Voltage-dependent Anion Channel 1-based Peptides Interact with Hexokinase to Prevent Its Anti-apoptotic Activity*

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In brain and tumor cells, the hexokinase isoforms, HK-I and HK-II, bind to the voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane. The VDAC domains interacting with these anti-apoptotic proteins were recently defined using site-directed mutagenesis. Now, we demonstrate that synthetic peptides corresponding to the VDAC1 N-terminal region and selected sequences bound specifically, in a concentration- and time-dependent manner, to immobilized HK-I as revealed by real time surface plasmon resonance technology. The same VDAC1-based peptides also detached HK bound to brain or tumor-derived mitochondria. Moreover, expression of the VDAC1-based peptides in cells overexpressing HK-I or HK-II prevented HK-mediated protection against staurosporine-induced release of cytochrome c and subsequent cell death. One loop-shaped VDAC1-based peptide corresponding to a selected sequence and fused to a cell-penetrating peptide entered the cell and prevented the anti-apoptotic effects of HK-I and HK-II. This peptide detached mitochondrial-bound HK better than did the same peptide in its linear form. Both cell-expressed and exogenously added cell-penetrating peptide detached mitochondrial-bound HK-I-GFP. These results point to HK-I and HK-II as promoting tumor cell survival through binding to VDAC1, thereby inhibiting cytochrome c release and apoptotic cell death. Moreover, VDAC1-based peptides interfering with HK-mediated anti-apoptotic activity may potentiate the efficacy of conventional chemotherapeutic agents.

Cancer cells are characterized by a high rate of glycolysis that serves as their primary energy-generating pathway (1). The molecular basis of this high rate of glycolysis involves a number of genetic and biochemical events, including overexpression of the mitochondria-bound hexokinase isoforms, HK-I and HK-II (1–5). In mammals, HK-I and HK-II are strategically located at the outer membrane of mitochondria, where they gain preferential access to mitochondrially generated ATP (6). In this manner, HK-I and HK-II drive the high glycolytic rates typical of tumor cells, even under aerobic conditions (1, 3). Such elevated levels of mitochondria-bound HK is suggested to play a pivotal role in promoting cell growth and survival in rapidly growing, highly glycolytic tumors and in protecting against mitochondria-mediated cell death (1, 4, 7). The elevated levels of HK-I and HK-II allow tumor cells to evade apoptosis, thereby allowing proliferation to continue (4). Indeed, overexpression of mitochondria-bound HK in the tumor-derived cell line U-937, in T-REx-293, or in vascular smooth muscle cells suppressed staurosporine-induced apoptotic cell death (8, 9). A decrease in apoptotic cell death and concomitant increase in cell proliferation have also been reported to be induced upon HK-II expression in NIH-3T3 (7), rat 1a fibroblasts (10), and WEHI 7.1 cells (11). In addition, binding of HK-II to mitochondria inhibited Bax-induced cytochrome c release and apoptosis (12).

Mitochondria-mediated apoptosis results in the efflux of a number of potential apoptotic regulators, such as cytochrome c, to the cytosol, triggering caspases activation and cell destruction. The molecular mechanism of HK-mediated protection against cell death involves HK-I and HK-II binding to the outer mitochondrial membrane protein, the voltage-dependent anion channel (VDAC) (8, 9, 13–16). Indeed, VDAC was shown to be up-regulated in cancer cells exhibiting elevated binding to HK-I and/or HK-II (17). Also, it has been recently shown that acute viruses, such as HEV, can protect infected cells from apoptotic death via enhanced VDAC and HK expression (18).

VDAC is a key channel protein that integrates cellular energy metabolism. VDAC has also been recognized as a key protein in mitochondria-mediated apoptosis, as the proposed target for the pro- and anti-apoptotic Bcl2 family of proteins and due to its function in the release of apoptotic proteins (19–21). Purified HK-I has been shown to directly interact with purified VDAC reconstituted into a planar lipid bilayer, leading to channel closure (8). HK-I also prevented opening of the permeability transition pore and suppresses the release of cytochrome c, thus inhibiting the mitochondrial phase of apoptosis (8), and thereby contributing to cell survival (12, 22).

Recent studies have shown that the interaction of HK with VDAC can be regulated via VDAC phosphorylation (23). It has also been shown that Akt activation partly inhibits apoptosis by promoting the binding of HK-II to mitochondria (23). This effect of Akt was shown to be mediated by negative regulation of glycogen synthase kinase 3β activity. Glycogen synthase

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2 The abbreviations used are: HK-I and HK-II, hexokinase isofrom I and II; VDAC, voltage-dependent anion channel; LP1, LP2, LP3 and LP4, VDAC1-based peptides; SPR, surface plasmon resonance; PI, phosphatidylinositol; GFP, green fluorescent protein; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; STS, staurosporine.
kinase 3β-phosphorylated VDAC is unable to bind HK-II, shifting the equilibrium toward cytosolic HK and thus allowing apoptosis induction (23). These results suggest that HK-I or HK-II, via their interaction with VDAC1, prevent key events in mitochondria-mediated apoptosis.

Despite these advances, the precise VDAC1 sequences interacting with HK-I or HK-II remain unknown. Recently, using site-directed mutagenesis, we identified VDAC1 domains that interact with HK-I (24), located in two cytoplasmic domains, according to previously proposed VDAC1 topology models (25, 26). However, according to recently published NMR-based VDAC1 structure (27, 28) only part of these domains are exposed to the cytosol. In addition, the amphipathic N-terminal domain of VDAC1 (24, 27, 28) was identified as the HK-I interaction site. These VDAC1 domains are involved in HK-mediated protection against cell death via the inhibition of cytochrome c release. In this study, we have used VDAC1-based synthetic and recombinant peptides to define the boundaries of VDAC1 sequences essential for HK-I and HK-II binding, and show their abilities to disturb HK-mediated protection against cell death.

EXPERIMENTAL PROCEDURES

Materials—Glucose 6-phosphate, Hepes, lactate dehydrogenase, leupeptin, mannitol, phenylmethylsulfonyl fluoride, propidium iodide, pyruvate kinase, staurosporine, sucrose, and Tris were purchased from Sigma. A Cibacron blue HiTrap™ Blue HP column was purchased from Amersham Biosciences. Monoclonal anti-VDAC antibodies came from Calbiochem-Novobiochem (Nottingham, UK). Monoclonal antibodies against actin and polyclonal goat antibodies against HK-I and HK-II were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-cytochrome c antibodies were obtained from BD Biosciences. Horseradish peroxidase-conjugated anti-mouse and anti-goat antibodies were obtained from Promega (Madison, WI).

Plasmids and Site-directed Mutagenesis—Five mVDAC1 peptide-encoding inserts were generated by standard PCR and cloned into the BamHI/EcoRI sites of the tetracycline-inducible pcDNA4/TO vector (Invitrogen), using mVDAC1 cDNA as a template and the primers indicated in Table 1. The N-terminal-, LP1-, LP2-, LP3-, and LP4-coding VDAC1 sequences (Fig. 1) were generated using primers F-N and R-N and F-1 and R-1 to F-4 and R-4, respectively (Table 1).

3. S. Abu-Hamad, D. Calo, N. Arbel, L. Arzoine, I. Israelson, N. Keinan, R. Ben-Romano, O. Friedman, and V. Shoshan-Barmatz, J. Cell Science, in press.

The four VDAC1 loop-based peptides, LP1 to LP4, were flanked by a tryptophan zipper motif, namely, the SWTWE amino acid sequence at the N terminus of the peptide and KWTWK sequence at the C terminus. Correct constructs were confirmed by sequencing. The pcDNA3-HK-II and pcDNA3-HK-I plasmids were kindly provided by J. E. Wilson (Michigan State University), and the HK-I-GFP fusion protein, in which GFP was connected to the HK-I C-terminal, was generated as described previously (24). Construction of the native and mutated mVDAC1 vectors, mVDAC1-, mVDAC1/E72Q-, mVDAC1/E202Q-, and mVDAC1/K109L-pcDNA4/TO, was described previously (24).

Peptides Synthesis—The synthetic N-terminal, LP1, LP2, LP3, and LP4 VDAC1-based peptides were synthesized by the Oligonucleotides and Peptide Synthesis Unit, Weizmann Institute, Rehovot, Israel. The LP4-Antp looped-shaped peptide, including the cell penetrating peptide, Antp, the peptide Antp, and other VDAC1-based peptides, were synthesized by GL Biochem (Shanghai, China). The N-terminal and LP4 peptides were water-soluble, whereas the other peptides were dissolved in Me2SO.

Cancer Cell Lines and Tumors—CT26 is a murine colon carcinoma cell line, B16 is a murine melanoma cell line, BCL1 is a murine B cell leukemia cell line, MCF7 represents a human breast carcinoma cell line, and MolT4 is a human T lymphoblastic leukemia cell line. All were purchased from ATCC (Rockville, MD) or provided by Dr. Eliezer Flescher (Tel Aviv University). T-REX-293 cells correspond to a transformed primary human embryonal kidney cell line expressing the tetracycline repressor (Invitrogen). Cell growth was maintained in a humidified atmosphere, at 37 °C with 5% CO2. CT26, B16, MCF-7, and T-REX-293 cells were maintained in Dulbecco’s modified Eagle’s medium (Biological Industries), supplemented with 10% fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate and nonessential amino acids (all from Biological Industries). MolT4 and BCL1 cells were maintained in RPMI 1640 medium (Biological Industries), supplemented with 10% fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Peripheral blood lymphocytes from venous blood of healthy donors were prepared by Ficoll-Hypaque (GE Healthcare) density gradient centrifugation, as previously described (30).

To generate BCL1 leukemia-bearing C57BL BALB/c mice (Tel-Aviv University), animals were inoculated intraperitoneally with BCL1 cells freshly extracted from BCL1-bearing mice. Four weeks later, the mice were killed under anesthesia, their

| Insert name | Oligonucleotides | Sequence (5’ to 3’) |
|-------------|------------------|--------------------|
| N           | F-N              | GCCAGATCTATGGCTGTCACCACCCACGTTA |
|             | R-N              | GCCAGATCTATGGCTGTCACCACCCACGTTA |
| LP1         | F-1              | CGAATTCTATGGCTGTCACCACCCACGTTA |
|             | R-1              | CGAATTCTATGGCTGTCACCACCCACGTTA |
| LP2         | F-2              | CGAATTCTATGGCTGTCACCACCCACGTTA |
|             | R-2              | CGAATTCTATGGCTGTCACCACCCACGTTA |
| LP3         | F-3              | GCCAGATCTATGGCTGTCACCACCCACGTTA |
|             | R-3              | GCCAGATCTATGGCTGTCACCACCCACGTTA |
| LP4         | F-4              | GCCAGATCTATGGCTGTCACCACCCACGTTA |
|             | R-4              | GCCAGATCTATGGCTGTCACCACCCACGTTA |
spleens were removed, and the cells were extracted by spleen dispersion. Red blood cells were disrupted using red blood cell lysis buffer (Sigma).

Cell Transfection—Logarithmically growing T-REx-293 cells were transiently co-transfected with plasmids pcDNA3-HK-II or pcDNA3-HK-I alone or with one of the following vectors: each of the mVDAC1-peptide-encoding pcDNA4/TO plasmids or the native or mutated mVDAC1-encoding pcDNA4/TO plasmid (or the corresponding control plasmid). Transfections were performed using FuGENE HD transfection reagent (Roche), according to the manufacturer’s instructions. After 48 h growth in the presence of tetracycline (1 μg/ml), cells were subjected to the desired treatment.

Acridine Orange/Ethidium Bromide Staining of Cells—To determine the extent of apoptosis, cells were subjected to staining with 100 μg/ml acridine orange and 100 μg/ml ethidium bromide (EtBr) in PBS (see figure legends).

Flow Cytometric Analysis of Cell Death—T-REx-293, Molt4, B16, and CT26 cells were incubated for 90 min with 20 μM LP4-Antp or Antp peptides in serum-free Dulbecco’s modified Eagle’s medium. MCF7 cells and peripheral blood lymphocytes were incubated with the peptides for 3 and 2 h, respectively. The cells were washed twice with PBS, and stained with propidium iodide (PI). The level of cell death, reflected by the percentage of PI-positive cells, was determined by a flow cytometer (FACScan, BD Biosciences) and ModFIT-lt2.0 software.

Cytochrome c Release Induced by Staurosporine (STS)—Control T-REX-293 cells and T-REX-293 cells expressing HK-I or HK-II and/or VDAC1-based peptides were grown on 60-mm dishes for 72 h in the presence of tetracycline (1 μg/ml). Thereafter, cells were exposed to STS (1.25 μM) for 4 h and assayed for cytochrome c release (see figure legends).

Isolation of Rat Brain, Mammary Cells, and Tumor-derived Mitochondria—Rat brain, mammary cells, and tumor-derived mitochondria were prepared by mechanical cell homogenization and differential centrifugation, as previously described (31).

Detachment of Mitochondria-bound HK and HK Activity Assay—To induce detachment of mitochondria-bound HK, increasing amounts of synthetic peptides (0.0025–0.2 mM) were added to isolated mitochondria (1.5–2 mg/ml) and released HK was assayed following its activity (32) or by immunoblotting using anti-HK-I antibodies.

Rat Brain HK-I Purification—HK-I was purified from rat brain mitochondria (8), with the modification of using a Cibacron Blue HiTrap™ Blue HP column and a AKTAbasic purifier chromatography system (Amersham Biosciences).

Real-time Surface Plasmon Resonance (SPR)—SPR, using the ProteOn-XPR36 system (Bio-Rad) was employed to study the interaction of VDAC1-based synthetic peptides with purified HK-I. Purified HK-I and rabbit IgG were immobilized onto a GLM sensor surface, according to the manufacturer’s instructions. The peptides were diluted in running buffer (150 mM NaCl, 0.005% Tween 20, 4% (v/v) Me2SO, 10 mM PBS, pH 7.4) and injected onto the sensor chip at varying concentrations (flow rate, 40 μl/min). Response units were monitored using the ProteOn imaging system and related software tools. Signals were normalized using appropriate controls.

Cell Penetration of fluorescein isothiocyanate (FITC)-labeled LP4-Antp and Antp Peptides—LP4-Antp and Antp peptides (1 mg) were labeled with FITC (Sigma) by incubation for 30 min with 50 μM FITC in 10 mM Tricine buffer, pH 8.7, at 37 °C. Unreacted reagent was removed by dialysis using membranes.
with a cut off of 1000 Da (Cellu. Sep H1, Membrane Filtration Products, Seguin, TX). T-REx-293 cells were incubated for 90 min with 20 μM FITC-labeled-LP4-Antp or Antp peptide in serum-free Dulbecco's modified Eagle's medium, washed with PBS, fixed with 4% formaldehyde, and viewed under a Zeiss LSM 510 meta-confocal microscope.

**LP4-Antp and Antp Peptide Treatment of HK-I Overexpressing Cells**—T-REx-293 cells were transiently transfected with plasmid pcDNA3-HK-I or control plasmid pcDNA3. 48 h after transfection, cells were incubated for 2 h with 20 μM of the LP4-Antp or the Antp peptide in serum-free Dulbecco's modified Eagle's medium and then exposed to STS (1.25 μM) for 2 h. Apoptosis was analyzed quantitatively by acridine orange/EtBr staining, as described above.

**Confocal Microscopy**—T-REx-293 cells (5 × 10⁶) were grown on poly-d-lysine-coated coverslips, and transfected with plasmid pEGFP-HK-I alone or with plasmid pEGFP-HK and pcDNA4/TO-LP4, stabilized with the tryptophan zipper motif. After 48 h, control cells were incubated for 90 min with a solution containing 0.4% Me₂SO or LP4-Antp (15 μM). Cells were fixed for 15 min using 4% paraformaldehyde prepared in PBS, rinsed for 30 min in PBS, and cell imaging was carried out by confocal microscopy (Olympus IX81).

**RESULTS**

We have demonstrated that the pro-survival protein HK-I, expressed at high levels in many types of cancer (1, 2, 5), interacted with VDAC1 and protected against cell death as induced by STS or VDAC1 overexpression (8, 9). We also demonstrated that E72Q-mVDAC1 no longer retains its capacity to bind HK-I (9, 24). The domains and amino acid residues involved in the interaction of VDAC1 with HK-I were also defined (9, 24). These domains, according to previously proposed VDAC1 membrane topology models (25, 26), are exposed to the cytosol. However, according to recently published NMR-based recombinant human VDAC1 structure (27, 28), these sequences are only partially exposed to the cytosol (Fig. 1). Based on certain mutations in VDAC1 found to diminish HK-I protection against apoptosis (24), VDAC1-based peptides were synthesized and their interactions with HK-I and HK-II, as well as the effects of their expression on HK-mediated anti-apoptotic activity, were studied.

**HK-II Overexpression Protects against Cell Death**—The anti-apoptotic effect of HK-II was tested by its overexpression in T-REx-293 cells expressing native or mutated mVDAC1 and induction of apoptosis by STS. STS-induced apoptotic cell death was reduced by 64% in cells overexpressing HK-II and native mVDAC1, as compared with the values obtained in control cells. However, the protective effect of HK-II was abolished in cells overexpressing E72Q-, E202Q-, or K109L-mVDAC1 (Fig. 2, A and B). The level of HK-II in cells transfected to express the protein was about 6-fold higher than in control cells (Fig. 2C). These results indicate that like HK-I (9, 24), the interaction of HK-II with VDAC1 involves glutamate and lysine residues located in 2 different regions of VDAC1. These results also show that although the cells express endogenous hVDAC1, the presence of mVDAC1 mutants, nonetheless, completely prevented the anti-apoptotic effect of HK-II. This is in agreement with previous studies (9) showing that no protection mediated by HK-I or ruthenium red against STS-induced apoptosis was observed in cells expressing E72Q-VDAC1, although endogenous VDAC1 was present in these cells. This indicates that E72Q-VDAC1 possesses a dominant negative effect and implies that VDAC1 molecules are capable of intermolecular interactions to form a homo-oligomer, involved in apoptosis induction (9).

**VDAC1-based Synthetic Peptides Interact with HK—**Five VDAC1-based peptides (labeled in gray in Fig. 1) (LP1, LP2, LP3, LP4, and the N terminus) were synthesized and their interactions with purified rat brain HK-I was demonstrated using SPR technology (Fig. 3). Purified HK-I or rabbit IgG were coupled to a SPR biosensor chip. Increasing concentrations (20 to 300 μM) of the VDAC1-based peptides were injected onto the
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sensor chips and binding to HK-I was monitored. The N-terminal, LP1, and LP4 peptides strongly bound to immobilized HK-I in a concentration- and time-dependent manner (Fig. 3). By contrast, two other VDAC1-based peptides, i.e. LP2 and LP3, did not interact with HK-I (Fig. 3). Binding of the LP1, LP4, and N-terminal peptides to HK-I was specific, because no signal was obtained with the IgG-immobilized chip (Fig. 3A, data shown only for LP1).

All HK-I-interacting VDAC1-based peptides associated relatively rapidly with the immobilized HK-I. However, whereas the LP1 and N-terminal peptides showed rapid dissociation, the LP4 peptide showed slow dissociation from the immobilized HK-I. The apparent binding affinities of the LP1, LP4, and N-terminal peptides to HK-I were derived from dose-response curves obtained using steady-state values and calculated to be 65, 70, and 140 μM for LP1, LP4, and N-terminal peptides, respectively (Fig. 3D, data shown only for the N-terminal peptide). The results thus demonstrate direct and specific interaction of selected VDAC1-based peptides with HK-I.

VDAC1-based Synthetic Peptides Detach Bound HK from Mitochondria—HK-I and HK-II are highly expressed in cancer cells, where over 70% of the protein is bound to mitochondria (3, 4). Brain mitochondria also express very high levels of mitochondria-bound HK-I (34). The effects of the synthetic VDAC1-based peptides on HK-I bound to rat brain mitochondria and on HK-I/HK-II bound to mitochondria iso-

latched from BCL1 leukemia cells were tested. The LP1 peptide, containing the Glu72 residue, was able to release mitochondria-bound HK from both rat brain and BCL1 mitochondria, as reflected in the appearance of HK activity in the supernatant (Fig. 4, A–C). The N-terminal peptide was also able to release HK from mitochondria from both sources, but to a lesser extent than LP1 (Fig. 4, A, B, and D). HK was released from rat brain mitochondria by both the LP1 and N-terminal peptides in a dose-response manner. Apparent affinities (K_D) of 2 and 20 μM were derived for the LP1 and N-terminal peptides from the saturation curve fits (Fig. 4, C and D). The LP2 and LP4 peptides also caused release of a small fraction of mitochondria-bound HK-I (Fig. 4A). By contrast, LP3 peptide did not detach HK from either mitochondrial preparation. The observations that the peptides induced detachment of mitochondria-bound HK suggest that a dynamic equilibrium exists between free and mitochondria-bound HK.

Expression of VDAC1-based Peptides in Cells Overexpressing HK-I or HK-II Prevents Their Anti-apoptotic Effects—The synthetic VDAC1-based peptides used in the SPR and HK detachment experiments are linear, flexible in solution, and most likely do not adopt the same loop-like structure as those predicted in the membrane topology model (Fig. 1). Thus, to better mimic the native loop-like structure, we designed VDAC1-based peptides flanked by the tryptophan zipper motif consisting of the amino acids sequences SWTWE at the N terminus and KWTWK at the C terminus of the peptide, inducing the formation of stable β-hairpins by tryptophan-tryptophan cross-strand pairing (35).

HK-I and HK-II overexpression was shown to prevent apoptotic cell death induced by STS or VDAC1 overexpression (Fig. 2 and Refs. 9 and 24). The results presented in Fig. 5 show that HK-I or HK-II overexpression protected against STS-induced apoptosis in T-REx-293 cells. When cells expressing HK-I or HK-II were also transfected with mammalian expression vectors encoding the VDAC1-based peptides, the expression of the N-terminal, LP1, LP2, or LP4, but not the LP3, peptides stabilized in a loop-form by an induced tryptophan zipper motif, no protection by HK-I or HK-II against STS-induced cell death was obtained (Fig. 5). These results suggest that the VDAC1 sequences represented by the recombinant loop-peptides, LP1, LP2, and LP4, or part thereof, as well as the N terminus recombinant peptide, interact with HK-I and HK-II and thus prevent interaction of these proteins with VDAC1, in turn preventing their protec-

FIGURE 3. Selected VDAC1-based peptides interact with HK-I in a specific and dose-dependent manner. Interaction of purified HK-I (E) with VDAC1-based peptides was revealed using real-time surface plasmon resonance. HK-I immobilized onto a GLM sensor surface was exposed to VDAC1-based peptides: LP1 (40, 100, and 200 μM), LP4 and N (C: 37.5, 75, and 150 μM), and LP2 and LP3 (B: 40, 100, and 200 μM). The peptides were run in parallel over surface strips of HK-I and responses (resonance units, RU), as a function of peptide concentration, were monitored using the ProteOn imaging system and related software tools. To control for nonspecific binding, the interaction of the peptides (200 μM) with surface strips of IgG was analyzed. Δ, N-terminal dose-response curve obtained using steady-state binding values (n = 3). An apparent affinity (K_D) of N-terminal peptide binding to HK-I, derived from the sigmoidal Hill plot fits of 140 μM was obtained. All experiments were carried out at 25 °C.

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**FIGURE 4. VDAC1-based peptides induce detachment of mitochondria-bound HK.** Mitochondria isolated from rat brain (A) or BCL1 leukemia tumor cells (B) were incubated without or with VDAC1-based peptides at the indicated concentrations. After a 1-h incubation at 25 °C, the samples were centrifuged (15,000 × g, 10 min) and soluble and mitochondria-bound HK fractions were separated. HK activity in the supernatant was measured spectrophotometrically at room temperature at 340 nm by coupling NADH oxidation by lactate dehydrogenase to the production of ADP by HK, and its subsequent phosphorylation by pyruvate kinase with phosphoenolpyruvate (1 mM) as the substrate. Lactate dehydrogenase and pyruvate kinase were added in excess, i.e.

**A** and **B**. To rule out mitochondrial contamination in the cytosolic fraction as the source of cytochrome c, the absence of VDAC was confirmed by immunoblotting (data not shown).

**D**. To demonstrate that the peptides can detach mitochondria-bound HK-I at the cellular level, we expressed the HK-I-GFP fusion protein alone or together with the LP1, LP2, LP3 or LP4 peptides or when incubated with a synthetic peptide LP4-Antp, and visualized HK-I-GFP cellular distribution (Fig. 7). Confocal fluorescence microscopy showed that in control cells, HK-I-GFP fluorescence is punctuated, as expected for a mitochondrial distribution (see also Ref. 24). However, when cells expressing HK-I-GFP were exposed to the synthetic peptide LP4-Antp, HK-I-GFP was detached from the mitochondria, as reflected in the diffuse fluorescence (Fig. 7A). Similarly, HK-I-GFP fluorescence in cells expressing the LP1 or LP2 but not the LP3 peptide was diffused throughout the cytosol (Fig. 7B). These results indicate that both expressed LP1, LP2, and LP4 but not the LP3 peptide, as well as the membrane-penetrating LP4-Antp peptide, detach or prevent HK-I binding to the mitochondria, as also demonstrated with isolated mitochondria (Fig. 4) and in the prevention of HK-I- or HK-II-mediated protection against STS-induced cell death (Fig. 5).

**E**. A schematic representation of the loop-shaped LP4-Antp peptide is shown in Fig. 6A. FITC-labeled LP4-Antp and Antp peptides were used to assess their cell penetration (Fig. 6B). Fluorescence microscope images of cells incubated with either peptide show that both penetrate the cell (Fig. 6B).

Next, we tested the effects of the LP4-Antp peptide on apoptosis induced by STS in T-REx-293 cells overexpressing HK-I (Fig. 6C). The LP4-Antp peptide prevented HK-I-mediated protection against cell death induced by STS. Although in cells exposed to the Antp peptide HK-I protected against STS-induced apoptosis by about 75%, in the presence of the LP4-Antp peptide the protection was only 13%. Moreover, the LP4-Antp peptide alone (i.e. in the absence of STS) induced apoptotic cell death. Like the LP4 peptide (Fig. 4), the LP4-Antp peptide also induced the displacement of HK-I from rat brain mitochondria. As demonstrated by Western blot analysis, incubation of isolated brain mitochondria with the loop-shaped LP4-Antp peptide but not the control Antp peptide resulted in the appearance of HK in the supernatant (Fig. 6D).

Finally, to demonstrate that the peptides can detach mitochondria-bound HK-I at the cellular level, we expressed the HK-I-GFP fusion protein alone or together with the LP1, LP2, LP3 or LP4 peptides or when incubated with a synthetic peptide LP4-Antp, and visualized HK-I-GFP cellular distribution (Fig. 7). Confocal fluorescence microscopy showed that in control cells, HK-I-GFP fluorescence is punctuated, as expected for a mitochondrial distribution (see also Ref. 24). However, when cells expressing HK-I-GFP were exposed to the synthetic peptide LP4-Antp, HK-I-GFP was detached from the mitochondria, as reflected in the diffuse fluorescence (Fig. 7A). Similarly, HK-I-GFP fluorescence in cells expressing the LP1 or LP2 but not the LP3 peptide was diffused throughout the cytosol (Fig. 7B). These results indicate that both expressed LP1, LP2, and LP4 but not the LP3 peptide, as well as the membrane-penetrating LP4-Antp peptide, detach or prevent HK-I binding to the mitochondria, as also demonstrated with isolated mitochondria (Fig. 4) and in the prevention of HK-I- or HK-II-mediated protection against STS-induced cell death (Fig. 5).
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**FIGURE 5.** Expression of VDAC1-based peptides in T-REx-293 cells prevented HK-I and HK-II-mediated protection against cytochrome c release and cell death induced by STS. T-REx-293 cells were transfected to express HK-I (A), HK-II (B), and/or the indicated VDAC1-based peptide. Apoptosis in the different cell types was induced by incubation with STS (1.25 μM) for 4 h and quantitatively analyzed by acridine orange/EtBr staining. Data represent mean ± S.E. (n = 2–4). Induction of VDAC1-based peptides expression by Tet (1 μg/ml) in the indicated transfected cells is also shown in B. C. cytochrome c release into the cytosol in the indicated transfected cells was analyzed following STS (1.25 μM) treatment for 4 h. Cells were harvested, washed twice with PBS, incubated for 1 h on ice in buffer (20 μl Hepes, pH 7.4, 250 mM sucrose, 1 mM EGTA, 1 mM EDTA, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride), and then lysed by a Dounce homogenizer (25 strokes). Homogenates were centrifuged (1,000 × g) at 4 °C for 5 min and the supernatants were re-centrifuged at 15,000 × g for 15 min at 4 °C. Aliquots (10 μl) of the resultant supernatants, designated as cytosolic fractions, were immediately boiled in SDS-PAGE sample buffer and resolved by SDS-PAGE on Tris-Tricine gels (13% polyacrylamide) and immunobotted using monoclonal anti-cytochrome c antibodies (1:1000), followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG. Secondary antibody binding was detected by chemiluminescence using a kit obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Although supernatants from the different treated cells were derived from an equal number of cells, protein concentrations in the total cell lysates were estimated and actin levels were analyzed by immunoblotting, using an anti-actin antibody. D. HK-I and HK-II levels in control cells and cells transfected to overexpress HK-I or HK-II and/or VDAC1-based peptide. Cell lysates (50 μg) were analyzed using polyclonal anti-HK-I and anti-HK-II antibodies, respectively. As a loading control, actin levels in the cell lysates were compared using an anti-actin antibody.

DISCUSSION

Recently (9, 24), we have identified those domains in VDAC1 involved in the interaction of the VDAC1 with HK-I. Relying on these identified VDAC1 sequences, we have now used synthetic peptides corresponding to these and other VDAC1 sequences and assessed their success in binding to and detaching of mitochondrial HK-I and HK-II. We also demonstrated their success in diminishing the protective activities of HK-I and HK-II against cell death, with the aim of introducing these VDAC1-based peptides into cancer therapy.

The Loop-shaped LP4-Antp Peptide Triggers Human and Murine Cancer Cell Death—To further assess the effect of the LP4-Antp peptide on cell viability, different human and murine cancer and transformed cell lines (T-REx-293, MCF7, Molt4, CT26, and B16 cell lines) were exposed to the loop-shaped LP4-Antp peptide and cell death was analyzed by flow cytometry of PI-positive cells (Fig. 8 and Table 2). As shown, the peptide-induced cell death in all these cell lines, each originating from a different type of cancer (or transformed cells, such as T-REx-293 cells) but not in human peripheral blood lymphocytes.

Interestingly, quantitative analysis of HK-I and HK-II levels in these cells showed that the higher the level of bound HK, the lesser the degree of cell death induced by the LP4-Antp peptide (Table 2). For example, the MCF7 cell line, which contains the highest level of bound HK, showed 24% cell death and only after 3 h of incubation, whereas Molt4 cells with 40% the HK level of MCF7 cells showed 65% cell death following a 1.5-h incubation with the same concentration of LP4-Antp peptide.

**FIGURE 6.** Loop-shaped LP4-Antp peptide penetrates into the cell and prevents HK-I anti-apoptotic activity. A, schematic presentation of the loop-shaped LP4-Antp synthetic peptide. The LP4 loop (yellow) is stabilized by the presence of a tryptophan zipper (Trp zipper, blue) and fused to the Antp peptide (purple). B, T-REx-293 cells were incubated for 90 min with 20 μM FITC-labeled LP4-Antp or Antp, washed with PBS, fixed with 4% formaldehyde, and viewed under a Zeiss LSM 510 meta-confocal microscope. Fluorescence (a), light microscope (b), and merged (c) images were recorded. C, T-REx-293 cells were transiently transfected with plasmid pcDNA3-HK-I or the control pcDNA3 plasmid. 48 h after transfection, the cells were incubated for 2 h with LP4-Antp peptide (20 μM) or the control Antp peptide (20 μM) and then exposed to staurosporine (STS) (1.25 μM) for 2 h. Apoptotic cell death was quantitatively analyzed by acridine orange/ethidium bromide staining. Data represent mean ± S.E. (n = 3). D, Western blot analysis of HK-I released from rat brain mitochondria following incubation with 10 and 20 μM LP4-Antp or Antp. G6P, Glu-6-P.
cells are indicated in black. Antp in age of PI-positive cells, determined by flow cytometry (control in gray). The cells were then stained with PI. The level of cell death, reflected by the percentage of PI-positive cells, was determined by flow cytometry. Data are mean ± S.E. of three to four independent experiments. The LP4-Antp-induced cell death was obtained by subtracting cell death in the control. Control indicates an average of the percentage of PI-positive cells incubated without (0.4% dimethyl sulfoxide) and with Antp peptide. The level of mitochondria-bound HK was analyzed by Western blot of isolated mitochondria using polyclonal anti-HK antibodies recognizing both HK-I and HK-II. Immunoblotting using anti-cytochrome c and anti-VDAC antibodies were used as loading controls.

Some of the VDAC1 amino acids, such as Glu72, mutation of which diminished HK-mediated protections against apoptosis, are located in the β-strands. Based on NMR study, it was proposed that Glu73 (counting from the first methionine) influenced the structural instability of the N-terminal segment (27) because Glu73, as a charged residue, was found to break the alternating pattern of hydrophobic and hydrophilic residues in strand β-4 and introduce structural instability of the N-terminal segment. Replacing Glu73 with glutamine or valine resulted in a stable conformation of the N-terminal part of the protein (27), and this may affect its accessibility to HK. As shown here and in previous studies (9, 24), substitution of Glu73 by glutamine has important consequences for the function of VDAC1, including abolishing ruthenium red- and HK-I-mediated inhibition of VDAC channel activity and ruthenium red- and HK-I-mediated protection against apoptosis.

In addition to the VDAC1 loops facing the cytoplasm, the N-terminal region of VDAC1 also interacts with HK. These finding thus suggest that the interaction of HK with VDAC1 is mediated by multiple interaction sites.

The interaction of the N terminus of VDAC1 with HK, as demonstrated by the loss of HK binding upon its removal3 as well as the results of this study, suggests that this region is accessible to HK and hence, exposed to the cytoplasm. This is in accord with the suggestion that the VDAC1 N-terminal region is a mobile component involved in the interaction with anti-apoptotic proteins.3 Various proposed VDAC1 membrane topology models predict differences in the location of the N terminus (25, 40, 41). Recent NMR-based studies of recombinant VDAC1 structure suggest that the N-terminal tail of VDAC1 is not part of the barrel wall but rather is located inside the pore, with only part of it in the form of α-helix (27, 28). However, because this segment is involved in voltage gating (42), it might adopt different conformations depending on cell conditions. Able to interact with HK, it is implied that this VDAC1 region is not permanently embedded in the pore.

Although the N-terminal region of HK has been defined as being essential for its anchoring to mitochondria (3, 8, 12, 43,
would have strong therapeutic potential because they affect mitochondria-bound HK, a characteristic of cancer cells, yet have no inhibitory effect on HK enzymatic activity (data not shown). In addition, because the VDAC1 sequence is highly conserved and found in all human cells, designed VDAC1-based peptides would be expected to be non-immunogenic and have no toxic effects. Moreover, VDAC1-based peptides, as pro-apoptotic compounds, can be used to potentiate the efficacy of conventional chemotherapeutic agents. Targeting VDAC1-based peptides to tumor cells overexpressing such anti-apoptotic proteins may prove an effective VDAC1-based cancer therapy, acting to minimize the self-defense mechanisms of cancer cells, thereby promoting apoptosis.

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