

Research article

Negative regulation of mitochondrial VDAC channels by C-Raf kinase

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Abstract

Background: Growth of cancer cells results from the disturbance of positive and negative growth control mechanisms and the prolonged survival of these genetically altered cells due to the failure of cellular suicide programs. Genetic and biochemical approaches have identified Raf family serine/threonine kinases B-Raf and C-Raf as major mediators of cell survival. C-Raf cooperates with Bcl-2/Bcl-X_L in suppression of apoptosis by a mechanism that involves targeting of C-Raf to the outer mitochondrial membrane and inactivation of the pro-apoptotic protein Bad. However, apoptosis suppression by C-Raf also occurs in cells lacking expression of Bad or Bcl-2.

Results: Here we show that even in the absence of Bcl-2/Bcl-X_L, mitochondria-targeted C-Raf inhibits cytochrome c release and caspase activation induced by growth factor withdrawal. To clarify the mechanism of Bcl-2 independent survival control by C-Raf at the mitochondria a search for novel mitochondrial targets was undertaken that identified voltage-dependent anion channel (VDAC), a mitochondrial protein (porin) involved in exchange of metabolites for oxidative phosphorylation. C-Raf forms a complex with VDAC *in vivo* and blocks reconstitution of VDAC channels in planar bilayer membranes *in vitro*.

Conclusion: We propose that this interaction may be responsible for the Raf-induced inhibition of cytochrome c release from mitochondria in growth factor starved cells. Moreover, C-Raf kinase-induced VDAC inhibition may regulate the metabolic function of mitochondria and mediate the switch to aerobic glycolysis that is common to cancer cells.

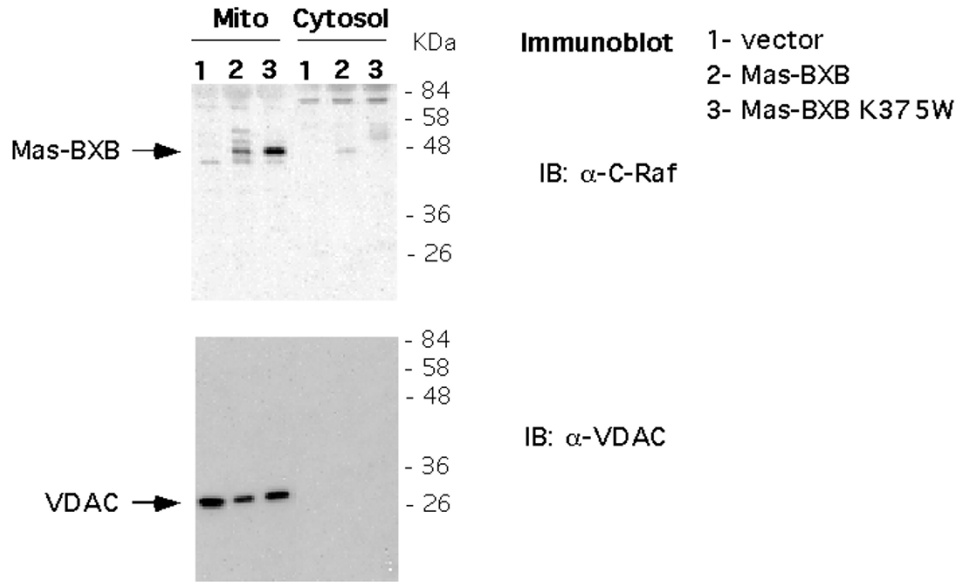
Background

Anti-apoptotic Bcl-2 family members are overexpressed in a large number of cancers and are responsible for resistance to anticancer drugs [1]. Oncogenic C-Raf contributes to the processes of cellular transformation and tumor development through its ability to suppress apoptotic cell death. Experimental evidence has been obtained, showing

that C-Raf maintains cell survival by (i) induction of auto-crine loops via the mitogenic cascade resulting in the activation of protein kinase B (PKB) [5,6], and (ii) cooperation with Bcl-2, which targets C-Raf to mitochondria and results in the inactivation of the pro-apoptotic protein Bad [3,7,8]. Additionally, Raf may suppress apoptosis by activation of NF-κB [9,10], and interaction with

Fig 1

A



B

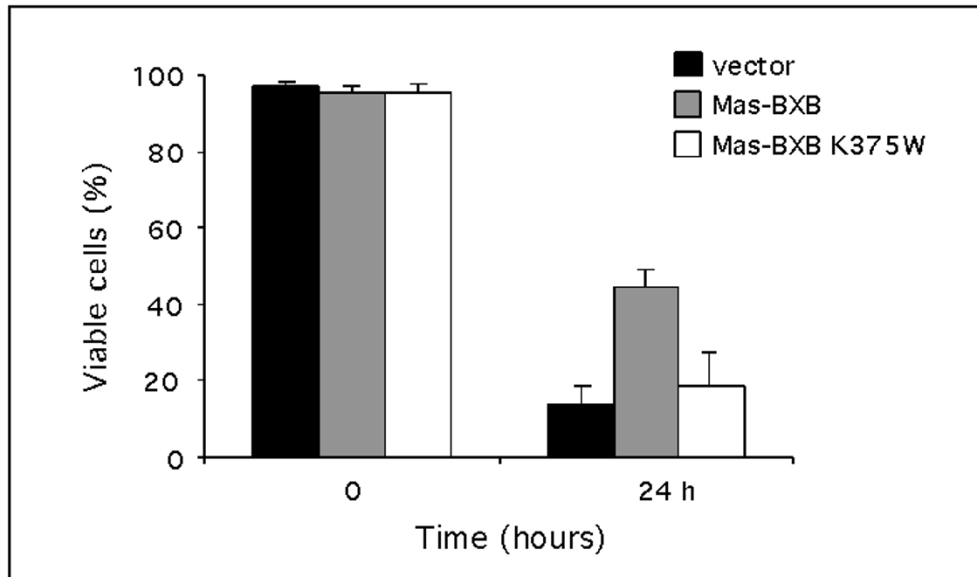


Figure 1

C-Raf modulates the mitochondrial membrane potential during apoptosis. A. Overexpression of C-Raf kinase in 32Dcl.3 cell lines. 32Dcl.3 cells expressing Mas-BXB fusion protein were maintained in IL3-containing medium. Expression of constitutively active Mas-BXB and inactive Mas-BXB K375W proteins was detected in mitochondrial but not cytosolic fractions using an anti-C-Raf antibody. Cell viability of 32Dcl.3 cell lines. 32Dcl.3 transfected cells were cultured without IL-3 for 24 hours and % viable cells was determined by trypan dye-exclusion.

IAP proteins (Sendtner, Troppmair and Rapp, unpublished results). The anti-apoptotic Bcl-2 family members expressed in the outer mitochondrial membrane inhibit the release of apoptogenic cytochrome c from mitochondria [11,12], antagonize the pore-forming activity of Bax [13], and directly interact with the mitochondrial permeability transition pore (PTP) that regulates mitochondrial membrane potential [14,15]. C-Raf induces phosphorylation of Bad, which in turn leads to sequestration of Bad in a complex with 14-3-3 in the cytosol [3,16]. However, C-Raf also suppresses apoptosis in cells that do not express Bad or are deficient for Bcl-2 [8] suggesting the existence of other targets for C-Raf at the outer mitochondrial membrane. Here we provide evidence that C-Raf regulates the mitochondrial voltage-dependent anion channel (VDAC) that can contribute to cell survival.

Results and Discussion

First we examined the effect of C-Raf on mitochondrial cytochrome c release. For this we used the expression plasmid pcDNA3-Mas-BXB, in which constitutively active C-Raf, BXB, was fused with the transmembrane domain of the yeast outer mitochondrial membrane protein Mas [17], allowing targeting of C-Raf to mitochondria independently of Bcl-2 [3]. BXB, the mutant of Raf used, lacks the N-terminal domain of C-Raf (residues 26–303) to prevent interaction with the small G protein Ras at the plasma membrane [18,19]. Upon transfection of interleukin 3 (IL-3) dependent 32Dcl.3 promyeloid cells, Mas-BXB was detected by western blotting almost exclusively in the mitochondrial fraction (Figure 1A). When deprived of IL-3 for 24 hours 32Dcl.3-Mas-BXB cells showed delayed cell death (Figure 1B) compared to 32Dcl.3 cells expressing the empty vector or a kinase dead version of mitochondrial Raf, Mas-BXB K375W, as previously shown [3]. IL-3 deprivation induced mitochondrial membrane depolarization observed in 32Dcl.3-vector and 32Dcl.3-Mas-BXB K375W cells is attenuated in 32Dcl.3-Mas-BXB cells and almost completely suppressed in 32Dcl.3 overexpressing Bcl-2 (Figure 2). These effects correlated with a partial blockage of cytochrome c release from mitochondria (Figure 3) and inhibition of pro-caspases 3 and 9 cleavage in 32Dcl.3-Mas-BXB, but not in 32Dcl.3-Mas-BXB K375W or vector transfected cells (Figure 3). Once in the cytoplasm cytochrome c binds with ATP to Apaf-1, which recruits and activates pro-caspase 9 [20]. Caspase 9 subsequently induces caspase 3 activation, which triggers the execution of apoptotic cell death. Taken together these data suggest that C-Raf inhibits apoptosis through mitochondrial membrane potential ($\Delta\Psi_m$) stabilization and inhibition of cytochrome c release.

VDAC, which is the most abundant protein of the outer mitochondrial membrane, provides a major channel for the movement of ions, ADP/ATP and metabolites in and

out of mitochondria [21]. It is a core component of the permeability transition pore (PTP) [21,22]. Opening of the PTP causes $\Delta\Psi_m$ disruption [22–24] and release of cytochrome c, most likely through VDAC/Bax channels [15,25,26]. Bcl-2 induces closure of these channels, thereby inhibiting the release of cytochrome c and $\Delta\Psi_m$ loss. To examine a potential interaction of C-Raf with cytochrome c releasing channels we first examined binding of C-Raf to Bax. Binding studies and the analysis of Bax phosphorylation in cotransfected 293 cells gave negative results (data not shown). The situation was different when VDAC was used instead of Bax. For this, constitutively active C-Raf (C-RafDD) and hemagglutinin-tagged VDAC (HA-VDAC) were transiently transfected into 293 cells and immunoprecipitation was performed 48 hours later using anti-C-Raf or anti-HA-antibody. C-Raf and VDAC coimmunoprecipitated from doubly transfected cells (Figure 4). Coimmunoprecipitation was also detected with cells coexpressing kinase inactive form of C-Raf together with HA-VDAC (data not shown), demonstrating that C-Raf kinase activity is not required for the interaction with VDAC. To narrow down the region of C-Raf binding to VDAC, HA-VDAC and the C-terminal half of C-Raf, BXB, were coexpressed in 293 cells. BXB and HA-VDAC were present in pull down experiments with either HA or C-Raf antibody, suggesting that the C-terminal part of C-Raf is sufficient for binding of VDAC (Figure 4). No interaction was detected between VDAC and the regulatory N-terminal part of C-Raf (data not shown).

After identifying VDAC as a new binding partner of C-Raf, we investigated, whether VDAC was phosphorylated by C-Raf. HA-VDAC immunoprecipitated from 293 cells was not phosphorylated in an *in vitro* kinase assay (Figure 5A), neither by recombinant full length active C-Raf (GST-C-RafDD) protein nor by the active recombinant C-terminal domain of C-Raf containing the catalytic region (BXB-DD), whereas both active kinases, but not inactive (GST-C-RafK375W) C-Raf, were able to phosphorylate MEK. To exclude indirect phosphorylation of VDAC by C-Raf *in vivo*, active C-Raf (C-RafDD or BXB) or the mitochondrial membrane targeted constitutively active Mas-BXB were cotransfected with VDAC into 293 cells. No phosphorylation of HA-VDAC was observed (Figure 5B), whereas Mas-BXB induced phosphorylation of HA-Bad as previously described [3]. We conclude that C-Raf binding does not induce phosphorylation of this mitochondrial protein.

To test for functional consequences of this interaction we examined the effect of C-Raf on VDAC channel activity. When reconstituted in a planar lipid bilayer membrane purified rat liver VDAC protein forms voltage-dependent and large conductance ion channels (1 nS in 0.3 M KCl) (Figure 6A). Rat liver VDAC protein was pre-incubated with recombinant GST-C-Raf or GST proteins before ana-

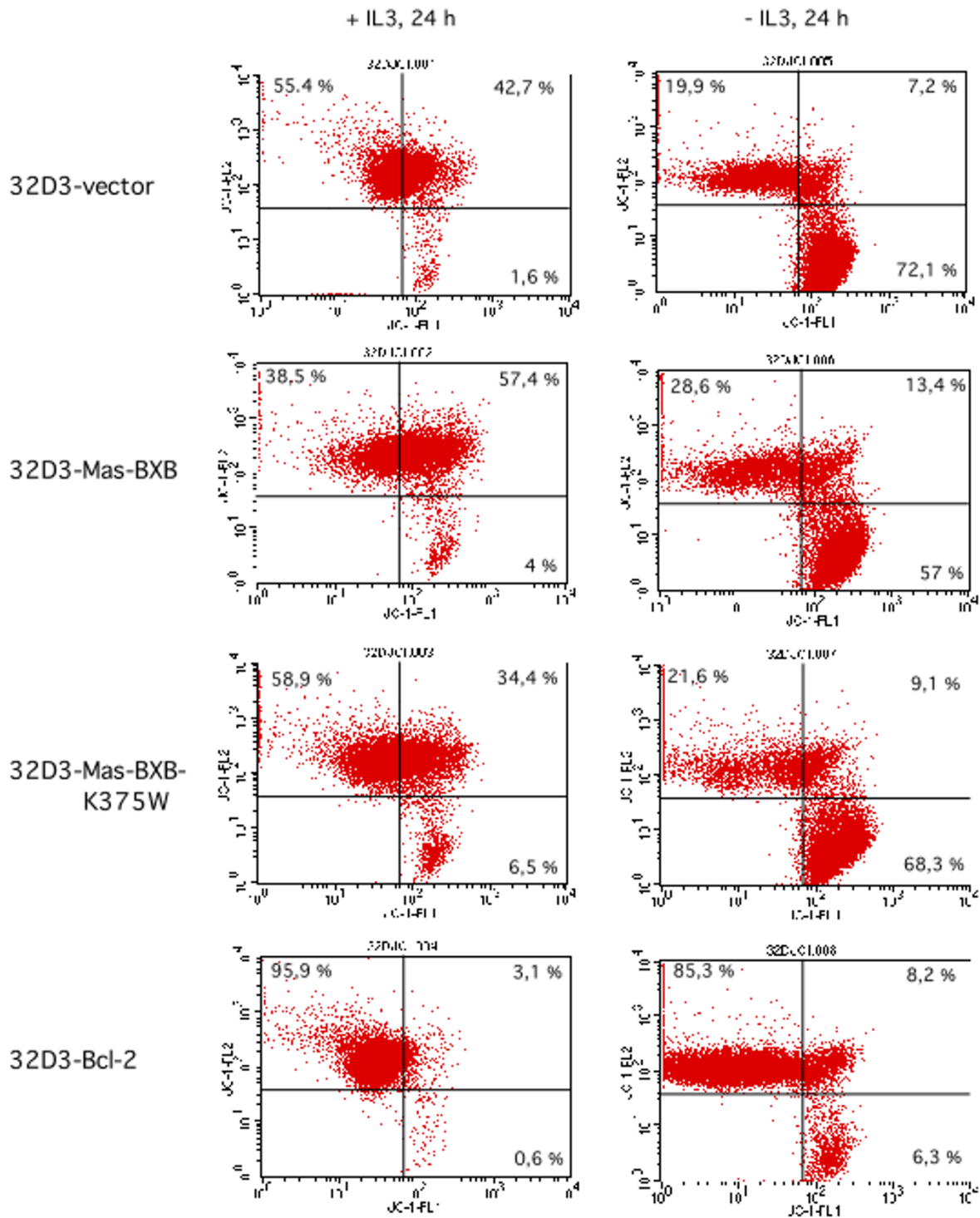


Figure 2
C-Raf delays the mitochondrial membrane depolarization occurring during apoptosis. 32Dcl.3 transfected cells were cultured with or without IL-3 for 24 hours, incubated with JC-1 cationic dye and washed with PBS before flow cytometry.

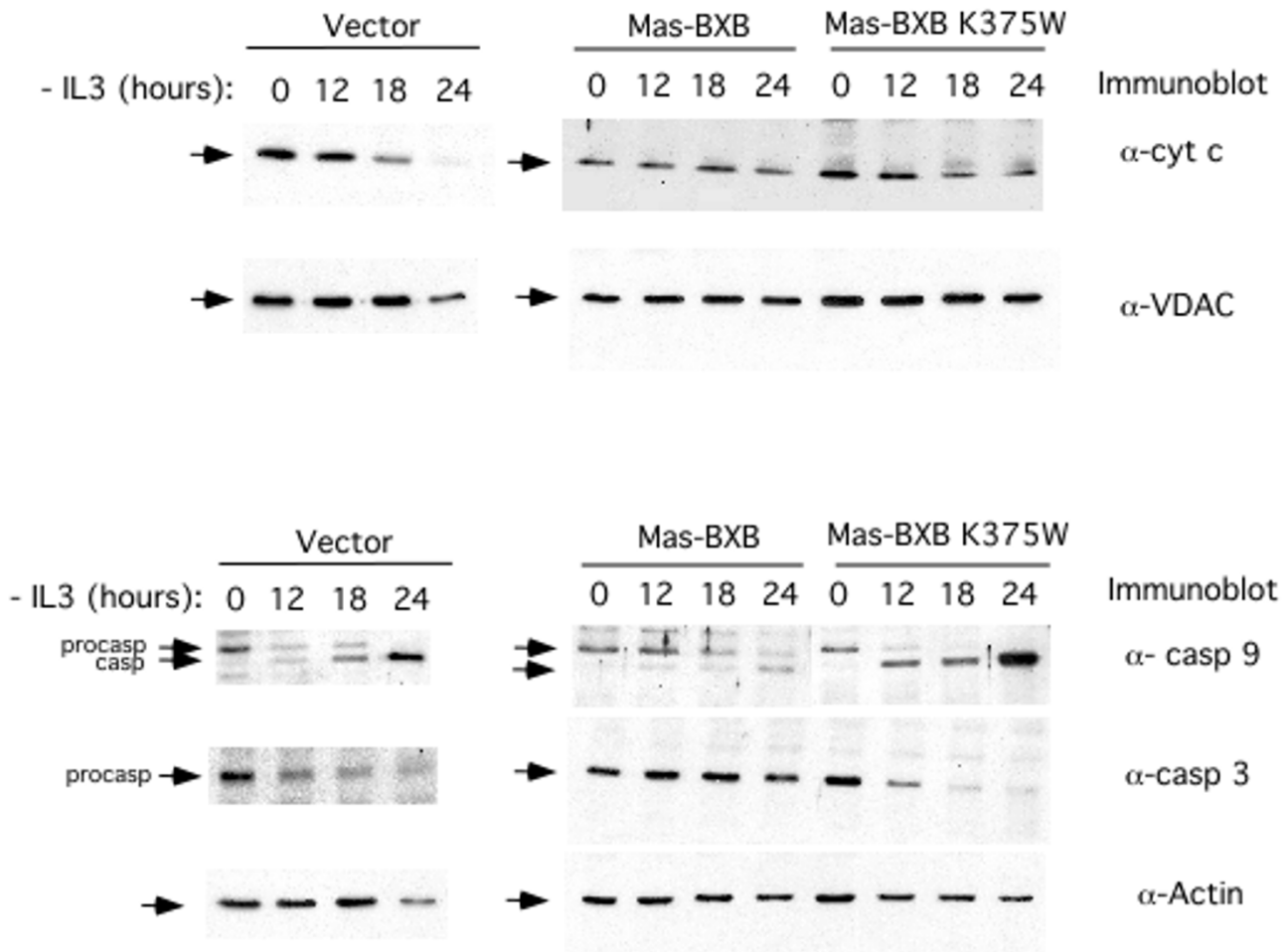


Figure 3

C-Raf inhibits the release of cytochrome c from mitochondria and caspases processing. 32Dcl.3 transfected cells were maintained without IL-3 for different time periods before isolating mitochondria and cytosol. Immunoblot analysis was performed on mitochondrial extracts for the detection of cytochrome c release and on cytosol for the analysis of procaspases 3 and 9 cleavage. Blots were stripped and reprobed with anti-VDAC or anti-actin antibodies as protein loading control.

lysing the VDAC channel activity. Recombinant active C-Raf protein (GST-C-RafDD) abolished the channel activity whereas GST or the inactive form of C-Raf (GST-C-RafK375W) (Figure 6A) or wild type protein (data not shown) had no effect. Inhibition of VDAC channel activity by C-Raf was specific since GST-C-RafDD did not alter the properties of Bcl-2 pores (Figure 6B). Moreover, the anti-apoptotic PAK1 kinase (active His-PAK1-T423E enzyme) and the MAPK pathway activating Src family kinase Lck (His-LckY505F kinase) did not modify the VDAC channel conductance (Figure 7). The interaction between VDAC and C-Raf is not sufficient for inhibition of channel activity since kinase dead C-Raf bound but did not inhibit VDAC. To check if C-Raf closed the VDAC channel or inhibited its reconstitution into the membranes, active or inactive C-Raf was added in lipid bilayer experiments to preformed rat liver VDAC channels. In these experiments

active C-Raf had no effect on already existing VDAC but blocked further reconstitution presumably by opposing membrane insertion (Figure 8). Stimulation of C-Raf kinase activity is paralleled by autophosphorylation, regulatory (S338, Y340/341) as well as feedback phosphorylation (multiple S/T phosphorylation sites) [27]. A negative net charge resulting from autophosphorylation (seen in the *in vitro* kinase assay, Figure 5A) and the presence of negatively charged glutamic acids in the mutant GST-C-RafDD protein (YY340/341-DD) might be the basis for the inhibitory effect of C-Raf on VDAC in contrast to inactive GST-C-Raf, which possesses near neutral charge. The negatively charged form of C-Raf may bind to the N-terminal amphipathic α -helix of VDAC and induce a conformational change of the channel. The C-Raf-VDAC dimer may no longer be able to insert into the membrane in an analogous fashion to the binding of an-

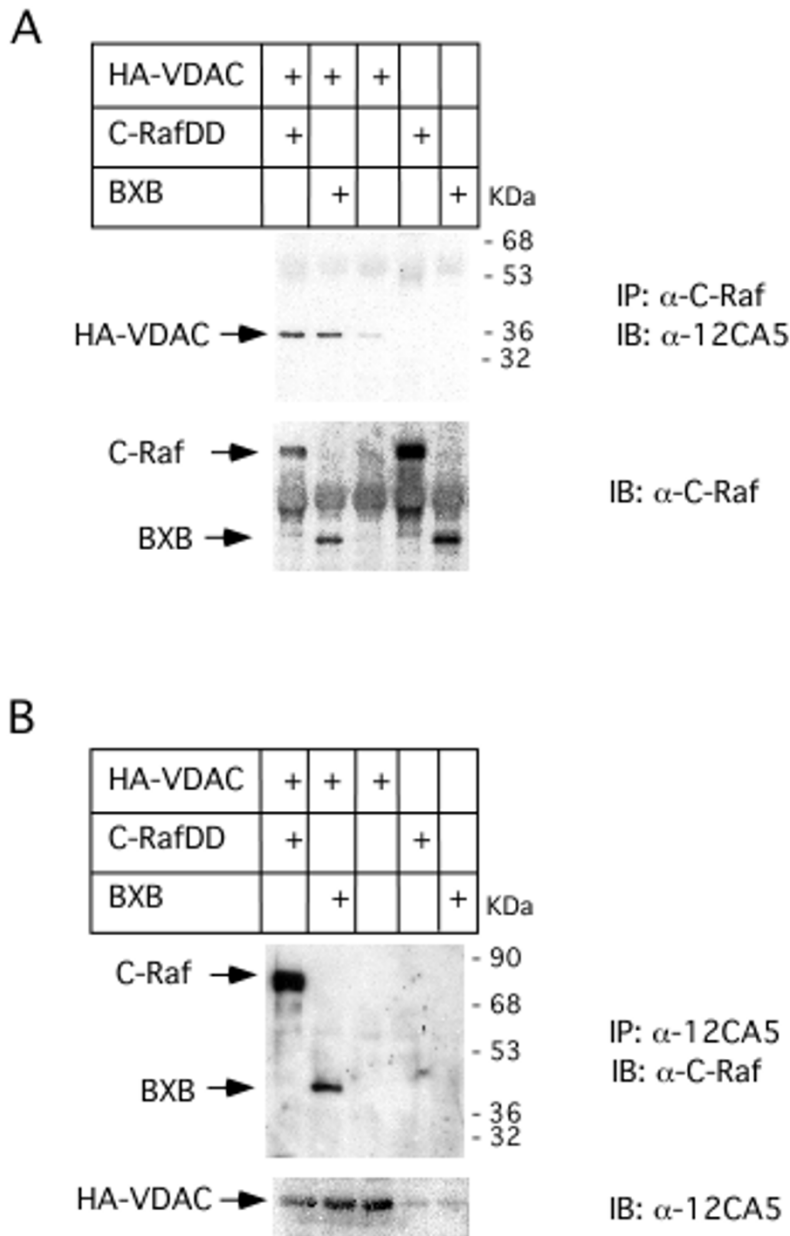


Figure 4

C-Raf interacts with VDAC. C-Raf and HA-VDAC proteins were transiently coexpressed in 293 cells and immunoprecipitated with anti-C-Raf (A) or anti-HA antibodies (B) before SDS-PAGE analysis. Immunoblot were probed with anti-C-Raf or anti-HA antibodies.

antibodies to VDAC [28,29]. Binding of active C-Raf kinase to VDAC may thus block cytochrome c release and apoptosis by decreasing the number of channels in the outer mitochondrial membrane. C-Raf shares with Bcl-2 [30,31] the ability to bind to VDAC and suppress cytochrome c release albeit by different mechanism, pore reduction versus pore closure. C-Raf and Bcl-2 could

cooperate to directly block the VDAC protein inhibiting the VDAC-induced depolarization and preventing functional PTP formation during apoptosis. Moreover, as regulation of respiration and cell survival [32] are coupled we cannot exclude the possibility of additional targets for C-Raf such as VDAC-bound oxidative phosphorylation- or glycolysis-enzymes. Our data, together with those show-

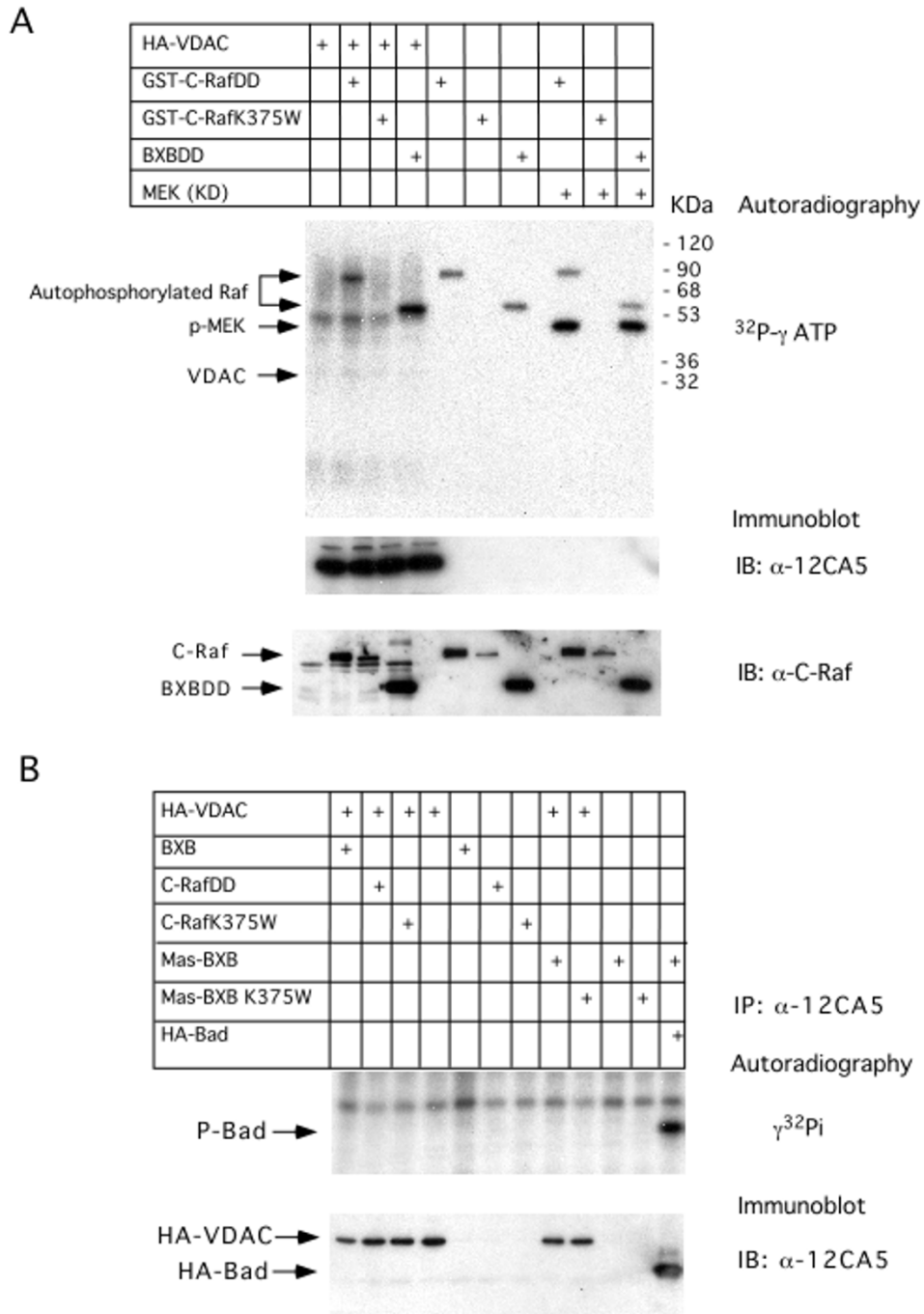


Figure 5
C-Raf kinase does not phosphorylate VDAC protein. **A.** *In vitro* kinase assay was performed using GST-C-Raf recombinant protein and HA-VDAC immunoprecipitated from 293 cells. HA-VDAC was incubated with recombinant active or inactive GST-C-Raf proteins in presence of radioactive ATP. As a positive control GST-C-Raf was incubated with MEK kinase dead substrate in the same conditions. The blots were also probed with anti-HA and anti-C-Raf antibodies. **B.** *In vivo* phospholabelling was performed by coexpressing HA-VDAC with different C-Raf constructs in 293 cells. Transfected cells were incubated with radioactive orthophosphate before HA-VDAC immunoprecipitation. HA-Bad was used as a positive control.

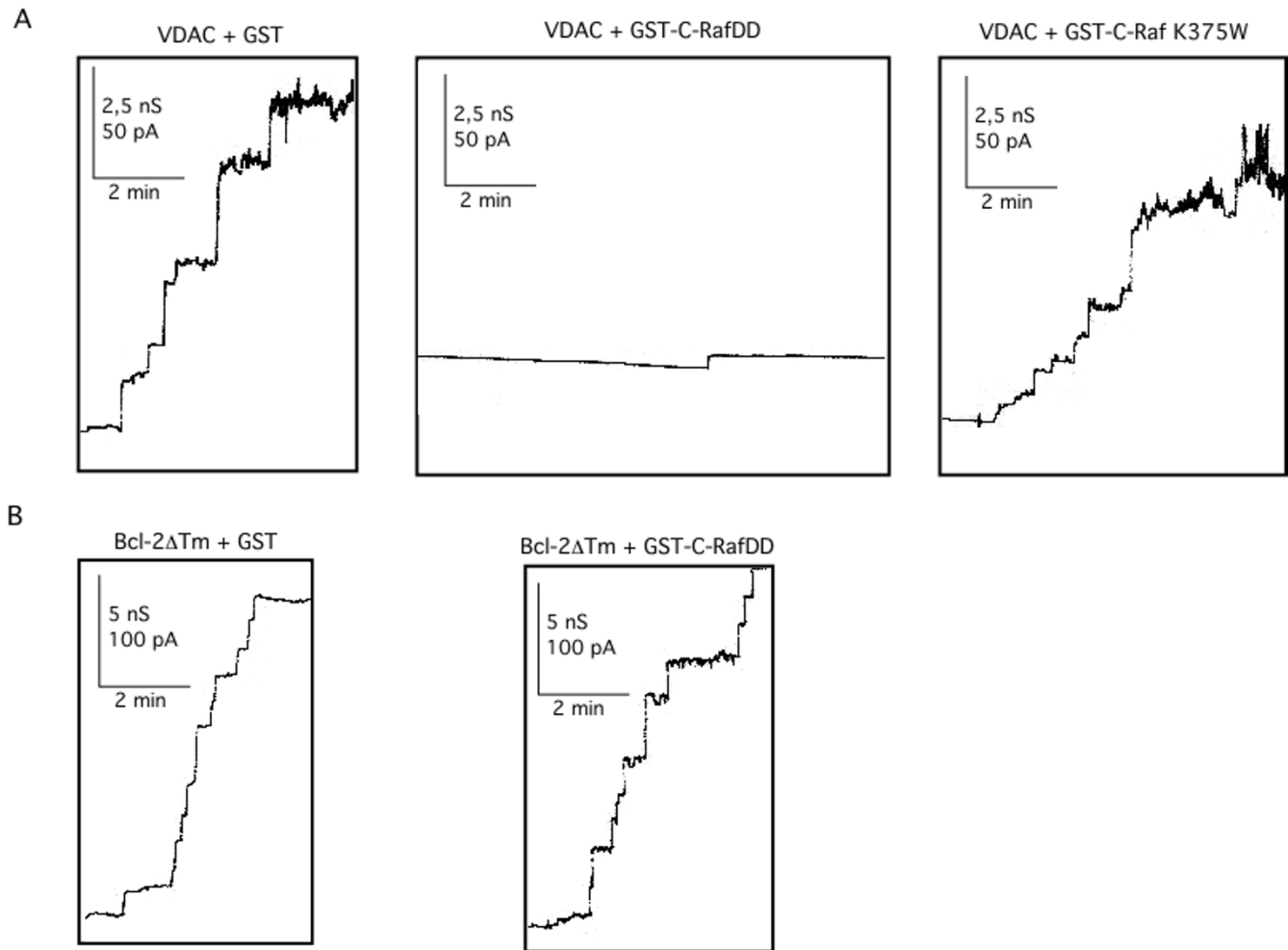


Figure 6

C-Raf kinase inhibits the VDAC channel function. Single channel recordings of purified rat liver VDAC protein in a DiphPC membrane. VDAC or Bcl-2ΔTm were preincubated with active GST-C-RafDD, inactive GST-C-RafK375W, or GST recombinant proteins before channel conductance measurement (A, B).

ing an involvement of A-Raf in controlling glycolysis (submitted data) provide evidence that Raf kinases are involved in mitochondrial function and regulation of cell metabolism, which might be an essential feature of their oncogenic potency.

Conclusions

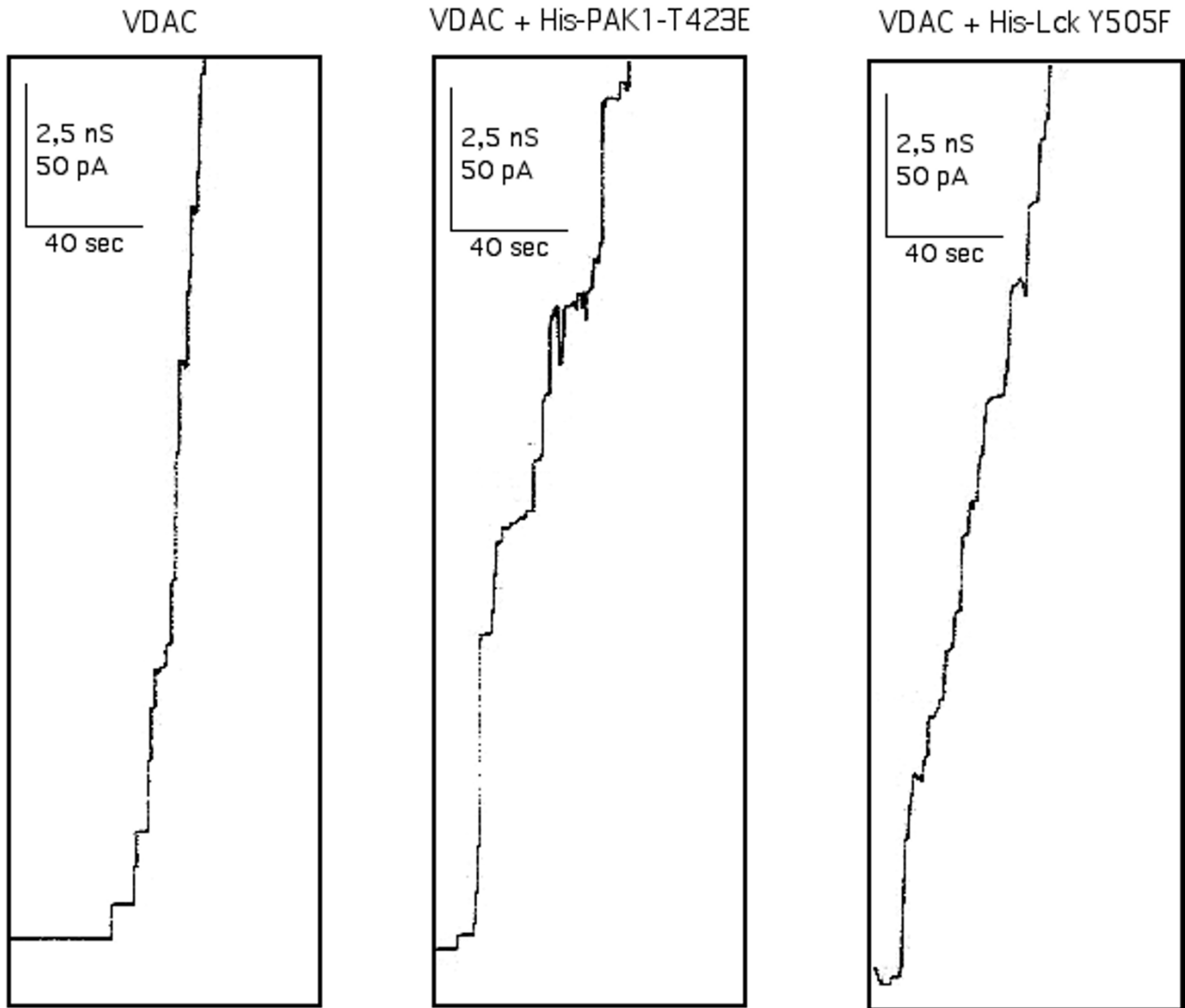
C-Raf has been demonstrated previously to interact with members of the Bcl-2 family that regulate traffic of apoptogenic proteins through the outer mitochondrial membrane. Here we identify the outer mitochondrial channel protein VDAC as binding partner and effector of C-Raf. We propose that this interaction may be responsible for the Raf-induced inhibition of cytochrome c release. Moreover, C-Raf kinase-induced VDAC inhibition may regulate

the metabolic function of mitochondria and mediate the switch to aerobic glycolysis that is common to cancer cells.

Materials and Methods

Plasmids

Human Bcl-2 cDNA encoding a protein lacking the COOH-terminal transmembrane domain (TM) (Bcl-2ΔC21: residues 219–239 in Bcl-2). cDNA coding for GST-Bcl-2ΔTm was expressed in pGEX-4T1 plasmid (gift of J. Reed). GST-C-RafYY340/341DD (active form of Raf), GST-C-RafK375W (inactive form of C-Raf), His-PAK1-T423E and His-LckY505F were cloned in pFastBac baculoviruses for expression in Sf9 insect cells.

Figure 7**Figure 7**

VDAC function is unaffected by active PAK1 or Lck kinase. As a negative control VDAC was pre-incubated with active His-PAK1-T423E or active His-LckY505F recombinant proteins (C). The aqueous phase contained 0.3 M KCl and the applied membrane potential was 20 mV.

Human VDAC1 cDNA was released from the pSK plasmid by *Bam*HI and *Sal*I restriction enzymes and subcloned into the *Bam*HI/*Eco*RV sites of pTracer-CMV plasmid to produce a HA-tag fusion protein. cDNAs coding for Mas-BXB, BXB or C-Raf were expressed in the pcDNA-3 expression plasmid (Invitrogen).

Cell transfection

Mouse promyelocytic 32Dcl.3 cells were stably transfected with pcDNA3 empty vector or pcDNA3-encoding active Mas-BXB or pcDNA-3-encoding inactive Mas-BXB K375W as previously described [3]. 293 cells were transiently transfected by a calcium phosphate method with pTracer-CMV-encoding HA-VDAC1 and/or pcDNA3 encoding C-Raf.

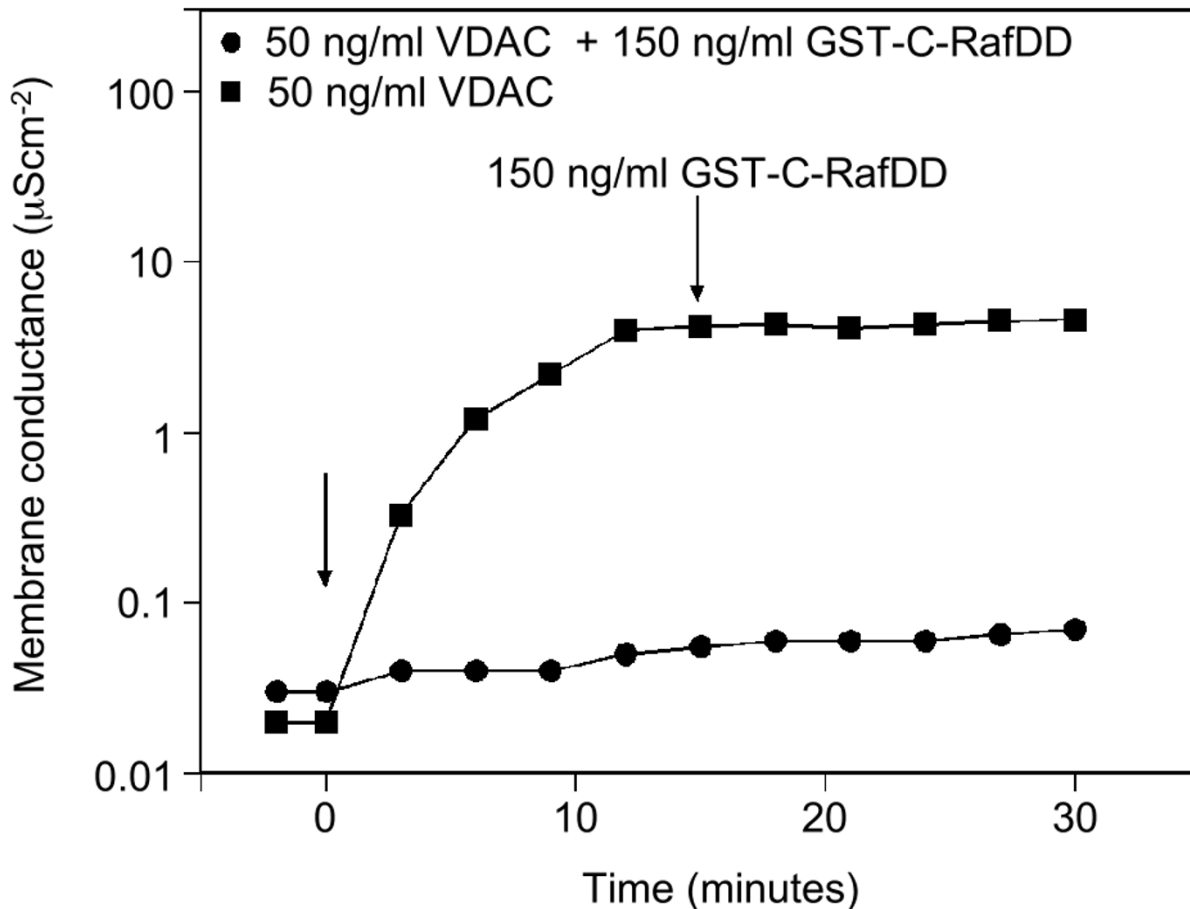


Figure 8

C-Raf blocks VDAC reconstitution into membranes. Membrane conductance as a function of time after the addition of 50 ng/ml VDAC (full squares) and 50 ng/ml VDAC pre-incubated with 150 ng/ml GST-C-RafDD (full points) to DiphPC membranes (left-hand side arrow). The aqueous phase contained 300 mM KCl supplemented with kinase buffer. 15 minutes after addition of VDAC, when the conductance was stationary 150 ng/ml GST-C-RafDD was added to both sides of the membrane (full squares, right-hand side arrow). The temperature was 20°C and the applied voltage was 20 mV. Note that the membrane conductance increase was inhibited when VDAC was preincubated with GST-C-RafDD (full squares), while this protein had no influence on already existing channels (full points).

Flow cytometry

For FACS analysis 32Dcl.3 cells lines were maintained in IL-3 deprived RPMI medium with 10% serum for 24 h. Cells were incubated with 7,5 µg/ml JC-1 for 15 min at room temperature, washed with PBS and subjected to FACS analysis.

Cell fractionation and immunoblotting

32Dcl.3 cells and transfectants were maintained in IL-3 deprived RPMI complete medium for 12 to 24 h. Cells were washed three times in phosphate-buffered saline solution and frozen at -70°C. Cell pellets were homogenized in buffer (10 mM HEPES pH 7.4, 0.3 M mannitol, 1 mM EGTA) containing protease inhibitors and centrifuged two times at 600 g for 5 minutes at 4°C. After a further centrifugation at 12 000 × g for 15 minutes at 4°C, cell pellets

contained-mitochondria enriched fraction were solubilised in the same buffer and a cytosolic fraction was obtained after centrifugation at 100000 × g for 1 h at 4°C. Proteins were resuspended in Laemmli buffer, applied to SDS-PAGE, transferred to a nitrocellulose membrane and analysed as described previously [6].

Immunoprecipitation

48 hours after transfection cells were lysed in NP-40 buffer (10 mM HEPES pH 7.4, 145 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 0.5 % NP-40) containing protease inhibitors. After centrifugation cell lysates were successively incubated with anti-hemagglutinin antibody (12CA5) or anti-C-Raf antibody and with protein A/G agarose beads. Immunoprecipitates were washed with NP-40 buffer, resuspended in Laemmli buffer and separated by SDS-PAGE. The pro-

teins were transferred to a nitrocellulose membrane and analysed as described previously [6].

Kinase assay

HA-VDAC was immunoprecipitated from transfected 293 cells and incubated with GST-C-Raf recombinant proteins in kinase buffer (25 mM Hepes, pH 7,4, 150 mM NaCl, 25 mM β -glycerophosphate, 1 mM DTT, 10 mM $MgCl_2$) containing 50 μ M ATP and 10 μ Ci [^{32}P]- γ ATP. Alternatively, GST-C-Raf was incubated with MEK as substrate instead of HA-VDAC. Phosphorylation of HA-VDAC or MEK was analysed by autoradiography. For the *in vivo* kinase assay transfected 293 cells were grown in DMEM medium with 0,3% serum for 24 h. Cells were cultured in phosphate free medium in the presence of 0,75 mCi [^{32}P] for 2 h before HA-VDAC immunoprecipitation.

Protein purification

GST-C-RafYY340/341DD, GST-C-RafK375W, His-PAK1-T423E and His-LckY505F were produced in Sf9 insect cells and purified as described [3]. *Escherichia coli* BL21 DE3(pLysS) were transformed with pGEX plasmids. After induction with isopropyl- β -D-thiogalactopyranoside (IPTG) GST-Bcl-2 Δ Tm protein was prepared from the bacterial soluble fraction incubated with glutathione-sepharose. To remove the GST-tag from Bcl-2 Δ Tm protein, beads were washed with lysis buffer (50 mM Tris, pH 8, 1 mM EDTA, 10 mM β -mercaptoethanol) containing 0,1 % Triton-X100 and 300 mM NaCl and incubated overnight at 4°C in buffer (50 mM Tris pH 8, 20 mM β -mercaptoethanol) containing thrombin (Sigma). Cleaved Bcl-2 Δ Tm was further purified on anion exchange Resource Q column (Pharmacia-Amersham) and the protein was eluted in a NaCl gradient. VDAC was purified from rat liver as described [33].

Lipid bilayer experiments

The channel-forming proteins were reconstituted into artificial lipid bilayer membranes in a teflon chamber [21,28]. The membrane was formed from a 1% (w/v) solution of diphytanoylphosphatidylcholine (DiphPC) (Avanti Polar Lipids, Alabaster, AL) in *n*-decane. VDAC or Bcl-2 Δ Tm proteins were added to the KCl buffer in both compartments of the chamber and the single-channel conductance of the pores was measured after application of a fixed membrane potential. To test the effect of C-Raf kinase on VDAC or Bcl-2 Δ Tm-formed channels, GST-C-RafYY340/341DD, GST-C-RafK375W or GST alone were incubated with channel-forming proteins in kinase buffer in presence of ATP for 30 min at 30°C. Samples were applied on both sides of the DiphPC membrane in KCl buffer and single-channel formation was measured. As a control GST, GST-C-RafYY340/341DD and GST-C-RafK375W were checked to not be able to form channels in artificial bilayer membranes. In control experiments

VDAC was first added to an artificial membrane. After reconstitution of several channels active and inactive C-Raf were added to the aqueous phase and their effect on VDAC was investigated.

Authors' contributions

V.L.: performance of tissue culture, survival and protein interaction analyses, performance and interpretation of the lipid bilayer experiments, manuscript preparation, J.T.: participation in the planning and discussion of the study, support to V.L. in the planning of experiments, manuscript preparation, R.B. performance and interpretation of the lipid bilayer experiments, U.R.R.: design and coordination of the study, manuscript preparation.

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