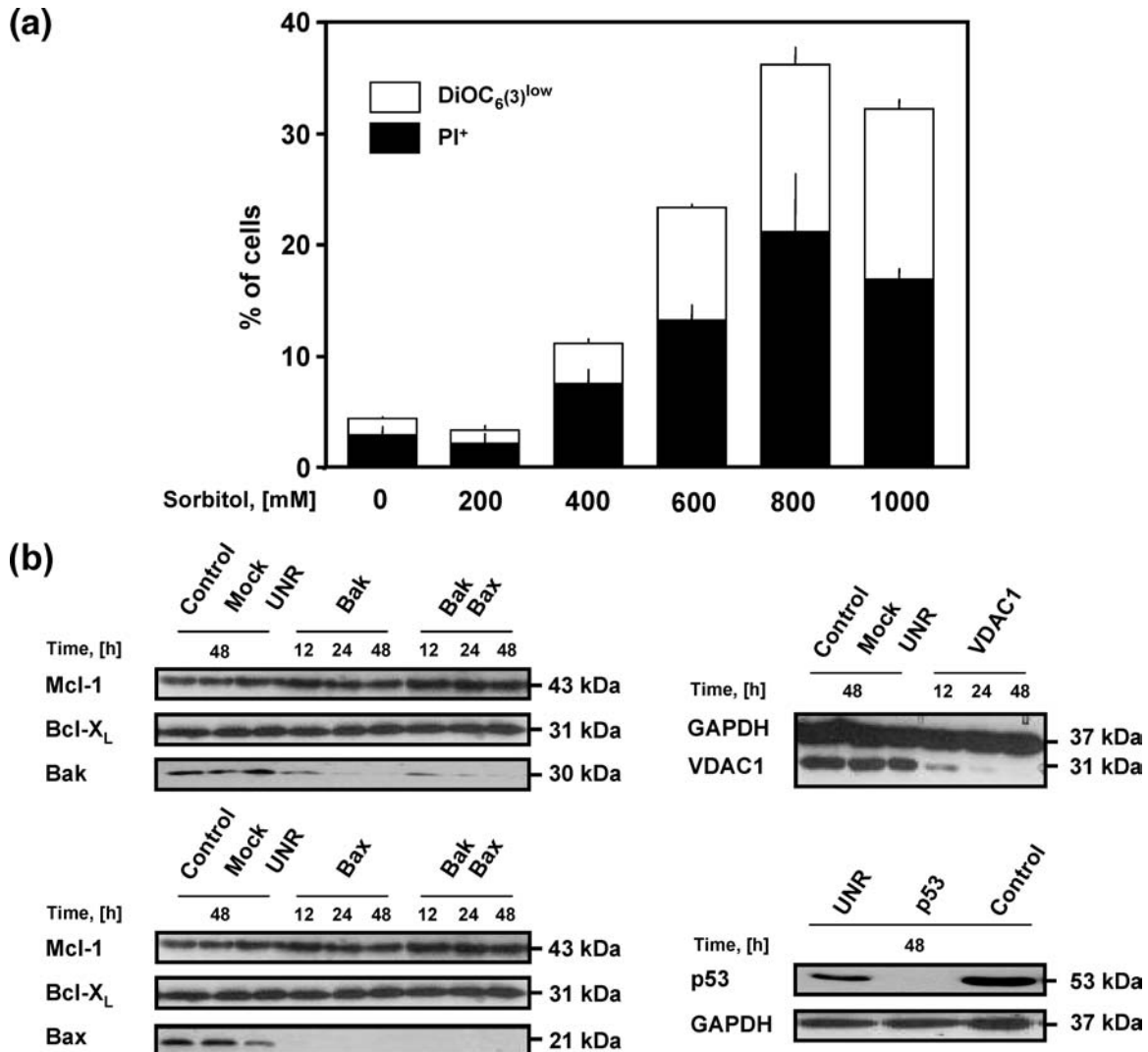
Fig. 6 *Involvement of the Bcl-2 protein family in hyperosmotic stress-induced death of A549 cells.* (a) A549 cells were treated with the indicated dose of sorbitol for 6 h, followed by the cytofluorometric assessment of mitochondrial membrane potential (DiOC$_6$(3) staining) and viability (PI staining). White columns illustrate the percentage of cells with a low mitochondrial membrane potential but still viable (DiOC$_6$(3)^{low}). Black columns indicate the percentage of cells with disrupted plasma membrane (PI$^+$). Data are mean of duplicate experiments $\pm$ SEM. (b, c) A549 cells were transfected with empty liposomes (Mock) or with siRNAs targeting Bax, Bak, Bcl-2, Bcl-X$_L$, p53, the voltage-dependent anion channel 1 (VDAC1) or an irrelevant "unrelated" control (UNR). (b) To check for the effects of siRNAs, total proteins were purified from transfected A549 cells at different time points (12, 24 or 48 h), separated according to molec-

ular weight on mono-dimensional SDS-PAGE, and finally analyzed by immunoblotting with the indicated antibodies. Antibodies specific for Mcl-1 and Bcl-X$_L$ or for GAPDH were employed as loading controls. (c) A549 cells transfected for 48 h with the indicated siRNAs were treated with 600 mM sorbitol for additional 6 h, then analyzed at FACS for mitochondrial membrane potential (DiOC$_6$(3) staining) and viability (PI staining). White columns depict the percentage of cells that have dissipated the mitochondrial membrane potential but are still viable (DiOC$_6$(3)^{low}). The percentage of cells with disrupted plasma membrane (PI$^+$) is illustrated by black columns. Data are mean of duplicate experiments $\pm$ SEM. Dashed lines indicate the range of statistical insignificance from control cells ($p > 0.05$, $\pm$ 3 SEM). For additional details please see also "Materials and methods"
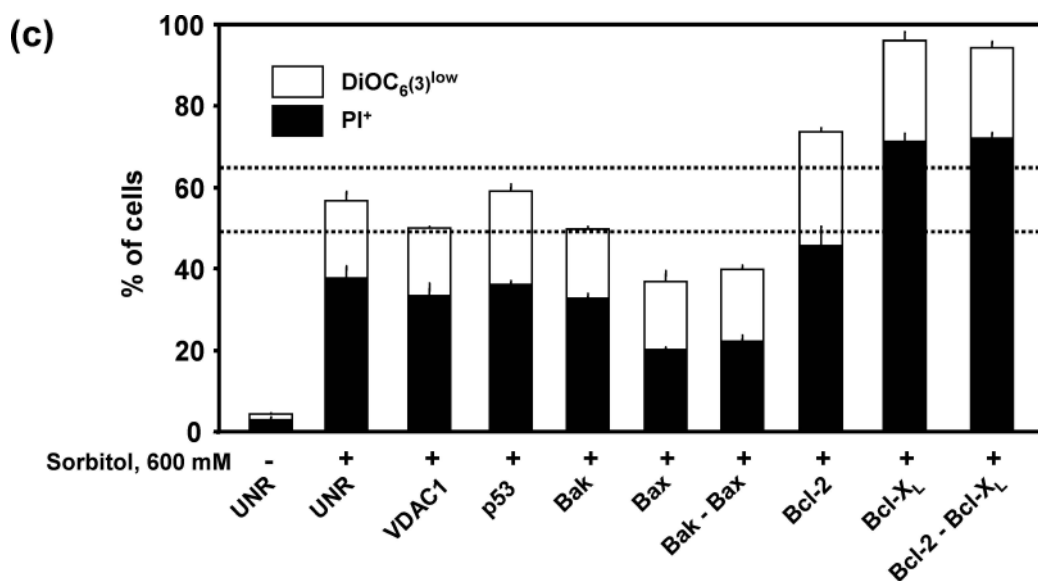
Fig. 6 (*Continued*).

Finally, the cytoprotective effect of Bcl-2 overexpression was explored in a fibroblast cell line (Rat-1) that was stably transfected with the abovementioned constructs encoding human WT Bcl-2, or Bcl-2 variants that selectively localize to mitochondria (Bcl-2 Acta) and hence co-localize with the mitochondrial marker HSP-60 or to the endoplasmic reticulum (Bcl-2 Cb5), which do not co-localize with HSP-60 (Fig. 8(a, b)) [23]. Similarly to HeLa cells, Rat-1 fibroblasts responded to sorbitol with the progressive activation of caspase-3, a dose-dependent dissipation of $\Delta\Psi_m$ and a reduction of viability (Fig. 8(c, d, e)). In this system, the overexpression of Bcl-2 Acta efficiently prevented the proteolytic maturation of caspase-3, while WT Bcl-2 and Bcl-2 Cb5 only retarded its activation (Fig. 8(c)). Of note, both WT Bcl-2 and Bcl-2 Acta (but not Bcl-2 Cb5) reduced the mitochondrial perturbation and the loss of cellular viability induced by sorbitol (Fig. 8(d, e)). Taken altogether, these results demonstrate that mitochondrion-targeted Bcl-2 specifically reduces the mitochondriotoxic and cytotoxic effects of hyperosmotic stress.

Conclusion

The data presented in this article strongly suggest that mitochondria play a major role in the lethal response to hyperosmotic stress. First, cells exposed to hyperosmotic stress manifest characteristic mitochondrial dysfunctions, in particular a loss of the mitochondrial transmembrane potential ($\Delta\Psi_m$), an increased generation of reactive oxygen species (ROS), and an at least partial permeabilization of the outer mitochondrial membrane, leading to the release of AIF (which can act as a caspase-independent death effector), in a cell type-specific fashion. Second, such mitochondrial changes occurred in some cell types (HCT116) in a caspase-independent fashion, because they were not accompanied by caspase activation and were not prevented by the pan-caspase inhibitor Z-VAD-fmk. In contrast, in other cell types (HeLa), the mitochondrial alterations occurred after the osmolyte-induced activation of caspases-8 and -3. Third, proteins belonging to the Bcl-2 family, which are known to exert a major effect on the apoptosis-associated functional and structural impairment of mitochondria, strongly influenced sorbitol-induced apoptosis. As a formal prove of the mitochondrial implication in the lethal process, we found that a mitochondrion-targeted Bcl-2 mutant could confer strong cytoprotection against cell death induced by hypertonic stress.

The differential sensitivity to cell death of the cell lines used in the present study, as well as the cell type-specific mitochondrial changes that we observed in response to hyperosmotic stress, may be related to differences in their proliferative and/or metabolic status [32]. Several observations suggest that proliferation and/or metabolic activity play a role in determining to which extent cells activate specific pathways to regulate cell function upon hypertonic stress [33, 34]. Moreover, it has been reported that changes occurring within the cell as a result of active metabolism/cell division likely promote the same alterations in intracellular water homeostasis resulting from the exposure to extracellular hypertonicity [35]. Thus, the osmotic stress response pathway appears to mediate cellular adaptation not only to overt extracellular hyperosmotic stress, but also to other processes that affect intracellular water homeostasis.

Although the data shown here strongly implicate mitochondria in sorbitol-induced apoptosis, a number of conundra remain to be resolved. Thus, it is not clear which signals
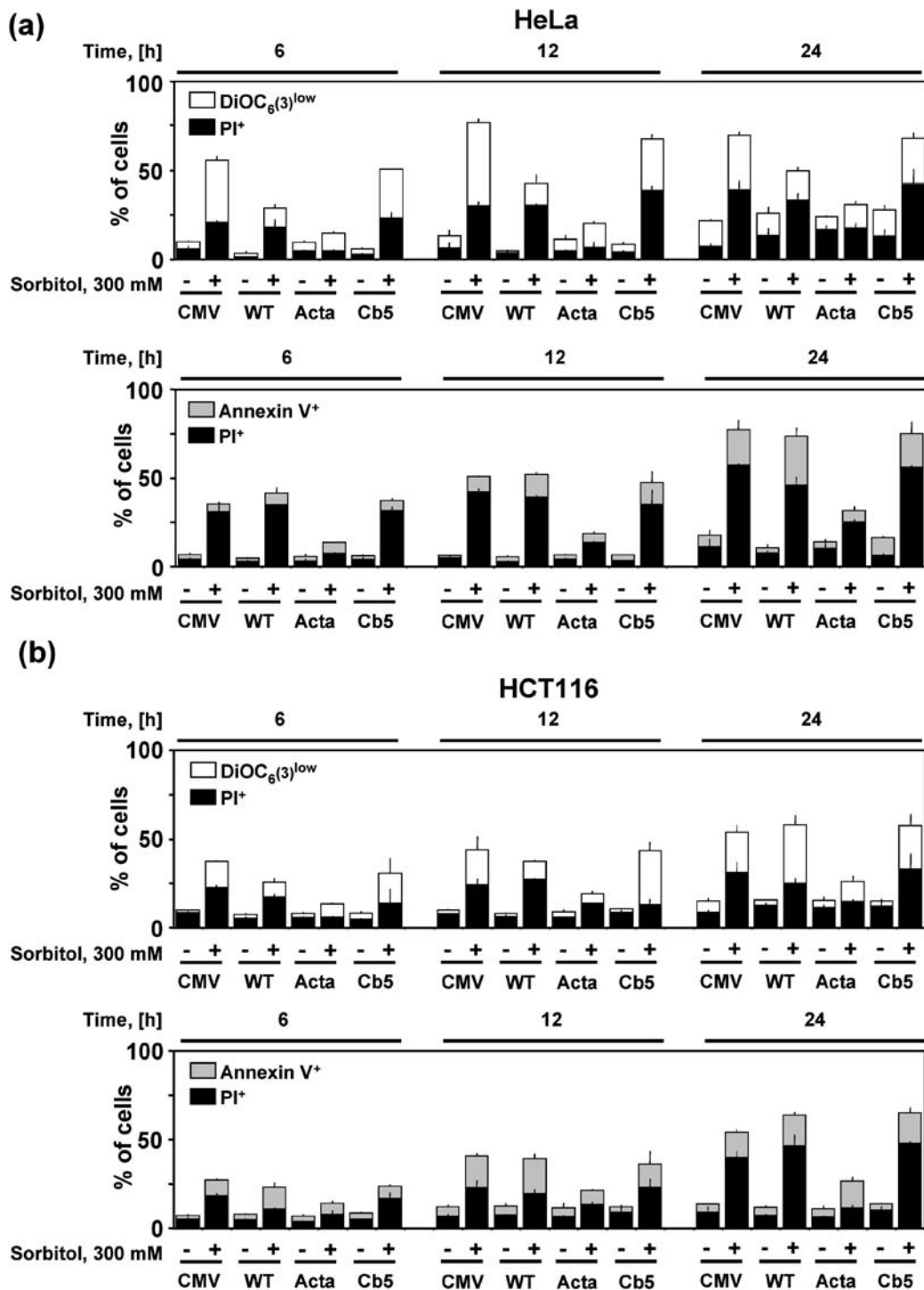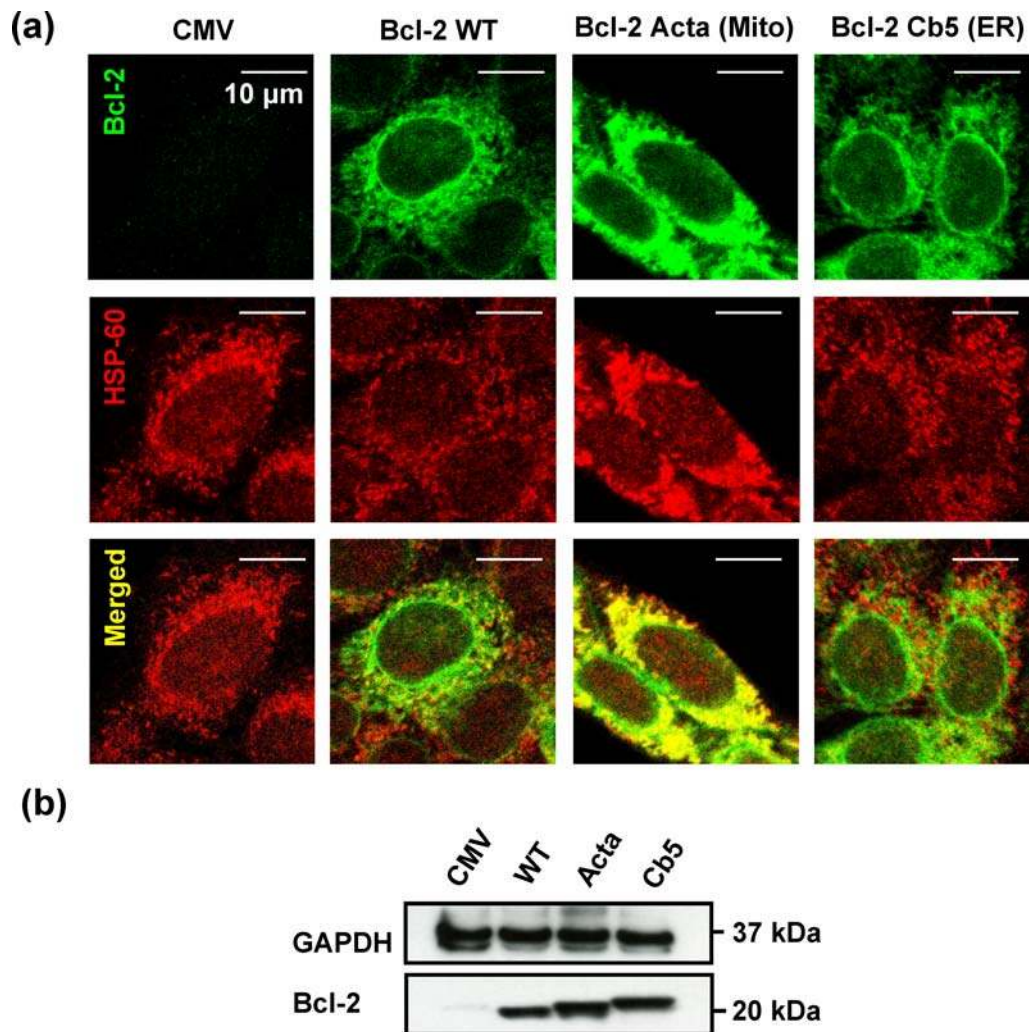
Fig. 7 *Bcl-2 targeted to mitochondria protects HeLa and HCT116 cells from hyperosmotic stress-induced cell death*. HeLa (a) and HCT116 (b) cells were transiently transfected with an empty control vector (CMV) or with a plasmid expressing Bcl-2, either in its wild-type configuration (WT) or fused to mitochondria- and endoplasmic reticulum-targeting peptides (Acta and Cb5, respectively). After 24 h, cells were treated with 0 (control) or 300 mM sorbitol for additional 6, 12 or 24 h, then stained for the cytofluorometric determination of mitochondrial membrane potential (DiOC$_6$(3) staining), PS exposure (Annexin V staining) and viability (PI staining). White columns report the percentage of cells that have a low mitochondrial membrane potential but are still viable (DiOC$_6$(3)^{low}). Black columns illustrate the percentage of cells with disrupted plasma membrane (PI$^+$). Light grey columns depict the percentage of cells characterized by PS on the outer leaflet of the plasma membrane (Annexin V$^+$). Data are mean of duplicate experiments $\pm$ SEM. For more detailed information please refer to the section "Materials and methods"

act upstream of mitochondria to elicit their permeabilization. Although we did not explicitly address the implication of the extrinsic pathway in sorbitol-induced apoptosis, our data point to an active involvement of the death receptor system, but only in HeLa, not in HCT116 cells. Caspases-8 and -3 were not activated following hyperosmotic stress in

HCT116 cells, arguing against a major participation of the death receptor-triggered caspase activation cascade in this model. Future studies will have to clarify at which stage of the apoptotic cascade the cell type-specific pathways elicited by hypertonic stress converge, and which are the molecular players that determine cell type-specific difference in the

Fig. 8 *Bcl-2 targeted to mitochondria protects Rat-1 fibroblasts from hyperosmotic stress-induced apoptosis*. (a–e) Rat-1 fibroblasts were transfected with an empty control vector (CMV) or with a plasmid expressing Bcl-2 in its wild-type configuration (WT) or fused to peptides targeting it to mitochondria (Acta) or to endoplasmic reticulum (Cb5). (a) Rat-1 cells transfected with the abovementioned constructs were submitted to immunofluorescence staining with antibodies specific for Bcl-2 and the 60 kDa heat-shock protein (HSP-60, which co-localizes with mitochondria), to ensure the correct localization of the Bcl-2 fusion proteins. (b) Proteins were extracted from Rat-1 fibroblasts transfected with the abovementioned plasmids, separated according to molecular weight on mono-dimensional SDS-PAGE, then analyzed by immunoblotting with antibodies specific for Bcl-2 and GAPDH (loading control). (c–e) Transfected Rat-1 fibroblasts were treated with 300 mM sorbitol for the indicated time then subjected to protein extraction for subsequent immunoblot (c) or to cytofluorometric analysis of mitochondrial membrane potential (DiOC$_6$(3) staining) and viability

(PI staining) (d–e). (c) After the treatment with 300 mM sorbitol for the indicated time, proteins were extracted, run on mono-dimensional SDS-PAGE, then analyzed by immunoblotting with antibodies specific for total caspase-3 and GAPDH (loading control). Caspase-3 activation corresponds to the progressive accumulation of the 17 kDa active caspase-3 fragment in parallel with a decrease of the pro-caspase-3 band (approx. 35 kDa). Only when Bcl-2 is targeted to mitochondria, sorbitol-induced caspase-3 activation is efficiently prevented. (d) Representative dot plots obtained after 6 h of treatment with 0 (control) or 300 mM sorbitol. X-axis, DiOC$_6$(3) staining; Y-axis, PI staining. (e) Kinetic response of Rat-1 fibroblasts to sorbitol treatment. White columns depict the percentage of cells which have dissipated the mitochondrial membrane potential but are still viable (DiOC$_6$(3)^{low}). The percentage of cells which have lost the integrity of plasma membrane (PI$^+$) is illustrated by black columns. Data are mean of triplicate experiments $\pm$ SEM. For further details please see the section "Materials and methods"

(c)

GAPDH — 37 kDa

Pro-caspase-3 — 35 kDa

Cleaved caspase-3 — 17 kDa

CMV　Bcl-2 WT　Bcl-2 Acta (Mito)　Bcl-2 Cb5 (ER)

Time, [h] 0 0.5 1 2 4 6　0 0.5 1 2 4 6　0 0.5 1 2 4 6　0 0.5 1 2 4 6

Sorbitol, 300 mM　Sorbitol, 300 mM　Sorbitol, 300 mM　Sorbitol, 300 mM

(d)

CMV　Bcl-2 WT　Bcl-2 Acta (Mito)　Bcl-2 Cb5 (ER)

Control

Sorbitol, 300 mM

PI vs $DiOC_6(3)$

(e)

% of cells

$DiOC_6(3)^{low}$

PI^+

Time, [h] 0 6 8 12 | 0 6 8 12 | 0 6 8 12 | 0 6 8 12

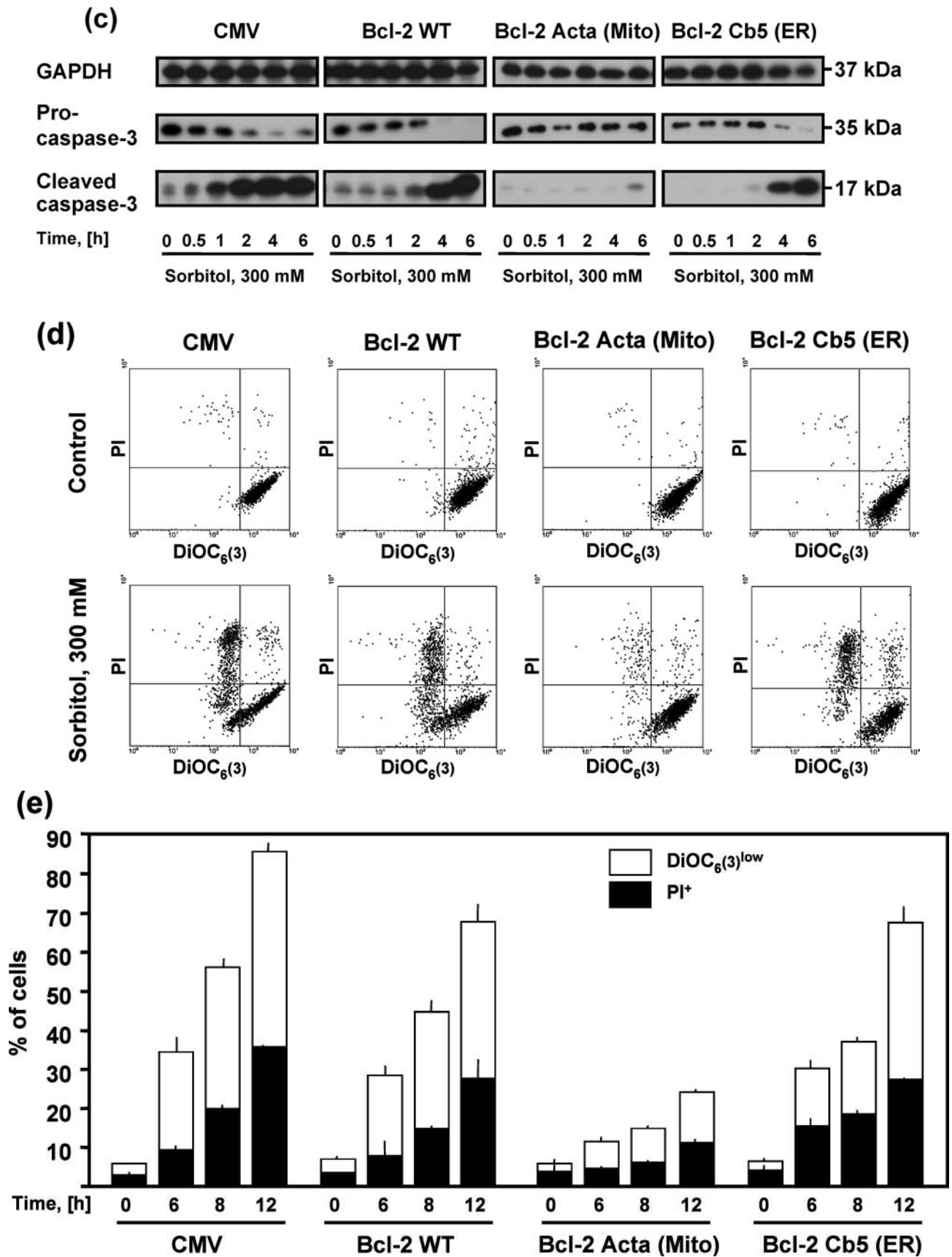CMV　Bcl-2 WT　Bcl-2 Acta (Mito)　Bcl-2 Cb5 (ER)

Fig. 8 (*Continued*).

response to hyperosmotic stress. For instance, it will be interesting to determine to which extent specific BH3 only proteins from the Bcl-2 family, lipid messengers and perturbations in ion or redox homeostasis contribute to trigger the mitochondrial phase of apoptosis in response to hyperosmotic stress.

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