Disruption of the VDAC2–Bak interaction by Bcl-x_S mediates efficient induction of apoptosis in melanoma cells

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The proapoptotic B-cell lymphoma (Bcl)-2 protein Bcl-x_S encloses the Bcl-2 homology (BH) domains BH3 and BH4 and triggers apoptosis via the multidomain protein Bak, however, the mechanism remained elusive. For investigating Bcl-x_S efficacy and pathways, an adenoviral vector was constructed with its cDNA under tetracycline-off control. Bcl-x_S overexpression resulted in efficient apoptosis induction and caspase activation in melanoma cells. Indicative of mitochondrial apoptosis pathways, Bcl-x_S translocated to the mitochondria, disrupted the mitochondrial membrane potential and induced release of cytochrome c, apoptosis-inducing factor and second mitochondria-derived activator of caspases. In melanoma cells, Bcl-x_S resulted in significant Bak activation, and Bak knockdown as well as Bcl-x_L overexpression abrogated Bcl-x_S-induced apoptosis, whereas Mcl-1 (myeloid cell leukemia-1) knockdown resulted in a sensitization. With regard to the particular role of voltage-dependent anion channel 2 (VDAC2) for inhibition of Bak, we identified here a notable interaction between Bcl-x_S and VDAC2 in melanoma cells, which was proven in reciprocal coimmunoprecipitation analyses. On the other hand, Bcl-x_S showed no direct interaction with Bak, and its binding to VDAC2 appeared as also independent of Bak expression. Suggesting a new proapoptotic mechanism, Bcl-x_S overexpression resulted in disruption of the VDAC2–Bak interaction leading to release of Bak. Further supporting this pathway, overexpression of VDAC2 strongly decreased apoptosis by Bcl-x_S. New proapoptotic pathways are of principle interest for overcoming apoptosis deficiency of melanoma cells.

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Apoptosis represents a safeguard mechanism for elimination of altered and mutated cells, and apoptosis deficiency is thus a characteristic feature of cancer cells further related to chemotherapy resistance.^{1,2} Overcoming of apoptosis deficiency in melanoma remains a particular target.³ Two major apoptosis pathways have been described in more detail. On one hand, extrinsic pathways are initiated by binding of death ligands (TNF- α , CD95L and TRAIL) to cell surface receptors, leading to the activation of initiator caspase (Csp) 8.⁴ On the other hand, intrinsic/mitochondrial apoptosis pathways are triggered by intracellular signals such as DNA damage and may also be activated by chemotherapeutic drugs.⁵ Key events in this pathway are depolarization of the mitochondrial membrane potential ($\Delta\psi/m$) and mitochondrial outer membrane permeabilisation (MOMP) resulting in release of mitochondrial factors such as cytochrome c, apoptosis-inducing factor (AIF) and second mitochondria-derived activator of Csp (Smac). Via the apoptosome, cytochrome c can activate initiator Csp9.⁶ Initiator Csp8 and 9 may activate effector Csp-3, which cleaves a large number of death substrates to set apoptosis into work.⁷

Mitochondrial apoptosis pathways are critically controlled by the family of pro- and antiapoptotic B-cell lymphoma (Bcl)-2 proteins.⁸ This superfamily shares homology in four

conserved regions termed Bcl-2 homology (BH) domains. Antiapoptotic proteins as Bcl-2, Bcl-x_L and myeloid cell leukemia (Mcl)-1 typically enclose all four BH domains (BH 1–4), whereas proapoptotic family members subdivide in the Bax/Bak group (BH 1–3) and the BH3-only group with Bad, Bid, Bik/Nbk, Bim, Noxa, Puma and others.^{6,9}

In present models, Bax and Bak drive MOMP and are neutralized by antiapoptotic family members. The BH3-only proteins contribute to the mutual regulation as sensitizers through inhibition of antiapoptotic family members or as direct activators of Bax.^{6,8} The steps of mutual regulation and neutralization are based on heterodimerization. Thus, the BH3 domain of proapoptotic Bcl-2 proteins encloses an amphipathic α helix, which binds into a hydrophobic groove formed by BH1, BH2 and BH3 of antiapoptotic family members.¹⁰ In a rheostat model, the balance of anti- and proapoptotic Bcl-2 proteins determines the fate of a cell.¹¹ In melanoma cells, apoptosis deficiency has been attributed to high expression of antiapoptotic Bcl-2 proteins.^{1,12,13}

Alternative splicing further increases the number of Bcl-2 proteins as well as the complexity of their regulation. The *bcl-x* gene gives rise to the antiapoptotic protein Bcl-x_L (long) and the proapoptotic Bcl-x_S (short).¹⁴ A dependency of Bcl-x_S-induced apoptosis on Bak has been described,^{15,16} however,

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Abbreviations: Bcl, B-cell lymphoma; BH, Bcl-2 homology domain; Mcl, myeloid cell leukemia; VDAC, voltage-dependent anion channel; AIF, apoptosis-inducing factor; Smac, second mitochondria-derived activator of caspases; Tet, tetracycline; Csp, caspase; PI, propidium iodide; AdV, recombinant adenovirus

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the pathway remained elusive. Besides the Bcl-2 family, also other proteins may be considered in the regulation of mitochondrial apoptosis.⁵ Thus, three isoforms of the voltage-dependent anion channel (VDAC1, VDAC2 and VDAC3) have been described, which mediate the exchange of metabolites through the mitochondrial membrane but have also distinct roles in apoptosis regulation.¹⁷ Interestingly, genetics and biochemical studies had indicated an antiapoptotic function for VDAC2 through binding and inhibition of the proapoptotic multidomain protein Bak,¹⁸ whereas VDAC1 serves proapoptotic functions by binding to Bcl-x_L.¹⁹

In this study, the mechanism of Bcl-x_S-induced apoptosis was investigated in melanoma cells. As the important finding, immunoprecipitation studies revealed interaction of Bcl-x_S with VDAC2, which resulted in a release of Bak from the VDAC2–Bak complex, thus explaining the Bak dependency of Bcl-x_S-mediated apoptosis.

Results

Efficient induction of apoptosis by recombinant adenovirus (AdV)-X_S. For investigating the efficacy and mechanism of Bcl-x_S-mediated apoptosis in melanoma cells, we constructed an adenoviral vector with the Bcl-x_S full-length cDNA under control of a tetracycline (Tet)-off promoter inserted into the adenoviral E1 region. The Tet/doxycycline-suppressed transactivator tTA was located in the adenoviral E3 region (Figure 1a). The construct AdV-X_S mediated high expression of Bcl-x_S in melanoma cells A-375, Mel-HO and Mel-2a, when doxycycline was omitted (on condition), whereas expression was abolished by doxycycline (off condition; Figure 1b).

Bcl-x_S overexpression resulted in strong induction of apoptosis in melanoma cell lines, as seen by reduced cell numbers, rounded and detached cells (Figure 1c) as well as by apoptotic cells with fragmented DNA, as quantified by flow cytometry (Figure 1d). Time kinetic analyses revealed an early induction of apoptosis at 24 h, which increased in a time-dependent manner to 30–45% at 72 h after transduction (Figure 1e). In contrast, cytotoxicity remained at a low level at early times and only slightly increased at 72 h, as determined by LDH release (Figure 1f). Comparative apoptosis induction in course of Bcl-x_S expression was obtained in the three melanoma cell lines by a DNA fragmentation ELISA (data not shown), and comparative values were also obtained in Mel-2a at 48 h by annexinV/propidium iodide (PI) staining (26%, Figure 1h) and annexinV single staining (35%, Figure 1i). In course of induced apoptosis, the cell numbers were strongly decreased by >50% at 72 h, as determined by the WST-1 assay (Figure 1g), and numbers of viable cells were decreased by 43% in Mel-2a at 48 h, as determined by calcein assay (Figure 1j).

High Bcl-x_S expression triggers Csps and mitochondria. Activation of the Csp cascade by Bcl-x_S was investigated in Mel-2a cells by western blot analyses for the initiator Csps 8 and 9 as well as the main effector Csp3. Some Csp processing appeared already at 24 h, which strongly increased at 48 h (Figure 2a). Involvement of the mitochondrial apoptosis pathway was determined by the mitochondrial

membrane potential ($\Delta\psi/m$)-dependent dye tetramethyl rhodamine methyl ester perchlorate (TMRM)⁺. It revealed decrease of $\Delta\psi/m$ in 43% of Mel-2a cells at 24 h after Bcl-x_S expression, which increased to 65% at 48 h (Figure 2b). The higher percentage of cells with decreased $\Delta\psi/m$ than cells with fragmented DNA (Figure 1e) suggested the mitochondrial response as an initial step in Bcl-x_S-mediated apoptosis.

Further proving the activation of mitochondrial pathways by Bcl-x_S, a significant release of cytochrome c, Smac and AIF was seen in Mel-2a at 48 h, as determined by western blotting of cytosolic extracts (Figure 2c). As seen in mitochondrial extracts, Bcl-x_S almost completely localized to the mitochondria at 48 h after transduction (Figure 2d). Bak was constitutively present in mitochondria and also moderate levels of Bax. Bcl-x_S did not induce further Bax translocation (Figure 2d) and also did not induce an altered expression of other Bcl-2 proteins, as proven by western blotting for Bcl-2, Mcl-1, Bax, Puma and Noxa, thus no indication of any indirect effect via altered expression of other Bcl-2 proteins (Figure 2e). Finally, no significant induction of the autophagosome-associated protein light chain 3 (LC3-II, 17 kDa) was seen at 24 h in melanoma cells, thus also no indication of an involvement of autophagy in the initial phase of Bcl-x_S-mediated cell death (Figure 2f).

Dependency of Bcl-x_S on pro- and antiapoptotic Bcl-2 proteins. The previously reported dependency of Bcl-x_S on Bak¹⁵ was also proven for AdV-X_S in HCT116 colon carcinoma cells with Bax knockout, Bak knockdown or double knockdown (Figure 3a). Whereas Bax knockout had only little effect on Bcl-x_S-induced apoptosis, Bak knockdown almost completely abolished apoptosis by Bcl-x_S at 24 h (Figure 3b).

The function of three different antiapoptotic Bcl-2 family members (Bcl-2, Bcl-x_L and Mcl-1) in Bcl-x_S-induced apoptosis was investigated. For Bcl-2, subclones of A-375 stably transfected with a Bcl-2 expression plasmid (A-375-Bcl-2) were compared with mock-transfected cells (A-375-Mock) (Figure 3c), for Bcl-x_L, transient transfection of A-375 cells was applied (Figure 3d), and Mcl-1 was downregulated by siRNA in A-375-Mock and A-375-Bcl-2 (Figure 3g). Whereas Bcl-2 has been described to preferentially inhibit Bax, Mcl-1 preferentially inhibits Bak and Bcl-x_L may bind and inhibit both Bax and Bak.⁶

Bcl-2 overexpression diminished the proapoptotic effects of AdV-X_S by 50% but completely prevented Bik/Nbk-induced apoptosis, which was subcloned in the same adenoviral background (Figure 3e). On the other hand, Bcl-x_S-induced apoptosis was completely abrogated by Bcl-x_L (Figure 3f). Finally, Mcl-1 knockdown further enhanced Bcl-x_S-induced apoptosis, seen both in A-375-Mock and in A-375-Bcl-2, as compared with scramble siRNA-treated cells (Figures 3i and h). Thus, all three antiapoptotic family members had a protective role, and the results were in agreement with Bcl-x_S-mediated apoptosis induction via Bak.

This decisive role of Bak was finally proven by N-terminus-specific antibodies for Bak and Bax (Bak-NT and Bax-NT), which indicate their activation. Clearly, Bak was activated by Bcl-x_S at 24 h (30% of cells), whereas Bax was only weakly activated and only at 48 h (Figure 4a). In contrast, Bik/Nbk showed an inverse picture, namely early activation of Bax with only weak activation of Bak at 48 h, in agreement with

apoptosis induction via Bax (Figure 4b). The dependency of Bcl-x_S on Bak in melanoma cells was finally proven by siRNA-mediated Bak knockdown (Figure 4c). This completely abrogated Bcl-x_S-induced apoptosis at 24 h, and only at 48 h some apoptosis was seen (Figure 4d), in relation with weak Bax activation.

Lacking interactions of Bcl-x_S with other Bcl-2 proteins.

We looked for interactions of Bcl-x_S with other Bcl-2 family members in melanoma cells by coimmunoprecipitation analyses. Therefore, A-375 was transiently transfected with Myc-tagged Bcl-x_S, Bcl-x_L or Bax expression plasmids, and overexpressed Myc-tagged proteins were immunoprecipitated

with anti-Myc microbeads. Apoptosis determined in parallel was enhanced by Myc-Bax and Myc-Bcl-x_S, whereas somewhat diminished by Myc-Bcl-x_L (data not shown). Overexpressed Bcl-x_S, Bcl-x_L and Bax were demonstrated in the supernatant fractions (S), and efficient immunoprecipitation was proven in the pellet fractions (P) with Bcl-x and Bax-specific antibodies (Figure 5a). No interaction was seen between Bcl-x_S and the Bcl-2 family members Bcl-2, Mcl-1, Bax, Bad or Noxa, as proven by empty P-fractions. As controls for Bcl-2 protein interaction, binding of Bcl-x_L to Bax and Bad as well as binding of Myc-tagged Bax to Bcl-2 and to endogenous Bax was seen (Figure 5a). The specific binding of Bcl-x_L to both Bax and to Bak was proven also after substitution of triton X-100 for the non-denaturing solvent CHAPS in the cell lysis buffer; also under CHAPS buffer conditions, no interaction was seen between Bcl-x_S and Bak or Bax (Figure 5b).

Owing to the only weak basal expression of Bcl-x_L in melanoma cells, a SK-Mel-13 cell clone stably transfected with a Bcl-x_L expression plasmid (SKM13-Bcl-x_L) was used for investigating the role of Bcl-x_L. These cells were transiently transfected with Myc-tagged Bcl-x_L, Bcl-x_S as well as with Bik/Nbk, for an additional control. Under conditions of Bcl-x_L overexpression, Bcl-x_S revealed a weak interaction with Bcl-x_L, whereas again no interaction was seen to Bcl-2 or Bax. As controls, interactions were seen of Bik/Nbk with Bcl-x_L and Bcl-2 as well as of Bax with Bcl-x_L (Figure 5c). Thus, no significant interaction of Bcl-x_S with other Bcl-2 family members was obtained that could explain Bcl-x_S-mediated apoptosis in melanoma cells.

Bcl-x_S disrupts the interaction of VDAC2 with Bak. When we looked for interactions of Bcl-x_S with other proteins, a notable interaction was identified with voltage-dependent channel protein 2 (VDAC2, Figure 6a). To distinguish between VDAC1 and VDAC2 binding, a VDAC2-specific antibody was compared with an antibody, which detects all three VDAC isoforms (VDAC1/2/3). After transient transfection and immunoprecipitation of Myc-tagged Bcl-x_L and Bcl-x_S, the nonselective antibody detected 30 kDa VDAC proteins that interacted either with Bcl-x_L or with Bcl-x_S, whereas the VDAC2 antibody detected a 30-kDa protein that exclusively interacted with Bcl-x_S (Figure 6a). This finding strongly suggests that VDAC1 interacted with Bcl-x_L, as previously reported,¹⁷ while VDAC2 interacts with Bcl-x_S.

To confirm the interaction between Bcl-x_S and VDAC2, we used a plasmid for overexpression of a Myc-tagged VDAC2. Specific precipitation of exclusively Myc-tagged VDAC2 was

ensured by immunoprecipitation with anti-Myc antibodies. Also under these strictly defined conditions, coimmunoprecipitation analysis revealed the specific interaction between Bcl-x_S and VDAC2 (Figure 6d).

The significance of this interaction in the regulation of apoptosis was proven in Mel-2a by immunoprecipitation of endogenous VDAC2 and Bak, respectively. After immunoprecipitation of VDAC2, the interaction between VDAC2 and Bak was clearly evident, when Bcl-x_S was off (Figure 6b), and after induction of Bcl-x_S, the binding between Bcl-x_S and VDAC2 was again reproduced. Highly suggestive for a mechanistic relation, induction of Bcl-x_S resulted in complete disruption of the VDAC2–Bak interaction, thus leading to a release of Bak (Figure 6b). The similar interaction was also seen in the reciprocal approach after immunoprecipitation of Bak. Thus, the interaction between Bak and VDAC2 was again clearly evident, when Bcl-x_S was off, whereas there was no interaction between Bak and Bcl-x_S. Again, Bcl-x_S overexpression disrupted the VDAC2–Bak complex (Figure 6c).

This regulation was also investigated after overexpression of VDAC2 (Myc-tagged). Again, both Bak and Bcl-x_S were bound to VDAC2, but due to an excess of VDAC2, no complete displacement of Bak from the complex was observed (Figure 6d). Again the specific role of VDAC2 was underlined, as a defined VDAC2 cDNA sequence had been used here for overexpression. The displacement of Bak from VDAC2 upon induced Bcl-x_S expression was also seen in A-375 melanoma cells after scramble siRNA transfection (Figure 6e, left panel), and the interaction of Bcl-x_S with VDAC2 appeared as independent of Bak expression itself, as also seen under conditions of Bak knockdown (Figure 6e, right panel).

VDAC2 abrogates Bcl-x_S-induced apoptosis. Proving the dominant antiapoptotic role of VDAC2 for Bcl-x_S, its overexpression strongly diminished Bcl-x_S-mediated apoptosis in A-375 melanoma cells at 24 h and at 48 h (Figure 7a). In contrast, Bik/Nbk-induced apoptosis was almost not affected by VDAC2 overexpression, underlining the selective antiapoptotic effect of VDAC2 on Bak (Figure 7a). In accordance, the reciprocal approach of VDAC2 downregulation by siRNA resulted in significant induction of apoptosis, reaching 15% even without Bcl-x_S expression (Figure 7b). Supporting the dependency of Bcl-x_S on VDAC2, the proapoptotic effects of Bcl-x_S were diminished after VDAC2 knockdown. Remaining proapoptotic activity despite VDAC2 knockdown may be related to additional activities of Bcl-x_S, as inactivation of Bcl-x_L (Figure 7b).

Figure 1 Apoptosis induction by strong and tightly controlled expression of Bcl-x_S. (a) The structure of Adv-XS is shown. The Bcl-x_S cDNA driven by a tetracyclin-controlled promoter (P_{TRE}) was subcloned into the Ad5 E1 region, and E3 had been replaced by the tetracyclin-suppressed transactivator (tTA) driven by a CMV promoter (P_{CMV}). The tTA mediates Tet-off regulation. Striped boxed indicate poly(A) regions. (b) Inducible Bcl-x_S expression is shown by western blotting in A-375, Mel-HO and Mel-2a at 48 h after transduction of Adv-XS (MOI = 50). Doxycycline was given for suppression (off condition) or was omitted (on condition). Equal protein loading was confirmed by β-actin, and Bcl-x_L-transfected SK-Mel-13 cells (SKM13-Bcl-x_L, C) are shown as control for Bcl-x_L expression. (c) Rounded cells indicating apoptosis are shown of Mel-2a at 48 h after transduction with Adv-XS under off and on conditions. (d) Examples of cell cycle analysis after PI staining indicating sub-G1 apoptotic cell populations in Mel-2a at 72 h of transduction. (e–g) Time course analyses of apoptosis (e, flow cytometry after PI staining), cytotoxicity (f, LDH release) and cell numbers (g, WST-1 assay) are shown for A-375, Mel-HO and Mel-2a cells at 24, 48 and 72 h after transduction with Adv-XS (50 MOI, + Dox = on, – Dox = off). As positive controls for induced cytotoxicity, cell lines were completely lysed by triton X-100 (T = 100%) or were treated with doxorubicin (D, 500 nM, 72 h). WST-1 values are expressed as percent of non-treated controls (= 100%). A luciferase-encoding adenovirus (Ad5-CMV-Luc) applied at the same MOI served as mock control (M). Mean values ± S.D.s of at least six individual values are shown, and statistical significance (P < 0.05) is indicated by asterisks. (h–j) In Mel-2a at 48 h of Bcl-x_S induction, apoptotic cells were determined by flow cytometric measurement of annexin V/PI staining (h) or by annexin V single staining (i), and numbers of viable cells were determined according to calcein staining (j). A shift to the right indicates annexin V-positive cells (i) and a shift to the left indicates calcein-negative (nonviable) cells (j)

Figure 2 Activation of Csp and mitochondria. (a) Processing of Csp-8, -9 and -3 is shown in Mel-2a at 24 and 48 h after transduction of AdV-XS (MOI = 50; on/off conditions). Equal protein loading (20 μg/lane) was confirmed by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Two independent experiments revealed comparable results. (b) Decrease of the mitochondrial membrane potential ($\Delta\psi/m$) is shown for Mel-2a at 24 and 48 h after transduction of AdV-XS. Overlays of on conditions (open graphs) and off conditions (gray) are shown. Two independent experiments revealed highly comparable results. (c and d) Cytosolic fractions (cyto) and mitochondrial fractions (mito) were isolated from Mel-2a cells at 48 h after transduction with AdV-XS (MOI = 50) and culturing under off and on conditions. Non-transfected cells (–) served as controls. (c) Cytosolic extracts demonstrate release of mitochondrial factors. Mitochondrial extracts served as control for mitochondrial proteins, and β-actin served as loading control. An antibody for mitochondrial VDAC proteins (VDAC1/2/3) was used for ruling out any contaminations of cytosolic extracts with mitochondria. (d) Mitochondrial extracts demonstrate mitochondrial translocation of Bcl-x_S, and cytosolic extracts served as controls. Here, expression of VDAC proteins confirmed equal mitochondrial protein loading. The whole experiment was performed two times, resulting in highly comparable results. (e) Expression levels of Bcl-2 proteins in total protein extracts were determined by western blotting in Mel-2a at 24 and 48 h after transduction with AdV-XS (on/off conditions). Equal protein loading (20 μg/lane) was confirmed by GAPDH. (f) Expression of LC3 was determined by western blotting in A-375, Mel-HO and Mel-2a at 48 h after transduction of AdV-XS (MOI = 50). Rapamycin-treated Mel-2a cells (Rapa, 10 μM, 24 h) served as positive control

Collectively, these data clarified the proapoptotic mechanism of Bcl-x_S, which selectively functions via the Bak pathway and is based on a disruption of the VDAC2-Bak interaction. Thus, released Bak triggers mitochondrial apoptosis pathways in melanoma cells. This model suggests dominant antiapoptotic functions of Bcl-x_L and Mcl-1 in this setting, whereas Bcl-2 functions on the other arm (Figure 7c).

Discussion

The superfamily of pro- and antiapoptotic Bcl-2 proteins is critically engaged in apoptosis through ruling the mitochondrial apoptosis pathways.⁶ The number of Bcl-2 family members and thus the complexity of regulation are further increased by differential splicing. Thus, the *bcl-x* gene splice

products enclose antiapoptotic Bcl-x_L (BH 1–4),²⁰ proapoptotic Bcl-x_{AK} that lacks a BH3²¹ and proapoptotic Bcl-x_S with a unique domain structure enclosing BH3, BH4 and a transmembrane domain.¹⁴ Apoptosis induction by Bcl-x_S had been previously shown in HeLa cells upon enforced splicing of Bcl-x_S²² as well as in lymphocytic leukemia cells, where Bcl-x_S was upregulated in drug-induced apoptosis.²³ For melanoma, we have previously demonstrated apoptosis induction *in vitro* and reduced tumor growth in mice in course of Bcl-x_S transfection.²⁴ Thus, targeting Bcl-x_S in cancer cells may serve as an additional therapeutic approach.

For evaluating its efficacy and unraveling of involved pathways, we constructed an adenoviral vector, which drives Bcl-x_S expression under Tet-off control. In this setting, Bcl-x_S revealed significant efficacy with up to 45% apoptosis induction in melanoma cells as well as activation of Csp of the extrinsic and intrinsic apoptosis pathways. The extrinsic initiator Csp-8 may be, however, also activated downstream of Csp-3,²⁵ which appears as suggestive for proapoptotic Bcl-2 proteins. Whereas cell survival and cell proliferation were strongly affected, no indications of early involvement of cytotoxicity or autophagy were seen, thus clearly suggesting apoptosis as the primary effect. Activation of mitochondrial apoptosis pathways by proapoptotic Bcl-2 proteins has been frequently reported,^{26,27} and also Bcl-x_S induced characteristic steps as decrease of mitochondrial membrane potential and release of cytochrome *c*, AIF and SMAC, thus characterizing Bcl-x_S as acting in parallel with other proapoptotic Bcl-2 proteins.

In mouse embryonic fibroblasts as well as in prostate carcinoma cells, a dependency of Bcl-x_S-induced apoptosis on the multidomain protein Bak has been described,^{15,16} which was approved here by Bak knockdown in colon carcinoma and in melanoma cells as well as by the use of Bax/Bak activation-specific antibodies. In contrast, many BH3-only proteins mediate apoptosis preferentially via Bax as Bik/Nbk and truncated Bid, or they activate both pathways as Bim.^{6,28} The variable dependency might depend on the interaction of these proteins with the different antiapoptotic Bcl-2 family members. Thus, Bcl-2 preferentially inhibits Bax, Mcl-1 inhibits Bak, and Bcl-x_L may bind and inhibit both proteins.²⁹ Thus, overexpression of Bcl-2 abrogated Bax-induced apoptosis in mouse embryonic fibroblasts³⁰ and in melanoma cells.¹³ In principle, all three antiapoptotic family members tested here (Bcl-2, Bcl-x_L and Mcl-1) revealed a suppressive function, as shown by overexpression and siRNA approaches. Nevertheless, complete abrogation of Bcl-x_S-induced apoptosis by Bcl-x_L and only partial inhibition by Bcl-2 were in agreement with apoptosis induction preferentially via Bak.

The dependency of Bcl-x_S-induced apoptosis on Bak but not Bax required a particular explanation, for which direct activation might be considered. Thus, direct interaction and activation of Bax has been reported for some BH3-only proteins as for Bim and Bid.^{6,30} A direct interaction of Bcl-x_S with Bak was, however, not seen in melanoma cells, as shown by reciprocal immunoprecipitation assays, which suggested an indirect effect. This could be based on interaction of Bcl-x_S with antiapoptotic Bcl-2 family members that can control Bak activity. Despite the particular role of Mcl-1, no direct interaction was seen with Bcl-

x_S. An interaction was, however, seen with Bcl-x_L, as also previously reported,³¹ but Bcl-x_L was only weakly expressed in most melanoma cell lines²⁴ and may prevent Bak-induced apoptosis only under conditions of its overexpression.

Other proteins that may contribute to the regulation of Bcl-2 proteins enclose VDAC1 and VDAC2. These two proteins serve opposite functions in apoptosis regulation. Thus, VDAC1 may drive mitochondrial channel formation,³² which is inhibited by the binding through Bcl-x_L.^{19,33} In contrast, VDAC2 exerts antiapoptotic functions by binding to the dimerization pocket of Bak, keeping Bak in its monomeric, inactive conformation.²⁶ Thus, higher susceptibility to death stimuli has been reported in VDAC2-deficient cells.^{18,34}

An interaction between VDAC2 and Bak was also seen here in melanoma cells. Of particular note, we demonstrate the specific interaction of Bcl-x_S with VDAC2 in melanoma cells, as proven by the use of different antibodies and the overexpression of a Myc-tagged VDAC2. Illuminating the mechanism of Bcl-x_S-induced apoptosis, reciprocal immunoprecipitation assays demonstrated that this interaction resulted in a release of Bak protein. A disruption of the Bak–VDAC2 complex has also been described for BH3-only proteins in mouse embryonic fibroblasts,^{18,26} however, BH3-only proteins also activate the Bax pathway, complicating the discrimination between both pathways. This pathway of Bak activation through its release from a complex with VDAC2 appears as a basic mechanism in apoptosis regulation. Its potency was demonstrated here by VDAC2 overexpression, which almost completely prevented Bcl-x_S-induced apoptosis. On the other hand, VDAC2 knockdown enhanced apoptosis by itself and the additional proapoptotic effects of Bcl-x_S were reduced. The selective inhibition of the Bak pathway by VDAC2 was obvious, as its overexpression remained without effect on Bik/Nbk-induced apoptosis.

Loss of Bax is a frequent event in cancer, whereas Bak expression usually persists.³⁵ Particularly for melanoma, Bcl-2 overexpression is highly characteristic, which may completely abrogate Bax-induced apoptosis.^{12,13} Thus, an efficient targeting of Bak could provide a suitable strategy for overcoming apoptosis deficiency of therapy-refractory tumors as melanoma. Bcl-x_S is of particular value for understanding the contribution and efficiency of the Bak-mediated pathway, as demonstrated here in melanoma cells.

In conclusion, the pathway of Bcl-x_S-induced apoptosis is unraveled, which is based on its interaction with VDAC2 to release Bak. The improved understanding of the role of VDAC2 in the regulation of Bcl-2 proteins may provide the basis for development of targeted therapies, which may act in addition to Bax-mediated strategies. Of particular importance will become the detailed characterization of the domain(s) in Bcl-x_S that bind to VDAC2. These are not necessarily identical to the BH3, which particularly mediates the interaction between Bcl-2 family members. The identification of a new proapoptotic domain may be finally used for the development of small molecules, in analogy to BH3 mimetics.

Materials and Methods

Cell culture and transfection. Three representative human melanoma cell lines were investigated: Mel-HO, Mel-2a and A-375.³⁶ Subclones of A-375 resulted from stable transfection of a pRES-Bcl-2 plasmid (A-375-Bcl-2) or an empty pRES

plasmid (A-375-Mock).¹³ The pIRES plasmid originated from Clontech (Palo Alto, CA, USA). SK-Mel-13 melanoma cells had been stably transfected with a pTet-responsive element (TRE)-Bcl-x_L plasmid (SKM13-Bcl-x_L), as previously described.²⁴ The pTRE-1 plasmid originated from Clontech (Heidelberg, Germany). For determination of the

Bax/Bak dependency, a cell culture model in HCT116 colon carcinoma cells was kindly provided by B. Vogelstein (John Hopkins Cancer Center, Baltimore, MD, USA). This is based on HCT116 parental cells (ATCC, Rockville, MD, USA), which express both Bax and Bak. Isogenic sublines with either Bax knockout or Bak knockdown as

well as Bax^{-/-}/Bak^{-/-}-double-knockdown cells had been generated.³⁷ Cell lines were cultured at 37 °C, 5% CO₂ in DMEM (Gibco, Karlsruhe, Germany), supplemented with 10% FCS and antibiotics.

For transient transfection, melanoma cells were seeded in six-well plates with 2×10^5 cells/well. At a confluence of 50%, cells were washed with Opti-MEM medium (Life Technologies, Carlsbad, CA, USA), followed by incubation with plasmid DNA (5 µg/ml) and 0.1% DMRIE-C (Life Technologies) in Opti-MEM (37 °C,

4 h). A detailed protocol for transient transfection had been given previously.³⁶ The following plasmids on the basis of pcDNA3 were used for expression of human full-length cDNA sequences attached to a 3' Myc-tag (pcDNA3-Bax-Myc, -VDAC2-Myc, -Nbik-Myc and -Bcl-x_s-Myc, Bcl-x_l-Myc). Another plasmid on basis of pCMV-Sport6 for expression of human, full-length VDAC2 cDNA without Myc-tag (pCMV-Sport6-VDAC2) was purchased from BioScience (Berlin, Germany; clone IRATp970E111D).

Figure 4 Activation of Bak by Bcl-x_s. (a and b) Activation of Bax and Bak was determined by flow cytometry after staining with conformation-specific antibodies (Bax-NT, Bak-NT) in Mel-2a at 24 h and 48 h after transduction of 50 MOI of AdV-XS (a) and of AdV-Nbk (b). Bars in the upper panels indicate the populations with activated Bax and Bak, respectively. (c) Expression of Bak and Bcl-x_s is shown in A-375 cells at 64 h after transfection of anti-Bak siRNA or scramble siRNA (Scr) and at 48 h after transduction with AdV-XS and culturing under on or off conditions. (d) Apoptosis induction was determined by quantification of sub-G1 cells in A-375 after AdV-XS transduction and anti-Bak siRNA treatment. Bar charts, mean values ± S.D.s of at least six individual values are shown, and statistical significance is indicated by asterisks

Figure 3 Dependency on Bak and inhibition by antiapoptotic Bcl-2 proteins. (a) Expression of Bax and Bak is shown in HCT116 parental cells and in subclones derived by Bak knockdown (Bax^{-/-}), Bak knockout (–/–/Bak) or double knockdown (–/–/–). Loading control: β-actin. (b) HCT116 cells were transduced with AdV-XS, cultured under off or on conditions, and percentages of apoptotic cell populations (sub-G1) were determined after 24 h. (c and d) Expression levels of Bcl-2, Bcl-x_l and Bcl-x_s were determined by western blotting in A-375-Mock and A-375-Bcl-2 (c) and in A-375 parental cells transiently transfected with Bcl-x_l (d). Proteins were isolated at 64 h after transfection and at 48 h after transduction with AdV-XS (50 MOI) and culturing under off or on conditions. Equal protein loading was confirmed by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (e) Percentages of apoptotic cell populations (sub-G1) were determined at 48 h of transduction with AdV-XS in subclones of A-375 stably transfected with Bcl-2 (A-375-Bcl-2) or mock-transfected (A-375-Mock). (f) Percentages of apoptotic cell populations (sub-G1) were determined in A-375 after transient transfection with a Bcl-x_l expression plasmid at 48 h after transduction with AdV-XS and growth under on and off conditions. (g) Expression of Mcl-1 and Bcl-x_s is shown in A-375-Mock cells at 64 h after siRNA transfection against Mcl-1 and at 48 h after transduction with AdV-XS as well as culturing under on or off conditions. Scramble siRNA-transfected cells as well as non-transduced and non-transfected cells are shown as controls (C). A parallel experiment with A-375-Bcl-2 showed a highly similar result (data not shown). (h) Representative histograms displaying sub-G1 cell populations (apoptotic cells) at 48 h after transduction are shown. (i) Quantification of apoptosis in A-375-Bcl-2 and A-375-Mock cells after siRNA transfection (Mcl-1 or scramble) at 48 h after transduction with AdV-XS and growth under on and off conditions. Non-transduced and non-transfected cells served as controls (C). Experiments were performed at least two times, which showed highly comparable results. Bar charts, mean values ± S.D.s of at least six individual values are shown. Statistical significance is indicated by asterisks

Figure 5 Immunoprecipitation analyses of Myc-tagged Bcl-2 proteins. (a) A-375 cells were transiently transfected with Myc-tagged Bcl-x_L, Bcl-x_S or Bax. Immunoprecipitates with anti-Myc antibody were analyzed by western blotting for Bcl-2 proteins. Mock controls were transfected with pcDNA3. Myc-tagged proteins with a higher molecular weight (M) are distinguished from endogenously expressed proteins (E). Supernatants not bound to the columns (S) were compared with the immunoprecipitated pellet fractions (P). (b) Binding to Bax and Bak was also analyzed, when cells were lysed without triton-X100 in CHAPS-containing cell lysis buffer. (c) Another experiment was performed with SK-Mel-13 cells stably transfected for Bcl-x_L overexpression (SKM13-Bcl-x_L). For further control, cells were here also transfected with Myc-Nbk. Each two independent experiments (a-c) gave highly comparable results

A siRNA approach against Mcl-1 was developed with the new sequence 5'-GCA AGA GGA UUA UGG CUA A-3'; the scramble siRNA had the sequence 5'-GCA GGA GCU AUG CUA CCA U-3'. These oligonucleotides were purchased from Eurofins MWG Operon (Ebersberg, Germany). Additional siRNA pools against Bak and VDAC2, respectively, were purchased from Santa Cruz (Bak, sc-29786; VDAC2 and sc-42357; Heidelberg, Germany). The annealed, double-stranded and desalted siRNAs were transfected in cells grown in 24-well plates using TurboFect reagent (Fermentas, Leon-Rot, Germany), according to the manufacturer's instructions.

Adenoviral construction. The Bcl-x_S full-length cDNA²⁴ was subcloned into the adenoviral vector pAd5-tTA, according to a strategy described previously.²⁸ In a first step, the cDNA was inserted into the TRE-containing pHVAd2 shuttle vector. The resulting TRE-Bcl-x_S expression cassette was then transferred into pAd5-tTA by homologous recombination, thereby replacing the E1 region and creating a new adenovirus, which was termed pAdV-XS according to other Bcl-2 protein adenoviruses reported previously (Figure 1a). The

Figure 6 Bcl-x_S disrupts the VDAC2-Bak interaction. (a) Immunoprecipitates with anti-Myc antibody were generated from A-375 cells transfected with Myc-tagged copies of Bcl-x_L, Bcl-x_S or Bax. Controls were mock-transfected (pcDNA3). Coimmunoprecipitation of any of the three VDAC proteins with Bcl-x_L or Bcl-x_S was proven by western blotting with a non-specific antibody (VDAC 1/2/3), and specific binding of VDAC2 was proven by a selective VDAC2 antibody. Cell lysis was performed with either triton X-100 or CHAPS-containing lysis buffer, as indicated. Interactions are shown by protein bands in immunoprecipitated pellet (P) fractions. (b) Mel-2a cells transduced with AdV-XS were cultured for 24 h under off or on conditions. Lysates were immunoprecipitated with anti-VDAC2 antibody, and immune complexes were analyzed by western blotting for Bcl-x_S and for endogenous VDAC2, Bax, Bcl-2 and Bak. Supernatant (S) and immunoprecipitated pellet (P) fractions were compared. (c) A reciprocal experiment is shown based on immunoprecipitation with anti-Bak antibody. (d) A coimmunoprecipitation analysis was performed after transfection of a Myc-tagged VDAC2 cDNA (plasmid pcDNA3-VDAC2-Myc). Mock-transfected cells received pcDNA3 empty plasmid. Selective VDAC2 immunoprecipitates were performed with an anti-Myc antibody. (e) VDAC2 immunoprecipitates were generated of A-375 cells after siRNA-mediated Bak knockdown or scramble siRNA treatment (Scr), and binding of Bcl-x_S was determined (a-e). For all immunoprecipitations, at least two independent experiments were performed, which always revealed the same result

transactivator tTA enables a Tet-off control of the gene downstream of TRE. The viral DNA was transfected in HEK293 cells, and adenoviral plaques corresponding to AdV-XS were propagated. Expression of Bcl-x_S after AdV-XS transduction was suppressed by addition of 1 µg/ml doxycycline to the culture

Figure 7 Model for Bcl-x_S-induced apoptosis. **(a)** Percentages of apoptotic cell populations (sub-G1) were determined in A-375 after transient transfection with a VDAC2 expression plasmid (pCMV-Sport6-VDAC2) and after transduction with AdV-XS (MOI = 50) and growth under on and off conditions for 24 h and at 48 h. Transduction started 16 h after transient transfection. Overexpression of VDAC2 and Bcl-x_S as determined by western blotting at 48 h after transduction is shown in the right panel. **(b)** Apoptosis induction (sub-G1 cells) is shown in A-375 after siRNA-mediated VDAC2 knockdown as well as at 24 and 48 h after Bcl-x_S induction. Transfection with siRNA was always 16 h before transduction. Expression of VDAC2 and Bcl-x_S is shown at 48 h after transduction in the right panel. Scramble siRNA-transfected cells (Scr) are shown as controls. Mean values ± S.D.s of at least six individual values are shown, and statistical significance ($P < 0.05$) is indicated by asterisks. **(c)** A proposed model, according to which Bcl-x_S activates Bak through neutralization of the antiapoptotic VDAC2 protein. Bcl-x_S-induced apoptosis is negatively controlled by Bcl-x_L and Mcl-1, whereas Bcl-2 controls Bax

medium (off condition), whereas omission of doxycycline resulted in promoter induction (on condition). A similarly constructed adenovirus for expression of Myc-tagged Bik/Nbk (Ad5-Myc-Nbk-tTA = AdV-Nbk) had been described previously²⁸ and was used here as control. A luciferase-encoding adenovirus (Ad5-CMV-Luc) served as mock control for adenovirus transduction and was applied at the same MOI.³⁸

Quantification of apoptosis, cytotoxicity and cell viability. For determination of percentages of apoptotic cells, cell cycle analyses were carried out according to Riccardi and Nicoletti,³⁹ and the fractions of cells with hypodiploid nuclei were determined. Cells were seeded in 24-well plates (50 000 cells/well), treated as indicated and harvested by trypsinisation. After washing with cold PBS, cells were incubated for 1 h in staining solution containing 40 µg/ml propidium iodide (PI, Sigma-Aldrich, Taufkirchen, Germany), 0.1% sodium citrate and 0.1% triton X-100. Nuclear DNA content was determined by flow cytometry (FACSCalibur and CellQuest software; Becton Dickinson, Heidelberg, Germany).

Alternatively, apoptosis was quantified by a DNA fragmentation ELISA (Cell death detection ELISA, Roche Diagnostics, Mannheim, Germany), which detects mono and oligonucleosomes formed in apoptotic cells. Cytotoxicity was determined in parallel by a cytotoxicity detection assay (Roche Diagnostics), which measures LDH activity in culture fluids. As positive controls for induced cytotoxicity, cells were completely lysed by triton X-100 or were treated with doxorubicin (500 nM, 72 h). These assays were performed according to protocols of the manufacturer with minor modifications.³⁶ The exposure of phosphatidyl serin on the cell surface as an early indication of apoptosis was detected by staining with annexinV-FITC (BD Pharmingen Biosciences, Heidelberg, Germany; #556419) and using PI counterstaining, as described previously.³⁷

For determination of cell proliferation and cell numbers, the WST assay (Roche Diagnostics) was used, and cell viability at the single-cell level was monitored by the life-cell labeling dye calcein-AM. Briefly, 10⁵ cells were incubated with calcein (4 µM; eBioscience, Frankfurt, Germany) in serum-free growth medium (60 min, 37 °C). After PBS washing, cell viability was determined by flow cytometry, comparing calcein-stained (viable) and non-stained (dead) cells. For determination of a decrease in mitochondrial membrane potential ($\Delta\psi/m$), the fluorescent dye

TMRM+ (Sigma-Aldrich) was used. Cells were harvested by trypsinisation and stained for 15 min at 37 °C with TMRM+ (1 µM), and changes of $\Delta\psi/m$ were determined by flow cytometry.

Western blot analysis. Detailed protocols for protein extraction and western blotting had been described previously.³⁶ As a standard, 10⁶ cells were harvested and lysed in a buffer containing 150 mM NaCl, 1 mM EDTA, 0.5% SDS, 0.5% Nonidet P-40, 2 mM PMSF, 1 µM leupeptin, 1 µM pepstatin, 10 mM Tris-HCl and pH 7.5. For analysis of cytochrome c and mitochondrial localization of Bcl-2 proteins, cytosolic and mitochondrial cell fractions were separated by a mitochondria/cytosol fractionation kit (Alexis, Grünberg, Germany). The following primary antibodies were used: procaspase-3 (Cell Signaling, Danvers, MA, USA; rabbit; 1:1000), cleaved Csp-3 (Cell Signaling; rabbit; 1:1000), Csp-8 (Cell Signaling; mouse; 1:1000), Csp-9 (Cell Signaling; rabbit; 1:1000), Bcl-x (Santa Cruz; mouse; 1:200), Bcl-2 (Santa Cruz; mouse; 1:200), c-Myc (Calbiochem, Nottingham, UK; rabbit; 1:500), Mcl-1 (Santa Cruz; rabbit; 1:200), Bax (Santa Cruz; rabbit; 1:200), Bak for western blotting (Assay Biotechnology Company, Sunnyvale, CA, USA; rabbit; 1:500; Bak for immunoprecipitation (Santa Cruz; rabbit; 1:500), Bad (Cell Signaling; rabbit; 1:1000), Puma (Epitomics, Burlingame, CA, USA; rabbit; 1:1000), LC3 (Novus Biologicals, Cambridge, UK; rabbit; 1:500), Noxa (ProSci Inc., Poway, CA, USA; rabbit; 1:500), cytochrome c (BD Biosciences, Heidelberg, Germany; mouse; 1:1000), VDAC1/2/3 (anti-porin 31 HL; Calbiochem; mouse; 1:500), VDAC2 (Santa Cruz; goat; 1:500), SMAC (Santa Cruz; mouse; 1:500), AIF (Santa Cruz; goat; 1:200), glyceraldehyde 3-phosphate dehydrogenase (Santa Cruz; mouse; 1:1000), β-actin (Sigma-Aldrich; mouse; 1:5000). As secondary antibodies, peroxidase-labeled goat anti-rabbit, goat anti-mouse and donkey anti-goat were used (Dako, Hamburg, Germany; 1:5000).

Assays for Bax/Bak activation. For analysis of Bax/Bak conformational changes related to their activation, primary antibodies specific for Bax/Bak N-terminal domains were applied in flow cytometry (Bax-NT, Upstate Biotechnology, New York, NY, USA, #06-499; Bak-NT, Merck, Darmstadt, Germany, #AM04). Melanoma cells (10⁵) were harvested by trypsinisation and fixed for 30 min with 4% paraformaldehyde in PBS. Cells were suspended in saponin buffer (1% FCS, 0.1% saponin in PBS) and incubated for 1 h at 4 °C in the dark with

antibodies Bax-NT (1 : 100) or Bak-NT (1 : 10). As secondary antibodies, goat anti-rabbit IgG (H + L)-FITC (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and goat anti-mouse IgG (H + L)-FITC (SouthernBiotech, Birmingham, AL, USA) were used. After washing and resuspension, cells were immediately measured by flow cytometry.

Immunoprecipitation. For immunoprecipitation analysis based on Myc-tagged proteins, melanoma cells (10⁶) were transiently transfected with the expression plasmids encoding for Myc-tagged Bcl-2 proteins. At 24 h after transfection of pcDNA3-Bax-Myc, -Nbk-Myc, -VDAC2-Myc, -Bcl-x_s-Myc, or at 48 h after pcDNA3-Bcl-x_s-Myc transfection, cells were washed with cold PBS and were resuspended in 1 ml of lysis buffer (Standard protocol, 150 mM NaCl, 50 mM Tris-HCl, pH 8 and 1% triton X-100). Selected experiments (Figures 6b and 5a) were performed by substitution of triton X-100 by 1% CHAPS. Immunoprecipitation of Myc-tagged proteins was carried out with the μ MACS c-Myc-tagged protein isolation kit following the protocol of the supplier (Miltenyi Biotec, Bergisch-Gladbach, Germany). Therefore, microbeads covered with anti-Myc antibodies were given to the lysate for binding the Myc-tagged proteins. Beads and bound proteins were captured on flow through magnetic columns, washed 4X with buffer 1 (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl and pH 8) and 1X with buffer 2 (20 mM Tris-HCl, pH 7.5). Proteins were then eluted with hot (95 °C) elution buffer (50 mM DTT, 1% SDS, 1 mM EDTA, 0.005% bromophenol blue, 10% glycerol, 50 mM Tris-HCl and pH 6.8).

For immunoprecipitation of VDAC2 and of Bak, the μ MACS protein A/G micro bead kit (Miltenyi Biotec) was used. Therefore, 2–4 μ g of the specific antibodies (VDAC2: Santa Cruz, goat, polyclonal; Bak: Santa Cruz; rabbit, polyclonal) were given to the lysates together with G protein-covered microbeads. Capturing of microbeads, washing and elution were as described above. Immunoprecipitates were investigated by western blotting.

Statistics. Experiments usually consisted of at least three values, and were performed at least two to three times. Means and S.Ds shown in bar charts of the figures were determined by enclosing all individual values (at least 6), and statistical significance was determined by Student's *t*-test and is indicated in the figures by asterisks (*P* < 0.05).

Conflict of Interest

The authors declare no conflict of interest.

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