Hexokinase-I Protection against Apoptotic Cell Death Is Mediated via Interaction with the Voltage-dependent Anion Channel-1

MAPPING THE SITE OF BINDING

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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. We have previously shown that HK-I decreases murine VDAC1 (mVDAC1) channel conductance, inhibits cytochrome c release, and protects against apoptotic cell death. Now, we define mVDAC1 residues, found in two cytoplasmic domains, involved in the interaction with HK-I. Protection against cell death by HK-I, as induced by overexpression of native or mutated mVDAC1, served to identify the mVDAC1 amino acids required for interaction with HK-I. HK-I binding to mVDAC1 either in isolated mitochondria or reconstituted in a bilayer was inhibited upon mutation of specific VDAC1 residues. HK-I anti-apoptotic activity was also diminished upon mutation of these amino acids. HK-I-mediated inhibition of cytochrome c release induced by staurosporine was also diminished in cells expressing VDAC1 mutants. Our results thus offer new insights into the mechanism by which HK-I promotes tumor cell survival via inhibition of cytochrome c release through HK-I binding to VDAC1. These results, moreover, point to VDAC1 as a key player in mitochondrially mediated apoptosis and implicate an HK-I-mVDAC1 interaction in the regulation of apoptosis. Finally, these findings suggest that interference with the binding of HK-I to mitochondria by VDAC1-derived peptides may offer a novel strategy by which to potentiate the efficacy of conventional chemotherapeutic agents.

Accumulating evidence indicates that the mitochondrionally bound isoforms of hexokinase, HK-I and HK-II, play pivotal roles in promoting cell growth and survival in rapidly growing, highly glycolytic tumors (1). As such, HK-I and HK-II were found to be overexpressed in many types of cancer, including colon, prostate, lymphoma, glioma, gastric adenomas, carcino-

mas, and breast cancers (2–5). The elevated levels of HK-I and HK-II allow tumor cells to evade apoptosis, thereby allowing proliferation to continue (6, 7). HK-I and HK-II dock onto the cytosolic surface of the outer mitochondrial membrane mainly through binding to the voltage-dependent anion channel (VDAC)4 (8). It has been proposed that binding of HK to mitochondria allows a continuous ATP flux, providing energy for the phosphorylation of glucose, and thus an increased glycolytic rate (7). VDAC, also known as mitochondrial porin, functions as the major channel allowing passage of nucleotides, ions, Ca2+ , and other metabolites between the intermembrane space and cytoplasm (9–11).

In vitro and in vivo studies have shown that HK-I and HK-II play a clear role in protecting against mitochondrially regulated apoptosis through direct interaction with mitochondria (3) and, more specifically, with VDAC (6). Several recent studies demonstrated that in tumor cells, HK-I (12–14) and HK-II (15, 16) not only augment cellular energy supply and levels of glucose 6-phosphate, an intermediate metabolic in many biosynthetic pathways, but also protect against cell death. The molecular mechanisms by which mitochondrially bound HK promotes cell survival are not, however, fully understood. Studies relying on purified VDAC, isolated mitochondria, or cells in culture suggest that the anti-apoptotic activity of HK-I occurs via its interaction with VDAC1 and modulation of the mitochondrial phase of apoptosis (6). HK-I interacts directly with VDAC to induce channel closure and prevent the release of cytochrome c. Moreover, HK-I overexpression in U-937 cells protected against apoptotic cell death induced by either staurosporine (6) or VDAC1 overexpression (17). It has also been shown that activation of glycogen synthase kinase 3β (GSK3β) induces the dissociation of HK-II from mitochondria via the phosphorylation of VDAC and that the cytotoxicity induced by chemotherapeutic drugs is increased when the binding of HK-II to mitochondria is disrupted (18).

Interactions of VDAC with other proteins, including creatine kinase (19), cytochrome c (20), the benzodiazepine receptor (21), the adenine nucleotide translocator (22), actin (23), and

4 The abbreviations used are: VDAC, voltage-dependent anion channel; HK, hexokinase; GFP, green fluorescent protein; FCS, fetal calf serum; PBS, phosphate-buffered saline; FACS, fluorescent-activated cell sorter; PLB, planar lipid bilayer; Tricine, N-(2-hydroxyethyl)glycine; PI, propidium iodide; STS, staurosporine.
the mHSP70 heat shock protein have also been proposed. VDAC is proposed to be a critical component of the mitochondrial phase of apoptosis, with its interaction with Bcl-2 family proteins and controlling the rate of release of intermembrane space proteins that activate the execution phase of apoptosis (10). For none of these proteins, however, has the interaction site(s) of VDAC been identified.

The tertiary structure of VDAC has not yet been solved. Several lines of experimental evidence point to the proposal that VDAC structure comprising a transmembrane β-barrel formed by 13 (9) or 16 (25) β-strands and an amphipathic N-terminal α-helix assigned by difference mapping as being exposed to the cytoplasm (25), crossing the membrane (9), or lying on the membrane surface (26).

Recently, we have demonstrated that a single mutation in VDAC1, i.e. glutamate 72 replaced by glutamine, inhibited HK-I interaction with VDAC1 and prevented HK-I-mediated protection against apoptotic cell death induced by overexpression of native VDAC1 (17). In this study, further analysis of those VDAC1 domains interacting with HK-I was carried out by site-directed mutagenesis of murine VDAC1. In doing so, we have localized two cytoplasmic domains in the VDAC1 protein that are required for interaction with HK-I and for HK-I-mediated protection against cell death via inhibiting release of cytochrome c.

**EXPERIMENTAL PROCEDURES**

**Materials**—Carboxymethyl (CM)-cellulose, cis-diammine-dichloroplatinum (II) (cisplatin), n-decane, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, leupeptin, mannitol, phenylmethylsulfonyl fluoride, soybean asolectin, stauroporine, poly-D-lysine, and propidium iodide (PI) were purchased from Sigma. Cibacron blue-agarose was purchased from Amersham Biosciences (Uppsala, Sweden). n-Octyl-β-D-glucopyranoside was obtained from Bachem AG (Bubendorf, Switzerland). Mito Tracker red dye CMXPos was purchased from Molecular Probes. Lauryl-(dimethyl)-amine oxide was obtained from Fluka (Buchs, Switzerland). Hydroxyapatite (Bio-Gel HTP) was purchased from Bio-Rad, and calf thymus DNA was purchased from Merck (Darmstadt, Germany). Monoclonal anti-VDAC antibodies came from Calbiochem-Novobiochem (Nottingham, UK). Monoclonal antibodies against actin and green fluorescence protein (GFP) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-cytochrome c antibodies were obtained from BD Biosciences Pharmingen. Horseradish peroxidase-conjugated anti-mouse antibodies were obtained from Promega (Madison, WI). Metafectene was purchased from Biotex (Munich, Germany). Cell growth medium RPMI 1640 and Dulbecco's modified Eagle's medium and the supplements fetal calf serum (FCS), L-glutamine, and penicillin-streptomycin were purchased from Biological Industries (Kibbutz Beit Haemek, Israel). Blasticidin and zeocin were purchased from InvivoGen (San Diego). Puromycin was purchased from ICN Biomedicals (Eschwege, Germany).

**Plasmids and Site-directed Mutagenesis—**mVDAC1 (obtained from W. J. Craigen, University of Houston) was cloned into the plasmid pEGFP-N1 (Clontech) for construction of mVDAC1-GFP. Site-directed mutagenesis of mVDAC1 was carried out *in vitro* by overlapping PCR amplification. Mutant mVDAC1 genes were constructed using the T7 and SP6 universal primers and those primers described in Table 1. Plasmid pEGFP-N1, carrying the wild-type mVDAC1 gene, served as the template for amplification of mutant mVDAC1 genes. Native or mutated mVDAC1 coding sequences were cloned into the BamH1 and EcoRV restriction sites of the pcDNA4/TO vector (Invitrogen) containing the zeocin resistance gene and two tetracycline operator sites within the human cytomegalovirus immediate-early promoter to allow for tetracycline-regulated expression of mVDAC1 in transfected cells. The HK-I-GFP fusion protein in which GFP was connected to the HK-I C-terminal (pEGFP-HK-I), was constructed using an EcoRI restriction site to introduce GFP into the 3′ (at the stop codon) of HK1 in plasmid pcDNA3.1 (provided by J. E. Wilson, Michigan State University) by site-directed mutagenesis with overlapping PCR amplification, using the following primers: 5′-CCCTTGGAGTCGCAGCTGGAGAATACCTTTGGAGTGACGAAA-3′ (forward) and 5′-GGCTTGGAGATCTCTCTTCGTCTTTCTTCCGGAAGGATGACGAAA-3′ (reverse). HK1 was excised from plasmid pcDNA3.1 by EcoRI and subcloned into pEGFP-N1. All constructs were confirmed by sequencing.

**Tissue Culture—**The U-937 human monocytic cells were grown under an atmosphere of 95% air and 5% CO2 in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 1000 units/ml penicillin, and 1 mg/ml streptomycin. Cells were plated at a density of 5.4 × 10^4 cells/cm^2^ in 24-well plates, washed once with PBS, and placed in serum-free medium. T-REX-293 cells, a transformed primary human embryonal kidney cell line (Invitrogen), were grown under an atmosphere of 95% air and 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS, 2 mM L-glutamine, 1000 units/ml penicillin, 1 mg/ml streptomycin, and 5 μg/ml blasticidin. Other cell lines used are stably transfected derivatives of T-REX-293 cells, a transformed primary human embryonal kidney cell line (Invitrogen), were grown under an atmosphere of 95% air and 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS, 2 mM L-glutamine, 1000 units/ml penicillin, 1 mg/ml streptomycin, and 5 μg/ml blasticidin. MCF7 (breast can-

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**TABLE 1**

| Mutant                        | Primer sequence |
|------------------------------|-----------------|
| E72Q-mVDAC1 Forward          | 5′-GACCTTTCACAGAATGGAC-3′ |
| E65Q-mVDAC1 Forward          | 5′-TTGGACCTTTAGGTCATGGG-3′ |
| K73L-mVDAC1 Reverse          | 5′-TTGGACCTTTAGGTCATGGG-3′ |
| D77N-mVDAC1 Forward          | 5′-GACACAGAAACCCCTGOGG-3′ |
| E202Q-mVDAC1 Forward         | 5′-GACACAGAAACCCCTGOGG-3′ |
| N75A-mVDAC1 Forward          | 5′-GACACAGAAACCCCTGOGG-3′ |
| G67A-mVDAC1 Forward          | 5′-GATGGACTGATA0CCCTGACG-3′ |
| Reverse                      | 5′-GATGGACTGATA0CCCTGACG-3′ |

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cer) cells were grown as U-937 cells except that RPMI 1640 was replaced with Dulbecco’s modified Eagle’s medium.

**Cell Transfection**—Logarithmically growing U-937 cells were resuspended in RPMI 1640 supplemented with 10% FCS, 1000 units/ml penicillin, and 1 mg/ml streptomycin at a concentration of 2.5 × 10^7 cells/ml. Cells were transfected with plasmids pEGFP, pEGFP-mVDAC1, pEGFP-mutated-mVDAC1, or with plasmid pcDNA4/TO containing native or mutated mVDAC1. Transfection was performed by electroporation with a single pulse of a Bio-Rad micropulser II with a capacitance extender.

**Acridine Orange/Ethidium Bromide Staining of Cells**—To determine cell viability, cells were subjected to staining with acridine orange (AcOr) and ethidium bromide (EtBr) in PBS as described previously (27). The AcOr/EtBr-stained cells were visualized by fluorescence microscopy (Olympus IX51), and images were recorded with an Olympus DP70 camera, using a SWB filter. In each independent experiment, in which early and late apoptotic cells were counted, ~300 cells were counted for each treatment.

**Cytochrome c Release Induced by STS or Cisplatin**—Control T-REx 293 cells and VDAC1-shRNA T-REx 293 cells stably expressing native mVDAC1 or E72Q-mVDAC1 under the control of tetracycline (1 μg/ml) were grown on poly-D-lysine (PDL)-coated coverslips, and transfection was performed as previously described (28). Briefly, PLB were pre-

**Flow Cytometry**—T-REx-293 cells (5 × 10^5) were transfected with pcDNA3.1-HK-I and 72 h later with plasmids encoding native-, E72Q-, or L201A-mVDAC1-GFP. 48 h later, the cells were exposed to STS (0.6 μM) for 30 h. The cells and their growth medium were collected (5000 × g for 10 min), with the cells washed twice with PBS, and resuspended in 60 μl of PBS to which 390 μl of cold 80% ethanol was added followed by incubation overnight at −20 °C. One day prior to FACS analysis, the samples were centrifuged (5000 × g for 10 min) and washed twice with PBS. The pellet was resuspended in 0.5 ml of RNase buffer containing 30 μg/ml RNase and 0.1% Triton X-100 in PBS and incubated overnight at 4 °C. The DNA-labeling fluorochromes, propidium iodide, was added to a final concentration of 15 μg/ml, and after 15 min of incubation, DNA content was analyzed using a BD FACSVantage SE flow cytometer (BD Biosciences).

**Confocal Microscopy**—T-REx-293 cells or hVDAC1-shRNA T-REx-293 cells were harvested, washed twice with PBS, and resuspended in 60 μl of cold 80% ethanol. After fixation, the cells were rinsed for 30 min in PBS. Cell imaging was carried out by confocal microscopy (Olympus IX81).

**VDAC Purification, Channel Recording, and Analysis**—Native and mutant mVDAC1 was extracted from mitochondria isolated from yeast expressing these proteins and purified by chromatography on hydroxyapatite followed by carboxymethyl (CM)-cellulose, when LDAO was replaced by β-OG (11). Reconstitution of purified VDAC into a planar lipid bilayer (PLB), current recording, and data analyses were all carried out as previously described (28). Briefly, PLB were prepared from soybean asolectin dissolved in n-decane (50 mg/ml). Only PLB with a resistance greater than 100 GΩ were used. Purified protein (about 1 ng) was added to the cis chamber after one or a few channels were inserted into the PLB, the excess protein was removed by perfusion of the cis chamber with 20 volumes of a solution to prevent further incorporation. Currents were recorded under voltage-clamp using a Bilayer Clamp BC-525B amplifier (Warner Instrument). The currents were measured with respect to the trans side of the membrane (ground). The currents were low-pass, filtered at 1 kHz (~3dB), using a Bessel filter (Frequency Devices 902), and digitized online using a Digidata 1200 interface board and pCLAMP 6 software (Axon Instruments). Sigma Plot 6.0 scientific software (Jandel Scientific) was used for curve fitting. All experiments were performed at 21–25 °C.

**RESULTS**

The interaction of HK-I with VDAC and protection against cell death induced by STS or VDAC1 overexpression has been
demonstrated (3, 6, 17). We showed that E72Q-mVDAC1 no longer retains its capacity to bind HK-I (17). To define the domains and additional amino acid residues involved in the interaction of VDAC1 with HK-I, further mutagenesis of mVDAC1 was carried out. The effects of these mVDAC1 mutations on HK-I protection against cell death were then analyzed by expression of native or mutated mVDAC1 in either U-937 cells, in hVDAC1-shRNA T-REx cells, where the endogenous VDAC1 level was suppressed (by ~85%) (27) or in the same cells overexpressing HK-I. The amino acids to be modified were selected based on sequences predicted to be exposed to the cytosol according to most VDAC1 membrane topology proposed models (9, 26). In addition, they were selected by a comparison to the Saccharomyces cerevisiae VDAC sequence known for its inability to bind HK-I (30).

Charged but Not Neutral Amino Acids in the Glu-72-containing Loop Are Essential for mVDAC1 Interaction with HK-I—The mVDAC1 residue Glu-72, implicated in HK-I binding (17), is not conserved in yeast VDAC1 as well as in Neurospora crassa VDAC, which is also unable to bind HK-I (30). Comparison of the amino acid sequences of the Glu-72-containing loop of human or murine VDAC1 with the same regions of S. cerevisiae or N. crassa VDAC reveals that in addition to Glu-72, the charged amino acids Glu-65, Lys-73, and Asp-77 are not conserved in the fungal proteins. To verify whether these amino acids participate in the mVDAC1-HK-I interaction, they were, respectively, replaced by Gln, Leu, and Asn.

Because VDAC1 overexpression induces cell death (17), U-937 cells were first transformed to overexpress HK-I and only then transfected with plasmids encoding native, E65Q, E72Q-, K73L-, or D77N-mVDAC1-GFP (Fig. 2, A and B). Apoptotic cell death induced by overex-
pression of native mVDAC1 or mVDAC1-GFP was dramatically reduced in cells overexpressing HK-I, dropping from about 75–80% to ~10% (Fig. 2B). On the other hand, as observed with E72Q-mVDAC1-expressing cells, no protective effect of HK-I against cell death was obtained in cells overexpressing E65Q-, K73L-, or D77N-mVDAC1-GFP (Fig. 2B). These results show that although the U-937 cells express endogenous hVDAC1, the presence of mVDAC1 mutants nonetheless completely prevented the anti-apoptotic effect of HK-I.

The anti-apoptotic effect of HK-I was also observed in other cell lines such as T-REx-293 cells. In this case, we used hVDAC1-shRNA-T-REx 293 cells, in which the endogenous human VDAC1 level was suppressed (by ~85%) using VDAC1-shRNA that specifically suppress the expression of human but not murine VDAC1 (27). Thus, hVDAC1-shRNA-T-REx 293 cells were transfected to stably express native, G67A-, E72Q-, or N75A-mVDAC1 under the control of tetracycline. The inhibited growth of the hVDAC1-shRNA-expressing cells (27) was restored by expression of G67A-, E72Q-, or N75A-mVDAC1, as induced by tetracycline (1 μg/ml) (data not shown). To induce cell death by mVDAC1 overexpression, cells stably expressing native and various mutants were exposed to high tetracycline concentrations (2.5 μg/ml) as well as to transiently expressed HK-I (Fig. 2C). In cells overexpressing HK-I, protection against apoptosis was observed only in cells overexpressing native, G67A-, or N75A-mVDAC1, but not in cells overexpressing E72Q-mVDAC1 (Fig. 2C). Immunoblot analyses using anti-HK antibodies of cell extracts confirmed the overexpression of HK-I in the transfected cells (Fig. 2D). These results indicate that HK-I interacts with the proposed VDAC1 cytosolic loop 1 (amino acids 62–80), the E72Q-containing loop, and point to the amino acid residues involved in this interaction.

**mVDAC1 Cytosolic Loop 2 Interaction with HK-I**—Because mutating charged amino acid residues in the proposed cytosolic loop 1 modified HK-I interaction with VDAC1, charged amino acids in the proposed cytosolic loop 2 (amino acids 107–122), Lys-109 and Lys-112, were replaced by Leu to verify their importance in VDAC1-HK-I interaction. In this case, the effects of HK-I overexpression on apoptotic cell death induced by STS in T-REx-293 cells expressing native, K109L-, or K112L-mVDAC1, as induced by tetracycline (1 μg/ml), were examined (Fig. 3A). HK-I overexpression protected against STS-induced apoptosis in cells expressing native, K109L-, or K112L-mVDAC1, reducing cell death from 60 to 12–20%. These results indicate that HK-I protects against cell death induced by STS and suggest that the proposed cytosolic loop 2 is not involved in the interaction of VDAC1 with HK-I.

**Negatively Charged Amino Acids in the Glu-202-containing Loop Are Essential for the Interaction of mVDAC1 with HK-I**—The function of the VDAC1 proposed cytosolic loop 4 (amino acids 186–227) in the interaction of the protein with HK-I was also verified. Glutamate 202 was mutated, because it was found to be essential for the interaction of ruthenium red with VDAC1 to prevent apoptotic cell death (31). When E202Q mVDAC1 was overexpressed in cells overexpressing HK-I, no protection against cell death was observed, in contrast to cells overexpressing the native mVDAC1 (Fig. 2C). Next, the amino acids in this loop: Glu-188, Lys-200, and Leu-201 were replaced by Gln, Leu, and Ala, respectively expressed in T-REx-293 cells expressing native or E72Q-mVDAC1. A, T-REx-293 HEK cells were transfected with pcDNA3.1-HK-I. After 72 h, these cells were transfected with pcDNA4/TO encoding native, E72Q-, K109L-, K112L-, E188Q-, K200L-, or L201A-mVDAC1, and protein expression was triggered by tetracycline (1 μg/ml) addition. 72 h after the second transfection, cells were exposed to STS (1.25 μM) for 5 h, and cell viability was analyzed by acridine orange/ethidium bromide staining (n = 3). Quantitative analysis of apoptosis in the different cells was performed by analysis of variance and t tests between control cells and other cells exposed to staurosporine; p < 0.001 (***" was considered statistically significant. Data shown are the mean ± S.E. B, release of cytochrome c induced by STS in T-REx-293 cells expressing native or E72Q-mVDAC1 was analyzed by immunoblotting using anti-cytochrome c antibodies.
results are in agreement with those obtained when apoptosis was analyzed by acridine orange/ethidium bromide staining (Figs. 2–4) or by cytochrome c release (Fig. 3). These results suggest that the interaction of HK-I with mVDAC1 involves the negatively charged amino acids in this loop together with charged residues in the Glu-72-containing loop; both are exposed to the cytoplasm according to a proposed topology model (9).

Cytochrome c Release Induced by STS or Cisplatin Is Inhibited by HK-I in Cells Expressing Native but Not E72Q-mVDAC1—Next, we tested whether HK-I inhibits cytochrome c release as activated by STS in T-Rex-293 cells expressing native or E72Q-mVDAC1. hVDAC1-shRNA-T-Rex-293 cells expressing mVDAC1 or E72Q-mVDAC1 under the control of tetracycline (1 µg/ml) were transfected to overexpress HK-I. The cytochrome c released from mitochondria to the cytosol during STS-activated apoptosis in these cells was then analyzed by immunoblotting, using anti-cytochrome c antibodies (Fig. 3B). HK-I overexpression inhibited cytochrome c release in cells expressing native but not E72Q-mVDAC1. Moreover, HK-I overexpression in MCF7 cells also inhibited apoptotic cell death (Fig. 5A) and cytochrome c release (Fig. 5B), as induced by STS or cisplatin. To rule out mitochondrial contamination in the cytosolic fractions as the source of cytochrome c, the presence of VDAC was ruled out by immunoblotting (data not shown). These findings correlate with the inability of HK-I to protect against cell death induced by STS in cells expressing E72Q-mVDAC1 (Figs. 3 and 4) or by overexpressing E72Q-mVDAC1 (Fig. 2), and is consistent with the inability of HK-I to interact with bilayer-reconstituted E72Q-mVDAC1 (Fig. 6).

E202Q-mVDAC1 Reconstituted into a Bilayer Displays a Weakened HK-I Binding Capacity—The interaction of HK-I with VDAC1 was also addressed in terms of the effect of HK-I on channel activity. Addition of HK-I at a holding potential of −10 mV had no effect on channel activity, even after 20 min of incubation. However, when the holding potential was switched for 1 min to +60 mV, HK-I inhibited channel activity in a time-dependent manner, stabilizing the channel in different conformational states with subsequently reduced conductance (Fig. 6A). Upon addition of the HK reaction product, glucose 6-phosphate (known to detach bound HK, Ref. 32), the HK-I-closed channel immediately recovered its maximal conductance (data not shown). Furthermore, not only positive high voltages but also high negative potentials converted VDAC1 to an HK-I-sensitive channel (not shown).

Because replacement of glutamate 202 with glutamine diminished HK-I protection against cell death (Fig. 2C), E202Q-mVDAC1 was reconstituted into a PLB to determine whether this residue indeed participated in HK-I binding to
mVDAC1 (Fig. 6B). Purified recombinant native, E72Q-, and E202Q-mVDAC1 were PLB-reconstituted, and the currents produced in response to voltages stepped from a holding potential of 0 to $-40 \text{ mV}$ were recorded before and 5 min after the addition of HK-I (Fig. 6B). Upon addition of purified HK-I to native mVDAC1, the channel conductance was reduced, and the channel was stabilized in a low-conducting state. On the other hand, HK-I had no effect on the conductance of E72Q-mVDAC1, even following incubation for up to 30 min. E202Q-mVDAC1 showed similar results as E72Q-mVDAC1, although with longer incubation times (>20 min), a HK-I-induced decrease in E202Q-mVDAC1 channel conductance was observed ($n = 5$). These results suggest that Glu-72 is essential for the interaction of VDAC1 with HK-I, while Glu-202 is most likely required for stabilization of the HK-I-VDAC1 interaction.

Finally, to demonstrate that HK-I does not interact with E72Q-mVDAC1 in the cell, we expressed the HK-I-GFP fusion protein in control cells and cells stably expressing native or E72Q-mVDAC1 and visualized its cellular distribution (Fig. 7). Confocal fluorescence microscopy showed that in control cells, HK-I-GFP fluorescence is punctuated and extensively co-localized with MitoTracker, a dye that specifically labels mitochondria in living cells. Similar distribution of HK-I-GFP was observed in cells stably expressing mVDAC1. On the other hand, HK-I-GFP fluorescence in cells expressing E72Q-mVDAC1 was diffused in the cytosol and not co-localized with MitoTracker. These results indicate that indeed, HK-I does not bind to the E72Q-mVDAC1 mutant as also demonstrated with the bilayer-reconstituted purified protein (Fig. 6).

**DISCUSSION**

It has been shown that tumor cells exhibit a high rate of glycolysis (7), now known to be due, in part, to the greatly increased expression of mitochondrially bound HK-I and HK-II in transformed cells (12–14). In vitro and in vivo studies have also shown that HK-I and HK-II play a clear role in protecting cells against mitochondrially regulated apoptosis through their direct interactions with VDAC (6, 15, 17, 18). Indeed, overexpression of HK-I in the tumor-derived cell line U-973 or vascular smooth muscle cells suppressed STS-, cisplatin-, or VDAC1 overexpression-induced apoptotic cell death (Figs. 2–5). HK-II-mediated protection against apoptotic cell death has also been reported in Rat1a fibroblasts (3), NIH-3T3 cells (33), and WEHI 7.1 cells (15). These results suggest that...
HK-I or HK-II, via their interaction with VDAC1, prevent key events in mitochondrially mediated apoptosis. It is well established that the N-terminal of HK is required for its interaction with the mitochondria (34–36) and VDAC (6). However, the VDAC1 sequences interacting with HK-I or HK-II remain largely unknown. As such, this study has addressed those VDAC1 amino acids and loops essential for HK-I binding and the resulting protection against cell death.

Two VDAC1 Loops Are Proposed to Form the Site of HK-I Interaction or Part Thereof—The tertiary structure of VDAC1 has not yet been solved. Computer modeling of the VDAC1 primary sequence has, however, led to the development of several models proposing the transmembrane organization of the protein, predicting a single α-helix and 13 or 16 trans bilayer β-strands (9, 37). These β-strands are connected by several peptide loops of different sizes on both sides of the membrane that possibly present sites of interaction for VDAC1-associated proteins, including HK-I and HK-II.

The finding that a single point mutation, E72Q or E65Q, abolished binding of HK-I to mutated VDAC1 reconstituted into a bilayer (Fig. 6) or expressed in T-REx-293 cells (Fig. 7) and prevented the protective effect of HK-I against cell death induced by overexpression of VDAC1 in U-937 or T-REx-293 cells (Ref. 17 and Figs. 2 and 3) points to the predicted Glu-72-containing loop serving as the HK-I-binding site, or part of it. This suggestion is further supported by the findings that modification of the VDAC1 glutamate 72 with DCCD prevented HK binding to mitochondria (8, 38), as well as HK-I-mediated inhibition of cytochrome c release (Fig. 3). Taking into account the voltage dependence of the interaction of both HK-1 (Fig. 6) and DCCD with VDAC1 leading to a decrease in VDAC conductance but not complete closing of the channel (39, 40) and the involvement of Glu-72 in this interaction, it is possible that the domain containing Glu-72 is not accessible unless conformational changes in VDAC are first elicited by exposure to high voltages. Indeed, various studies have proposed that the Glu-72-containing loop is part of the voltage sensor described as a mobile region in yeast and N. crassa VDAC (41, 42).

According to one of the proposed VDAC1 topology models, Glu-72 is located in the middle of the first cytoplasmic loop (9). Therefore, six other amino acids in this proposed loop were mutated, and the effects of such mutations on HK-I binding to purified VDAC1 and HK-I protection against cell death were analyzed. The finding that other charged amino acid residues, i.e. Glu-65, Glu-72, Lys-73, and Asp-77, are essential for HK-I binding to VDAC1 and protection against cell death (Fig. 2) strongly points to this loop as interacting with HK-I. A second Glu-202- and Glu-188-containing proposed mVDAC1 loop was also found to be involved in the interaction of HK-I with mVDAC1 (Fig. 6B) and in the protection against cell death produced by HK-I (Fig. 3). The finding that the inability of E202Q-mVDAC1 to bind HK-I is overcome after a relatively long incubation time suggests that the Glu-202-containing loop is required for stabilization of the interaction between HK-I and VDAC1. It should be noted that as Glu-72, Glu-202 was also found to be essential for the interaction of ruthenium red with VDAC1 and protecting against cell death (31).

The association of HK-I or HK-II with VDAC1 was suggested to involve a hydrophobic 15 amino acid sequence found in the N-terminal regions of HK-I and HK-II, serving an anchoring role at an early step of the interaction (34, 35). Indeed, removal of this HK-I N-terminal region eliminated interaction of the protein with VDAC (6, 35). The role of the N-terminal HK-I sequence in the mitochondrial anchoring of the protein was also demonstrated using anti-HK-I-N-terminal peptide polyclonal antibodies, able to inhibit HK-I binding to mitochondria (43). Our results, however, uncover the amino acids and domains in VDAC1 involved in the association with HK-I namely the Glu-72- and Glu-202-containing VDAC1 loops.

The requirement of the negatively charged glutamate 65, 72, 188, and 202 residues may be related to the dependence of HK binding to mitochondria on Mg2+ ions, proposed to encourage direct electrostatic interactions between HK and the surface of the outer mitochondrial membrane via phospholipids or other components (44, 45). Mg2+ has been proposed to form a bridge between repulsive negative charges on both HK-I and the outer mitochondrial membrane (45) or may present a structurally specific bridge linking HK and VDAC. In our experiments addressing the interaction of HK-I with VDAC1 (in bilayer or in isolated mitochondria) no Mg2+ was, however, present or required, yet HK-I nonetheless bound to VDAC1.

It has been shown that Akt activation inhibits apoptosis partly by promoting the binding of HK-II to the mitochondria (3). This effect of Akt was shown to be mediated by negative regulation of GSK3β activity. GSK3β protein-phosphorylated VDAC is unable to bind HK-II that dissociates from the mitochondria and thus allows apoptosis induction (18).

Cisplatin Activation of Cytochrome c Release Is Mediated by VDAC1—The cytotoxicity of cisplatin has generally been attributed to its ability to form inter- and intra-strand nuclear DNA cross-links (46). However, recent studies suggest that mitochondria are also critical targets of cisplatin (47, 48). Cisplatin was found to induce mitochondrial dysfunction in renal proximal tubular cells (RPTC), and the release of cytochrome c from the mitochondria to the cytosol (49) from mitochondria isolated from cells of the cisplatin-sensitive head and neck cancer cell line, PCI-13 (48). In addition, an up-regulation of Bcl-2 was found to inhibit cisplatin-induced apoptosis of renal proximal tubular cells in rat (50). Moreover, it has been demonstrated that cisplatin interacts directly with VDAC. It was demonstrated that the level of cisplatin bound to VDAC is more than 200-fold higher than the amount bound by total cellular proteins (48). In addition, when cells of the cervix squamous cell carcinoma (A431) cell line were exposed to cisplatin, the level of VDAC1 was found to be down-regulated by a factor of three, consistent with activation of mechanisms acting to protect cells from drug-induced damage (24).

Our results demonstrating that cisplatin induces cytochrome c release and apoptotic cell death that can be inhibited by HK-I (Fig. 5) strongly suggest that cisplatin activates the mitochondrially mediated pathway. Moreover, the inability of HK-I to protect against cisplatin-induced cytochrome c release in cells expressing mutated E72Q-mVDAC1 (Fig. 5) suggests that VDAC1 is involved in cisplatin induction of apoptosis. Thus, taken together, these findings indicate that the mito-
chondrially dependent pathway plays a critical role in the cytotoxic action of cisplatin.

To conclude, the interaction of HK-I with the two proposed VDAC1 cytosolic loops resulted in HK-I-mediated inhibition of cytochrome c release (Figs. 3 and 4 and Ref. 6) and in the protective effect of HK-I against apoptotic cell death (Figs. 2–4 and Refs. 6, 17). Therefore, inhibition of HK-I or HK-II interaction with VDAC may initiate mitochondrial apoptotic signaling cascades. Moreover, these results clearly reveal the involvement of VDAC1 in mitochondrially mediated apoptosis. Thus, these findings could lead to the use of peptides representing the two VDAC1-HK-I-binding Site

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