In self-defence: hexokinase promotes voltage-dependent anion channel closure and prevents mitochondria-mediated apoptotic cell death

Heftsi AZOULAY-ZOHAR, Adrian ISRAELSON, Salah ABU-HAMAD and Varda SHOSHAN-BARMATZ[1]

Department of Life Sciences, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel

In tumour cells, elevated levels of mitochondria-bound isoforms of hexokinase (HK-I and HK-II) result in the evasion of apoptosis, thereby allowing the cells to continue proliferating. The molecular mechanisms by which bound HK promotes cell survival are not yet fully understood. Our studies relying on the purified mitochondrial outer membrane protein VDAC (voltage-dependent anion channel), isolated mitochondria or cells in culture suggested that the anti-apoptotic activity of HK-I occurs via modulation of the mitochondrial phase of apoptosis. In the present paper, a direct interaction of HK-I with bilayer-reconstituted purified VDAC, inducing channel closure, is demonstrated for the first time. Moreover, HK-I prevented the Ca^{2+}-dependent opening of the mitochondrial PTP (permeability transition pore) and release of the pro-apoptotic protein cytochrome *c*. The effects of HK-I on VDAC activity and PTP opening were prevented by the HK reaction product glucose 6-phosphate, a metabolic intermediate in most biosynthetic pathways. Furthermore, glucose 6-phosphate re-opened both the VDAC and the PTP closed by HK-I. The HK-I-mediated effects on VDAC and PTP were not observed using either yeast HK or HK-I lacking the N-terminal hydrophobic peptide responsible for binding to mitochondria, or in the presence of an antibody specific for the N-terminus of HK-I. Finally, HK-I overexpression in leukaemia-derived U-937 or vascular smooth muscle cells protected against staurosporine-induced apoptosis, with a decrease of up to 70 % in cell death. These results offer insight into the mechanisms by which bound HK promotes tumour cell survival, and suggests that its overexpression not only ensures supplies of energy and phosphometabolites, but also reflects an anti-apoptotic defence mechanism.

Key words: apoptosis, hexokinase, mitochondria, permeability transition pore, voltage-dependent anion channel (VDAC).

INTRODUCTION

Cancer cells are characterized by a high rate of glycolysis, which serves as their primary energy-generating pathway [1,2]. The molecular basis of this high rate of glycolysis involves a number of genetic and biochemical events [2], including overexpression of mitochondrial-bound isoforms of hexokinase (HK-I and HK-II) [2–4]. The bound enzyme is less susceptible to inhibition by its product, glucose 6-phosphate (G-6-*P*), a metabolic intermediate precursor in most biosynthetic pathways [5].

Accumulating evidence indicates that HK (both HK-I and HK-II) plays a pivotal role in cancer by promoting cell growth and survival [6,7]. Recent studies [8] indicate that, in malignant cancer, HK-II not only improved the cell's energy supply, but also protected against cell death. HK-II has also been shown to inhibit Bax-induced cytochrome *c* release and apoptosis in HeLa cells [7]. It has also been proposed that elevated binding of HK-I and/or HK-II to mitochondria in tumour cells is due to an increase in the expression of the VDAC (voltage-dependent anion channel) [9].

VDAC, an integral protein of the mitochondrial outer membrane also known as mitochondrial porin, is a large channel that transports anions, cations [10,11], Ca^{2+} [12], adenine nucleotides [13] and other metabolites [14] into and out of mitochondria. VDAC is also believed to be a constituent of the mitochondrial PTP (permeability transition pore). Current models suggest that a PTP is formed at contact sites between the inner and outer membranes by association of VDAC in the outer membrane with the ANT (adenine nucleotide translocator) in the inner membrane, cyclophilin D in the matrix, and possibly other proteins [15,16]. Regulated opening of the PTP leads to a rapid loss of mitochondrial membrane potential, organellar swelling and release of the apoptogenic protein cytochrome *c*. This, in turn, activates caspases (death-driving proteolytic proteins) [17] and leads to apoptotic cell death [17–19].

HK-I and HK-II share high functional similarity and differ only in tissue distribution [20]. Both isoforms interact via a hydrophobic 15-amino-acid sequence in their N-terminal region with mitochondrial VDAC [21–23]. The occurrence of an interaction between HK-I and VDAC is supported by various pieces of evidence: the enrichment of both HK-I and VDAC at the contact site fraction [23], binding of HK-I to VDAC reconstituted into liposomes [24], and co-purification of HK-I with the VDAC–ANT complex [23]. However, none of the findings demonstrated a direct interaction of HK-I with VDAC; nor did they reveal the exact molecular mechanism of this interaction.

Here we demonstrate that HK-I interacts with isolated mitochondria, and prevents the opening of the PTP and PTP-mediated cytochrome *c* release. Moreover, HK-I binds directly to bilayer-reconstituted VDAC and induces closure of its channel. These results suggest that HK-I, by interacting with VDAC, prevents key events in mitochondria-mediated apoptosis.

EXPERIMENTAL

Materials

ADP, ATP, atractyloside, BSA, CM-cellulose, CsA (cyclosporin A), G-6-*P*, G-6-*P* dehydrogenase, Hepes, leupeptin, mannitol, n-decane, PMSF, soybean asolectin, staurosporine, sucrose, Tris, Reactive Red–agarose and Triton X-100 were purchased from

Abbreviations used: ANT, adenine nucleotide translocator; CsA, cyclosporin A; GFP, green fluorescent protein; G-6-*P*, glucose-6-phosphate; HK, hexokinase; LDAO, lauryl(dimethyl)amine oxide; PLB, planar lipid bilayer; PTP, permeability transition pore; VDAC, voltage-dependent anion channel.

[1] To whom correspondence should be addressed (e-mail vardasb@bgumail.bgu.ac.il).

Sigma (St. Louis, MO, U.S.A.). Cibacron Blue–agarose was purchased from Amersham Biosciences (Uppsala, Sweden), and $^{45}Ca^{2+}$ was purchased from NEN® Life Science Products (Boston, MA, U.S.A.). n-Octyl β-D-glucopyranoside was obtained from Bachem AG, and LDAO [lauryl(dimethyl)amine oxide] was obtained from Fluka. Hydroxyapatite (Bio-Gel HTP) was purchased from Bio-Rad Laboratories (Hercules, CA, U.S.A.), and Celite was from BDH (Poole, Dorset, U.K.).

Monoclonal anti-VDAC antibodies raised against the N-terminal region of 31HL human porin [25] (clone no. 173/045; cat. no. 529538-B) were from Calbiochem-Novobiochem (Nottingham, U.K). Monoclonal antibodies against rat brain HK were kindly provided by Dr John E. Wilson (University of Michigan, Ann Arbor, MI, U.S.A.), and polyclonal anti-HK-I antibodies (M-15) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A). Monoclonal antibodies against cytochrome *c* were obtained from Pharmingen (San Diego, CA, U.S.A.). Horseradish peroxidase-conjugated goat anti-mouse antibodies were from Protos Immunoresearch (San Francisco, CA, U.S.A.). Alkaline phosphatase-conjugated goat anti-mouse IgG was obtained from Promega (Madison, WI, U.S.A.).

Mitochondrial preparation

Mitochondria were isolated from rat liver using published procedures [26].

Ca^{2+} accumulation

Ca^{2+} uptake by freshly prepared rat liver mitochondria (0.5 mg/ml) was assayed for 1–20 min at 30 °C in the presence of 225 mM mannitol, 75 mM sucrose, 120 μM $CaCl_2$ (containing 3×10^4 c.p.m./nmol $^{45}Ca^{2+}$), 5 mM Hepes/KOH, 5 mM succinate and 0.1 mM P_i, pH 7.0. $^{45}Ca^{2+}$ uptake was terminated by rapid Millipore filtration followed by a wash with 5 ml of 150 mM KCl [12].

Mitochondrial swelling

Ca^{2+}-induced large-amplitude mitochondrial swelling was assayed in freshly prepared mitochondria under the same conditions as for Ca^{2+} accumulation, except that the temperature was 24 °C. CsA-sensitive mitochondrial swelling was initiated by the addition of Ca^{2+} (0.2 mM) to the sample. Absorbance changes at 520 nm were monitored every 15–20 s with an Ultraspec 2100 spectrophotometer.

Release of cytochrome *c*

Mitochondria (0.5 mg/ml) were incubated with or without 1 unit/ml HK-I in a solution containing 150 mM KCl, 25 mM $NaHCO_3$, 5 mM succinate, 1 mM $MgCl_2$, 3 mM KH_2PO_4 and 20 mM Hepes, pH 7.4 [8]. Swelling was monitored after the addition of 200 μM Ca^{2+}, and following complete swelling the samples were centrifuged and mitochondrial pellets (25 μg) or supernatant (40 μl) were subjected to SDS/PAGE and Western blot analysis using monoclonal antibodies against cytochrome *c*.

Purification, proteolytic treatment and activity of rat brain HK-I

Rat brain HK-I was purified according to Wilson [27]. Briefly, crude brain mitochondria containing tightly bound HK-I were incubated with 1.2 mM G-6-*P* and 0.5 mM K-EDTA to release the enzyme into the soluble fraction. Further purification of HK-I was achieved by affinity chromatography on Cibacron Blue–agarose (Pharmacia). The purified enzyme was concentrated and washed in 0.5 mM K-EDTA, 10 mM glucose, 1 mM dithiothreitol and 10 mM K-Hepes, pH 7.8, using an Amicon device and a 50 000 Da molecular mass cut-off membrane (Spectrum; type C). The concentrated enzyme (up to 1 mg/ml; 30–60 units/mg) was frozen in liquid nitrogen and stored at − 80 °C until use.

For proteolytic treatment, HK-I was incubated for 1 h on ice with trypsin or chymotrypsin at a protease/HK-I ratio of 1:4 (w/w) in a solution containing 10 mM glucose, 5 mM Mg-Hepes and 20 mM K-Hepes, pH 7.8. Proteolysis was terminated by addition of a 5-fold excess (w/w) of trypsin inhibitor or 0.5 mM PMSF. In the case of chymotrypsin treatment, removal of the N-terminal hydrophobic peptide of HK-I was confirmed by Western blot analysis using a specific monoclonal antibody.

HK-I activity was measured spectrophotometrically at room temperature at 340 nm by coupling NADH formation to the production of G-6-*P* by HK-I and its oxidation by G-6-*P* dehydrogenase. The reaction mixture (1 ml) contained 4 mM Mg-Hepes, 1 mM K-EDTA, 0.6 mM NAD^+, 10 mM glucose, 1 mM ATP, 1 mg/ml BSA and 20 mM K-Hepes, pH 7.8. A 100-fold excess of G-6-*P* dehydrogenase over HK-I was employed.

Binding of rat brain HK-I to mitochondria

Mitochondria (0.5–2 mg/ml) were incubated on ice for 1 h with HK-I (0–2 unit/ml) in 0.1 ml of iso-osmotic medium supplemented with 5 mM Mg-Hepes, pH 7.8, and 1 mg/ml BSA. Soluble and mitochondria-bound HK-I fractions were separated and analysed for free and bound HK-I activity respectively [28]. HK-I binding was defined as the percentage activity present in the bound fraction relative to the total activity in the bound and free fractions combined. In some cases, HK-I binding was assayed under conditions of mitochondrial swelling.

Protein determination

Mitochondrial protein was determined by the Biuret method [29], while HK protein concentration was determined according to Bradford [30], using ovalbumin as a standard.

VDAC purification

VDAC was extracted from mitochondria with 3 % Triton X-100 and purified using chromatography on hydroxyapatite and Reactive Red–agarose in the presence of 0.3 % Triton X-100 [31]. For the study of the interaction of HK-I with VDAC, a novel procedure for the purification of VDAC using the detergent β-octyl glucoside was developed. Briefly, rat liver mitochondria (200 mg of protein) were incubated for 30 min at 0 °C (at 5 mg/ml) in a solution containing 10 mM Tris, pH 7.0, 0.15 mM PMSF, 0.5 μg/ml leupeptin and 0.05 % LDAO. After centrifugation at 44 000 ***g*** for 20 min, the supernatant was discarded and the pellet was resuspended at 5 mg/ml in the above solution containing 2 % LDAO. After centrifugation at 44 000 ***g*** for 30 min, the LDAO-extracted VDAC was applied to a dry hydroxyapatite/celite (2:1, w/w) column (0.08 g/mg of protein) and eluted with a buffer containing 10 mM Tris, pH 7.4, 50 mM NaCl, 20 mM sodium phosphate and 2 % LDAO. The VDAC-containing fractions (identified by Coomassie Blue staining) were collected, diluted 5-fold with 10 mM Tris, pH 7.4, and loaded on to a CM-cellulose column pre-equilibrated with 10 mM Tris, pH 7.4, and 0.5 % n-octyl β-D-glucopyranoside. The loaded column was then washed with the same equilibration buffer, and VDAC was eluted with the same buffer containing 0.4 M NaCl.

VDAC recording and analysis

Reconstitution of the purified VDAC into a PLB (planar lipid bilayer), single channel current recording and data analyses were carried out as described previously [12]. Briefly, a PLB was prepared from soybean asolectin dissolved in n-decane (50 mg/ml). Purified VDAC (approx. 1 ng) was added to the chamber defined as the *cis* side. After one or a few channels were inserted into the PLB, excess protein was removed by washing the *cis* chamber with 20 vol. of solution to prevent further incorporation. Currents were recorded under a voltage clamp using a Bilayer Clamp BC-525B amplifier (Warner Instruments, Hamden, CT, U.S.A.). Currents were measured with respect to the *trans* side of the membrane (ground). The currents were low-pass-filtered at 1 kHz using a Bessel filter (Frequency Devices, Haverhill, MA, U.S.A.), and digitized on-line using a Digidata 1200-interface board and PCLAMP 6 software (Axon Instruments Inc., Union City, CA, U.S.A.).

Gel electrophoresis and immunoblot analyses

SDS/PAGE was performed according to Laemmli [32] and gels were stained with Coomassie Brilliant Blue. For immunostaining, the membranes were blocked with 5 % (w/v) non-fat dry milk and 0.1 % Tween-20 in Tris-buffered saline, incubated with monoclonal anti-VDAC antibodies (1:7000) or anti-HK-I antibodies (1:2000), and then with alkaline phosphatase-conjugated anti-mouse IgG or horseradish peroxidase-conjugated anti-mouse IgG as a secondary antibody (1:10 000). Using the former, the coloured reaction product was developed with 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium, while a Super Signal West Pico kit for an enhanced chemiluminiscent substrate (Pierce, Rockford, IL, U.S.A.) was used for the detection of horseradish peroxidase.

Tissue culture

The U-937 human monocytic cell line was grown under an atmosphere of 95 % air/5 % CO_2 in RPMI 1640 supplemented with 10 % (v/v) fetal calf serum, 1 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Exponentially growing U-937 cells were resuspended in RPMI 1640 supplemented with 10 % fetal calf serum and 1 mM L-glutamine at a concentration of 2.5×10^7 cells/400 μl. The vascular smooth muscle cell line was grown under the same conditions as described for U-973 cells, except that the medium was minimum essential medium. Transfection with the mammalian expression vectors pEGFP, pcDNA3 or pcDNA3-HK-I (provided by Dr J. E. Wilson, University of Michigan) was performed by electroporation with a single pulse from a Bio-Rad Micropulser II with a capacitance extender unit (200V, 950 μF). Transfection efficiencies were 68–72 %, as assessed by GFP (green fluorescent protein) expression. Transfected cells were incubated on ice for 10 min before and after transfection, and then resuspended in 20 ml of RPMI 1640 supplemented with 10 % fetal calf serum, 1 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. The cells were then grown for 72 h prior to the induction of apoptosis by the addition of 1.25 μM staurosporine.

Treatment of cells

Cells were plated at a density of 5.4×10^4 cells/cm^2 in 24-well plate, washed once with PBS and placed in serum-free medium. The cells were then either left untreated or treated with staurosporine (1.25 μM) to induce apoptosis. Cells were analysed for viability after 3, 5 and 7 h.

Acridine Orange/ethidium bromide staining of cells

To determine cell viability, cells were subjected to Acridine Orange/ethidium bromide staining. Cells were centrifuged at 1500 ***g*** for 5 min at room temperature and then resuspended in a 25 μl of medium, to which 2 μl of a solution containing 100 μg/ml Acridine Orange and 100 μg/ml ethidium bromide in PBS was added. The cells were then visualized by fluorescence microscopy (Olympus BX60, equipped with a CCD camera), using a blue filter.

RESULTS

HK-I interacts specifically with VDAC to induce channel closure

Highly purified (> 98 %) mitochondrial VDAC (see Figure 1C), isolated from liver mitochondria using CM-cellulose and β-octyl glucoside, was reconstituted into a PLB and its channel activity was studied under voltage-clamp conditions [12,33]. Current passing through the VDAC in response to voltages stepped from a holding potential of 0 mV to the potentials indicated in each current trace was recorded prior to the addition of HK-I. At relatively small membrane potentials (− 10 mV), the conductance remained constant for up to 120 min of recording (Figure 1). However, upon addition of purified HK-I, the open channel was stabilized in its low-conducting state (Figure 1A, I). HK-I-promoted VDAC closure was observed at all voltages tested, with the VDAC adopting a constant conductance regardless of the voltage gradient applied (Figure 1B). HK-I modified VDAC conductance only when added to the *cis* side of the bilayer in which VDAC was reconstituted, indicating, as expected, that HK-I interacts with the cytosolic face of VDAC [22].

The specificity of the interaction of HK-I with VDAC was demonstrated by several observations. Yeast HK had no effect on VDAC conductance (Figure 1A, III). In addition, HK-I subjected to chymotryptic (Figure 1A, II) or tryptic (results not shown) digestion failed to modify VDAC conductance. It has been shown that treatment of HK-I and HK-II with trypsin or chymotrypsin results in removal of the N-terminal hydrophobic peptide of HK-I that is responsible for its binding to mitochondria [21]. Indeed, protease-treated HK-I did not cross-react with a specific monoclonal antibody against the HK-I N-terminus (Figure 1C), indicating the requirement of the HK-I N-terminal region for its interaction with VDAC. Furthermore, HK-I was able to close the VDAC in the presence of yeast HK (Figure 1A, III) or chymotrypsin-treated HK-I (Figures 1A, II and 1A, III).

The effect of HK-I on VDAC was reversed by the HK reaction product G-6-*P*, a central metabolic intermediate precursor in biosynthetic pathways. Furthermore, addition of G-6-*P* led to the re-opening of channels closed by HK-I (Figure 1A, III), supporting a reversible interaction of HK-I with VDAC.

HK-I prevents opening of the PTP

Opening of the mitochondrial PTP is thought to be one of the checkpoints that mediate cell entry into apoptosis [15–19,34–36]. Since VDAC is a proposed component of the PTP, the effects of HK-I on PTP opening, as monitored by Ca^{2+} accumulation and swelling of energized mitochondria, and by PTP-mediated release of cytochrome *c*, were addressed. When freshly prepared mitochondria were allowed to generate a membrane potential, transient Ca^{2+} accumulation was observed (Figure 2A). This suggests that mitochondria undergo a permeability transition and lose accumulated Ca^{2+} via the PTP [12,15,18]. Addition of HK-I prior to PTP induction, or even after Ca^{2+} accumulation had reached its peak, yet before PTP activation, prevented the release

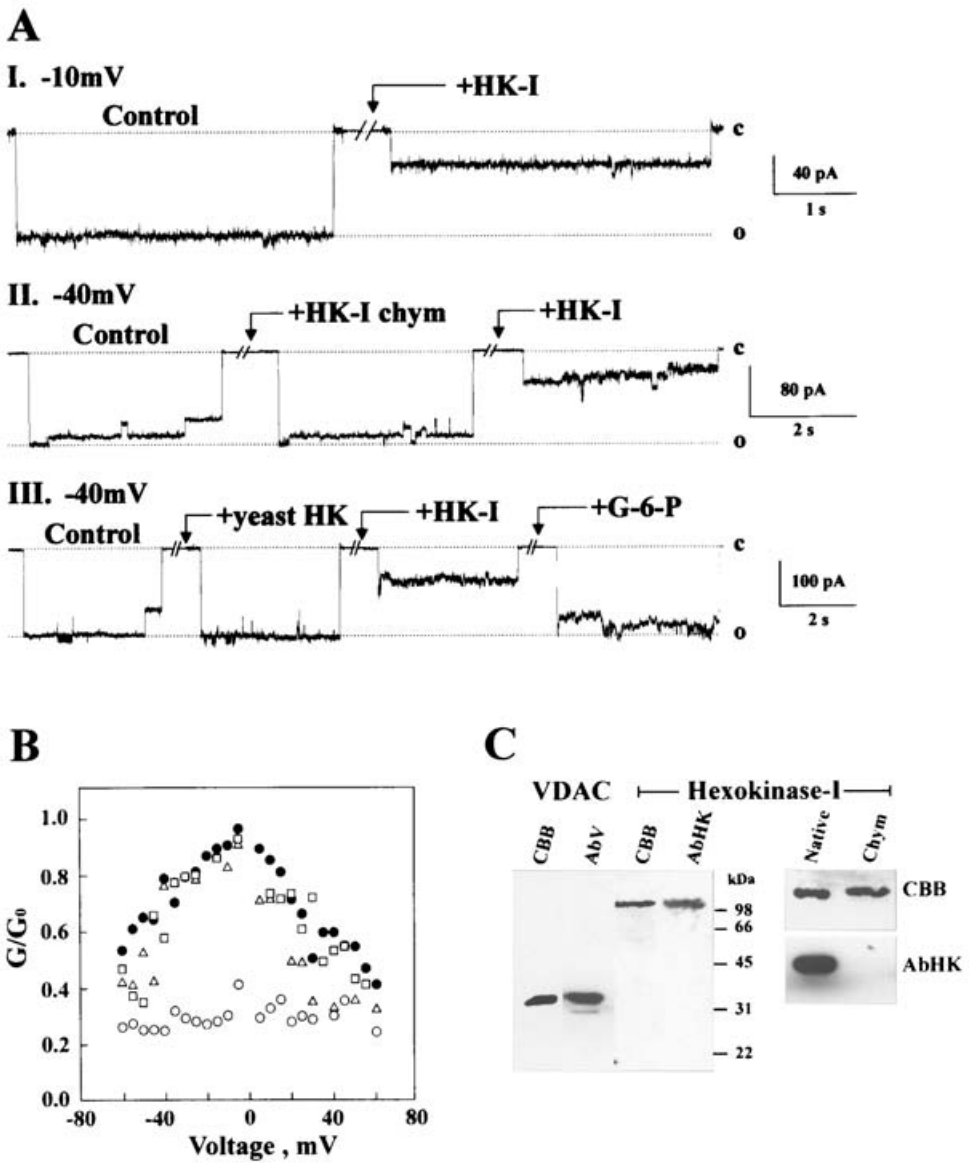

Figure 1 HK-I interacts specifically with the VDAC and induces channel closure

(**A**) Purified VDAC was reconstituted into a PLB, and channel currents through VDAC were recorded. Panel I shows currents through the VDAC in response to a voltage step from 0 to − 10 mV, recorded before and 2 min after the addition of HK-I (28.6 m-unts/ml). Panels II and III show similar experiments as in I, except that voltage was stepped from 0 to − 40 mV and, where indicated, chymotrypsin-treated HK-I or yeast HK respectively (each 28.6 m-units/ml) was added. Channels closed by HK-I were re-opened by G-6-*P* (1.0 mM). The dotted lines indicate current levels in the open (o) and closed (c) states. (**B**) Multi-channel (approx. 10 channels) recordings of the average steady-state conductance of VDAC before (●) and after the addition of HK-I (○), yeast HK (□) or chymotrypsin-treated HK-I (△) as a function of voltage. Relative conductance was determined as the ratio of conductance at a given voltage (G) to maximal conductance (G_0). (**C**) Coomassie Brilliant Blue (CBB) staining and immunoblots (Ab) of purified VDAC, HK-I and chymotrypsin-treated HK-I (Chym). Immunostaining was conducted using monoclonal antibodies raised against either the N-terminus of VDAC (AbV) or rat brain HK (AbHK). The positions of marker proteins are indicated.

of accumulated Ca^{2+} (Figure 2A). In the presence of HK-I, mitochondrial swelling, as monitored by the change in absorbance following Ca^{2+} addition, was also prevented (Figure 2B).

Specificity of the effect of HK-I in preventing PTP opening and cytochrome *c* release

The specificity of the effect of HK-I on PTP opening was reflected by several findings. HK-I-mediated inhibition of PTP opening was prevented by an antibody specific to the N-terminus of HK-I (Figure 3A). The failure of non-binding species of HK, such as yeast HK or trypsin- or chymotrypsin-treated HK-I (Figure 3B), to prevent PTP opening also confirmed the specificity of the HK-I effect, and the requirement of the N-terminal region of HK-I for its interaction with VDAC.

Upon induction of the PTP, cytochrome *c* was released from mitochondria (as revealed by Western blot analysis). HK-I, however, inhibited this release (Figure 3C). These effects of HK-I are similar to those of CsA, a well known inhibitor of the PTP (Figures 2B and 3C). Indeed, the CsA-sensitive mitochondrial swelling, transient Ca^{2+} accumulation and release of cytochrome *c*, events that reflect PTP opening and are almost universal features

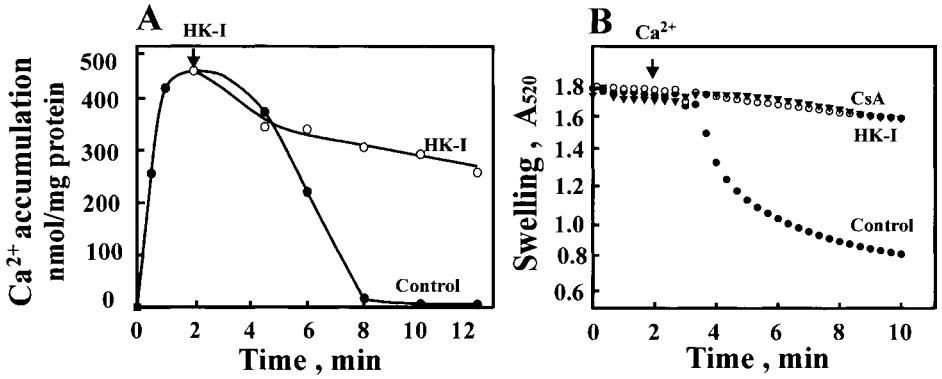

Figure 2 HK-I prevents PTP opening

PTP opening in isolated mitochondria was followed by measuring Ca^{2+} accumulation (**A**) and mitochondrial swelling (**B**). (**A**) Ca^{2+} accumulation was assayed as described in the Experimental section, in the absence or the presence of HK-I (1 unit/ml). (**B**) Ca^{2+}-induced mitochondrial swelling was assayed in the absence and the presence of HK-I as in (**A**), except that the temperature was 24 °C and the phosphate concentration was 150 μM and where indicated CsA (10 μM) was present. Swelling was initiated by the addition of Ca^{2+} (200 μM) to the sample, and was monitored by following the change in absorbance at 520 nm.

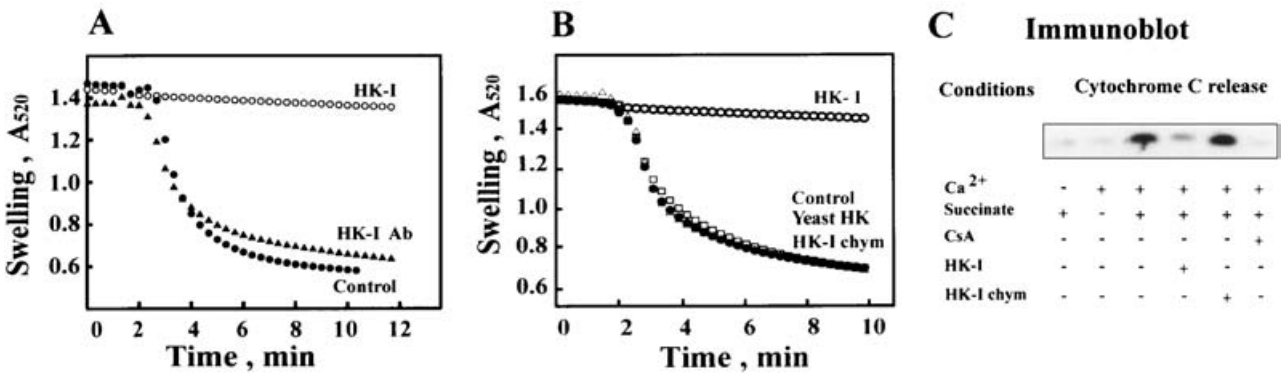

Figure 3 Specificity of the prevention by HK-I of PTP opening and release of cytochrome *c*

(**A**) Mitochondrial swelling was assayed in the presence (○) or absence of HK-I (1 unit/ml; ●) or HK-I preincubated with monoclonal antibodies (Ab) against the N-terminal 11 amino acids of HK-I (▲). (**B**) Mitochondrial swelling was assayed in the absence or presence of chymotrypsin-treated HK-I (HK-I-chym; □) or yeast HK (△). (**C**) Cytochrome *c* release was assayed under conditions that induced (i.e. presence of succinate or Ca^{2+}) or did not induce (i.e. absence of succinate and Ca^{2+}) PTP opening, and in the absence or presence of HK-I (1 unit/ml), chymotrypsin-treated HK-I (1 unit/ml), yeast HK or CsA (10 μM). Released cytochrome *c* was assayed using a monoclonal anti-cytochrome *c* antibody, as described in the Experimental section.

of apoptotic cell death [20], were all inhibited specifically by HK-I.

Correlation between HK-I binding and prevention of PTP opening and its susceptibility to G-6-*P*

The binding of HK to mitochondria is of potential benefit, since bound HK can exclusively utilize mitochondrially synthesized ATP, and is less susceptible to inhibition by its product G-6-*P* [5]. As shown in Figure 4, G-6-*P* prevented the closure of the PTP by HK-I in a concentration-dependent manner. Moreover, G-6-*P* was able to re-open a PTP closed by HK-I (Figure 4B). Thus G-6-*P* was able to re-open not only HK-I-closed bilayer-reconstituted VDAC (Figure 1), but also HK-I-closed PTP, suggesting a reversible interaction of HK-I with VDAC, and thereby with PTP.

The concentrations of G-6-*P* required for the prevention of PTP closure by HK-I were approx. 50-fold higher than those required for the inhibition of soluble HK-I activity (IC_{50} values of 5 and 0.1 mM respectively; Figure 4C). Thus, in agreement with earlier studies [5], binding of HK-I to mitochondria conferred resistance to G-6-*P*.

Since our results suggested that a protein–protein interaction between HK-I and VDAC serves to modulate PTP opening, the correlation between HK-I binding to mitochondria and inhibition of PTP opening was analysed. Indeed, HK-I binding to isolated mitochondria and the subsequent prevention of PTP opening (as a function of HK-I concentration) were well correlated. Both interactions showed strong positive co-operativity (Figure 4D). This high co-operativity is thought to reflect the assembly of HK-I or HK-II tetramers prior to, or during, mitochondrial binding [37,38], and could suggest that modulation of the PTP by HK-I requires oligomerization of the former before or during its interaction with VDAC.

Effects of known PTP modulators on the prevention of PTP opening by HK-I

The effects of PTP ligands on its modulation by HK-I are demonstrated in Figure 5. Atractyloside, a known inhibitor of the ANT (a proposed component of the PTP [39]) and promoter of PTP opening, did not prevent HK-I-induced PTP closure when added either before of after HK-I addition (Figure 5A). This indicates

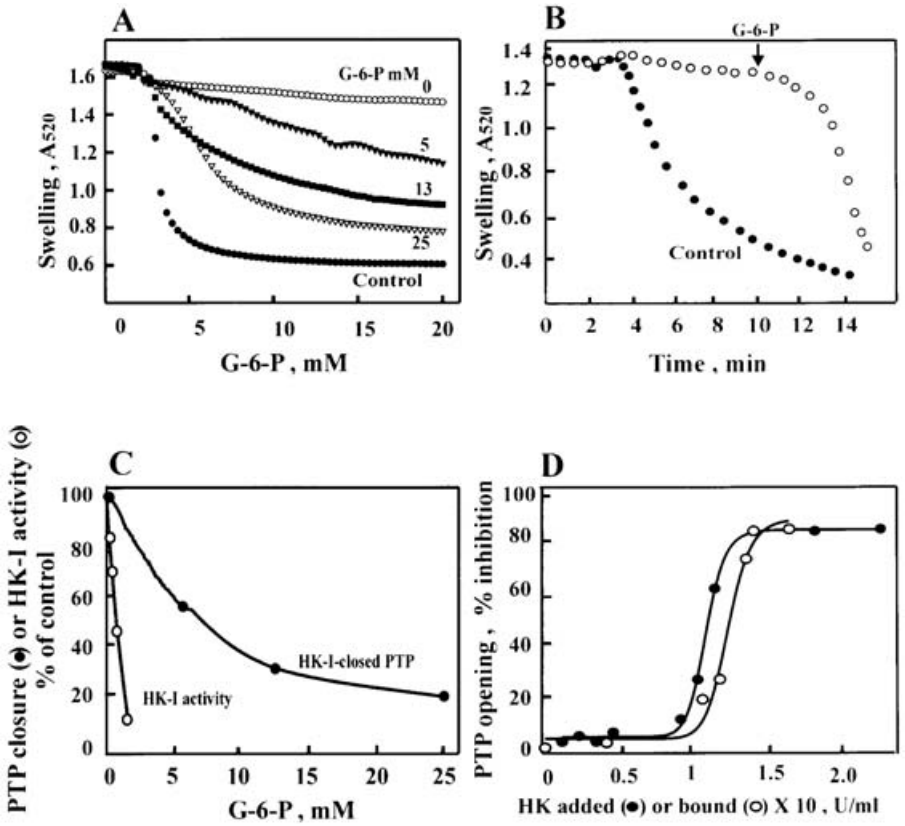

Figure 4 Modulation by G-6-*P* of HK-I binding to mitochondria and prevention of PTP opening

(**A**) PTP opening (mitochondrial swelling) was assayed in the absence (●) or the presence of HK-I (1 unit/ml) and the indicated concentrations of G-6-*P* (in mM). (**B**) PTP opening was assayed in the presence of HK-I; at the indicated time, G-6-*P* (1.5 mM) was added, and the re-opening of the PTP was followed. (**C**) The data in (**A**) were re-plotted as the percentage of PTPs closed as a function of G-6-*P* concentration (●). Control (100 %) represents completely closed PTP (in the presence of HK-I), and the degree of PTP closure in the presence of both HK-I and G-6-*P* was determined after 15 min. In the presence of HK-I, half-maximal PTP opening was obtained at 5 mM G-6-*P* (i.e. $IC_{50} = 5$ mM). Inhibition of soluble HK-I activity by different concentrations of G-6-*P* is also presented (○; $IC_{50} = 0.1$ mM). (**D**) The correlation between HK-I binding and prevention of PTP opening is demonstrated by monitoring Ca^{2+}-induced mitochondrial swelling and HK-I binding in the same sample. PTP opening (○) was induced by Ca^{2+} in the absence and the presence of the indicated concentrations of HK-I; after 15 min, samples were centrifuged and the membrane-bound HK-I (●) was determined as described in the Experimental section.

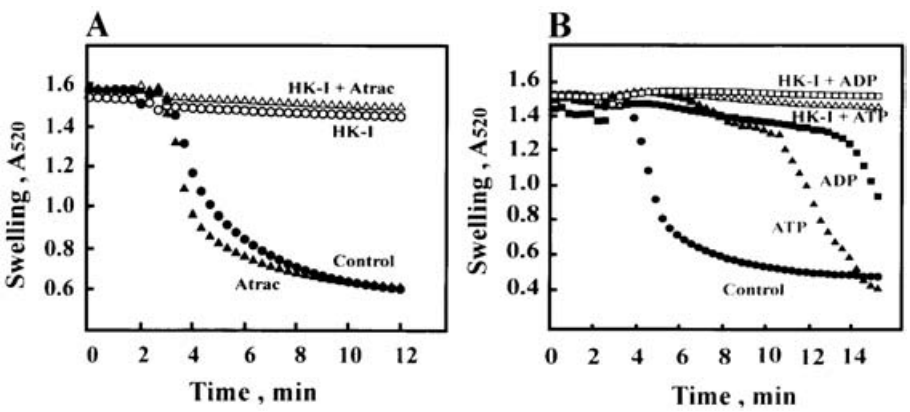

Figure 5 Effects of known PTP ligands on inhibition of PTP opening by HK-I

Ca^{2+}-induced mitochondrial swelling was assayed in the presence of HK-I (1 unit/ml) and the indicated compounds. (**A**) Mitochondrial swelling was assayed in the presence of a relatively low concentration of Ca^{2+} (50 μM), in the absence (●) or in the presence of atractyloside (▲; 10 μM) and/or HK-I (1 unit/ml; △, ○). (**B**) Ca^{2+}-induced mitochondrial swelling was assayed as in Figure 2(B), except that ATP (▲; 1.5 mM), ADP (■; 1.5 mM) and/or HK-I (△, □; 1 unit/ml) were present as indicated.

that HK-I binding to the VDAC determines the closed/open state of the PTP. Finally, as shown previously [40,41], the presence of ADP or ATP delayed PTP opening by approx. 5 to 10 min (Figure 5B). HK-I, in the presence of either nucleotide, stabilized the PTP in the closed conformation, and no PTP opening was observed even after 40 min.

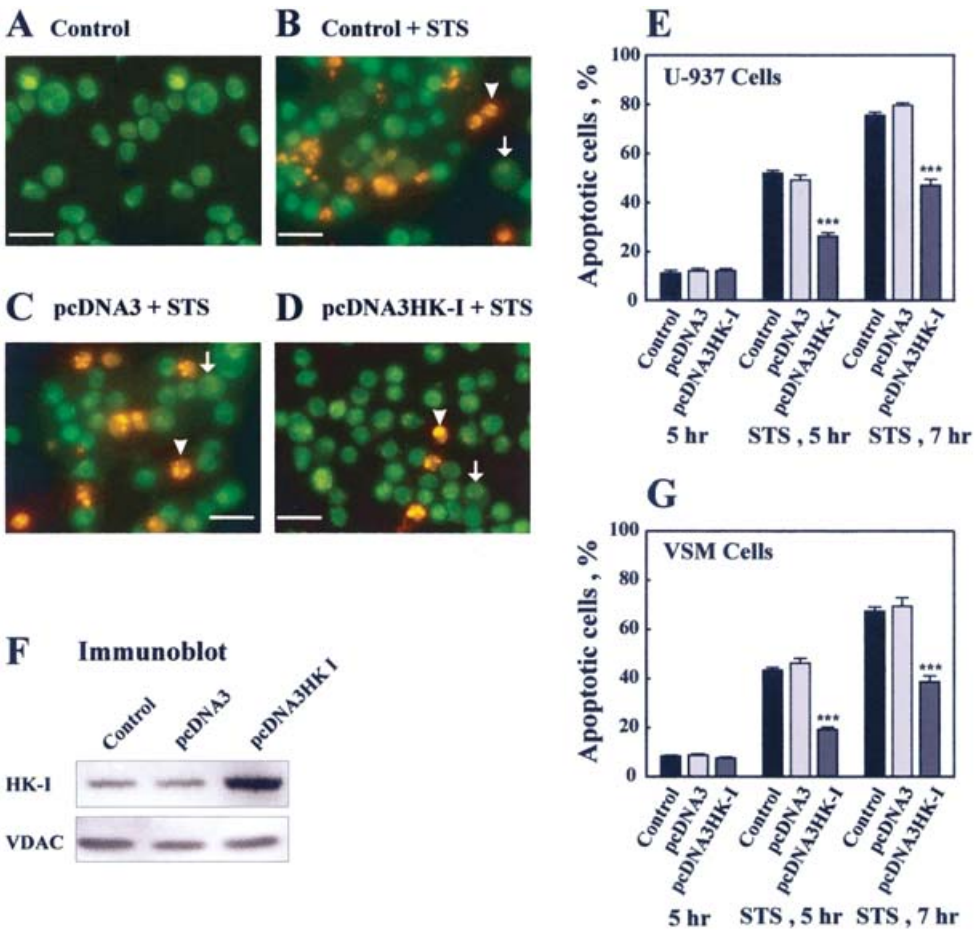

Figure 6 Overexpression of HK-I in U-937 or vascular smooth muscle cells suppresses staurosporine-induced apoptotic cell death

U-937 human monocytic cells were grown and transfected with the different plasmids as described in the Experimental section. U-937 cells before (**A**) and 5 h after (**B**–**D**) exposure to 1.25 μM staurosporine (STS) are shown: (**B**) control cells, (**C**) pcDNA3-transfected cells, (**D**) pcDNA3-HK-I-transfected cells. Arrows indicate cells in an early apoptotic state, represented as a degraded nucleus, but with an intact cell membrane (stained by Acridine Orange only). Arrowheads indicate a late apoptotic state, as shown by the presence of a degraded nucleus and a non-intact cell membrane (stained by both Acridine Orange and ethidium bromide). Cell morphology was assayed by transmission microscopy, and levels of transfection of 68–72 % (GFP expression) were measured by fluorescence microscopy. Cells were stained with Acridine Orange/ethidium bromide to analyse apoptotic cells. Scale bar, 50 μm. (**E**) Quantitative analysis of apoptosis in the different cells was assessed 5 and 7 h after their exposure to staurosporine (1.25 μM) by ANOVA and *t* test; $P < 0.001$ was considered statistically significant (***). Data are means $\pm$ S.E.M. ($n = 6$). In each independent experiment, approx. 200 cells were counted in each treatment. (**G**) Similar experiment as in (**E**), using vascular smooth muscle (VSM) cells (***$P < 0.001$, $n = 3$). (**F**) Western blot analysis of HK-I overexpression in U-937 cells. Mitochondria were isolated from control, pcDNA3- and pcDNA3-HK-I-transfected cells, and aliquots (10 μg of protein) were analysed for HK-I levels, using polyclonal antibodies against the N-terminus of HK-I (N-19; Santa Cruz, Biotechnology). The relative amount of HK-I in comparison with VDAC levels (also estimated using specific antibodies) is indicated.

Expression of HK-I in cell culture prevents staurosporine-induced apoptosis

Since cancer cells are characterized by overexpression of HK-I and HK-II [2], we tested whether HK-I overexpression in leukaemia-derived U-937 cells would suppress apoptosis induced by staurosporine. Accordingly, U-937 cells were transfected with plasmids containing GFP or HK-I, or with no insert. Apoptosis was then induced in the different cells by exposure to staurosporine (Figure 6). Upon exposure of control or plasmid-transfected cells to staurosporine for 5 or 7 h, 50 % and 80 % respectively of the cells underwent apoptosis, whereas 12 % of the cells not exposed to staurosporine died. When U-937 cells were transformed to express HK-I, a decrease in the degree of staurosporine-induced apoptotic cell death of approx. 67 % was observed after 5 h, with a decrease of 46 % after 7 h (Figure 6E). Taking into account the fact that only approx. 70 % of the cells were transfected, the resistance to staurosporine-induced apoptosis in cells overexpressing HK-I was estimated to be approx. 95 % and 66 % after 5 and 7 h respectively of exposure to staurosporine. In such cells, HK-I levels in the mitochondrial fraction were increased 5–6-fold relative to those in control cells, and in comparison with the levels of VDAC (Figure 6F).

The anti-apoptotic effect of HK-I overexpression was also observed in a non-cancer vascular smooth muscle cell line, where apoptotic cell death was decreased by approx. 69 % and 50 % after 5 and 7 h respectively of exposure to staurosporine (Figure 6G).

DISCUSSION

In the present study, we have characterized the modulation of the mitochondrial phase of apoptosis by HK-I using purified HK-I, purified VDAC, isolated mitochondria and HK-I-overexpressing tumour-derived cells in culture. Our results show that purified HK-I interacts directly with purified VDAC reconstituted into a PLB, inducing channel closure. Interaction of HK-I with

mitochondria prevented the opening of the PTP as well as the accompanying release of cytochrome *c*. These findings provide clear evidence that HK-I interacts directly with the VDAC, preventing PTP opening and thereby the mitochondrial phase of apoptosis.

Mitochondria are recognized as the site of regulation of the execution of cell death initiation, especially apoptosis [16–19,34–36,42]. The mitochondrial phase of apoptosis involves the opening of the PTP and the selective release of pro-apoptotic proteins responsible for apoptotic cell death from the inter-membrane space [15,18,43–45]. The molecular composition and regulation of the PTP are yet not fully understood. Current models suggest that the VDAC and ANT, embedded in the mitochondrial outer and inner membranes respectively, are constituents of the PTP [15,18,44]. Whereas HK-I or HK-II has no physical contact with the ANT, it binds to the VDAC, as demonstrated in this and other studies [24,46]. It should be noted, however, that attempts to demonstrate physical interactions between HK-I or HK-II and the VDAC by cross-linking have failed so far. This may reflect the complex and dynamic nature of the interaction. Still, the results presented here provide strong evidence for the VDAC being part of the PTP, and for the modulation of the PTP through the interaction of HK-I with the VDAC, leading to its closure.

Ultimately, closure of the PTP-associated VDAC by HK-I through direct protein–protein contacts would prevent opening of the PTP from and release of the pro-apoptotic protein cytochrome *c* (Figures 2–5), thereby blocking the gateway to apoptosis. This proposal was confirmed at the cellular level, whereby overexpression of HK-I in the tumour-derived cell line U-973 or in vascular smooth muscle cells suppressed staurosporine-induced apoptotic cell death by approx. 68 % (or by approx. 97 % when taking into account a cell transfection efficiency of 70 %) (Figure 6). A decrease in apoptotic cell death and an increase in cell proliferation have also been reported following HK-II expression in NIH-3T3 [6] and Rat 1a [47] cell lines.

Binding of HK-I to the mitochondria and modulation of the PTP showed high co-operativity (Figure 4D), consistent with previous results on HK-I binding to mitochondria. This can be interpreted as reflecting the formation of HK-I tetramers prior to, or during, binding to mitochondria [28,37]. Interestingly, oligomerization of Bax, a pro-apoptotic protein, before its interaction with mitochondria (most likely with the VDAC [48]), as well as the ability of HK-II to hinder such an interaction [8], have been reported. These effects of HK-I and HK-II point to the central role played by these enzymes in protecting cells from death via their interaction with the VDAC and modulation of the mitochondrial phase of apoptosis.

By binding to the VDAC, HK gains preferential access to mitochondrially generated ATP [2], thereby leading to greatly increased rates of aerobic glycolysis [49]. Aerobic glycolysis provides a number of metabolic intermediates used in biosynthetic reactions that provide building blocks for fatty acid synthesis, DNA replication and protein production. The increased levels of G-6-*P* generated by the enhanced amount of HK-I supports NADPH production and, thereby, many other biosynthetic reactions, including those involved in antioxidant defence [50]. Thus the expression of high levels of HK-I or HK-II in transformed cells was interpreted as an adaptation to maintain the heavy energetic demands of such cells. Our studies, however, also demonstrate that HK-I prevented PTP opening by direct interaction with the VDAC, stabilizing it in the closed state, even when one of the other major components of the PTP, i.e. the ANT, was stabilized by atractyloside in the open conformation (Figure 5).

The results presented here provide new insight into the mechanism whereby mitochondria-bound HK promotes tumour cell growth and survival. We propose that overexpression of HK-I in malignant cells serves to ensure a supply of energy and phosphometabolites for the proliferating cell, and also reflects a defence mechanism. In this defence mechanism, HK-I prevents apoptotic cell death by inhibiting opening of the PTP and release of the pro-apoptotic cytochrome *c*, as well as by interfering with the ability of the pro-apoptotic Bax protein to bind to mitochondria and induce release of cytochrome *c* [8], thus encouraging cell survival [7].

Regardless of the nature of regulation of the PTP, VDAC, as a component of the PTP, offers a suitable target for pharmacological intervention designed to correct abnormalities associated with dysregulation of mitochondrial homoeostasis observed under conditions of oxidative stress, toxaemia or apoptosis. Furthermore, HK, as recently suggested [2], is a key player in the growth and survival of many types of cancer, and hence represents an ideal prospect for therapeutic intervention in view of its interaction with and modulation of VDAC activity, as demonstrated here.

This research was supported by a grant from the Israel Science Foundation, administered by The Israel Academy of Science and Humanities. We thank Mr Erez Garty for VDAC purification using n-octyl β-D-glucopyranoside, and Dr Jerry Eichler for reading the manuscript and providing valuable suggestions.

REFERENCES

1 Arora, K. K. and Pedersen, P. L. (1988) Functional significance of mitochondrial bound hexokinase in tumor cell metabolism. Evidence for preferential phosphorylation of glucose by intramitochondrially generated ATP. J. Biol. Chem. **263**, 17422–17428
2 Pedersen, P. L., Mathupala, S., Rempel, A., Geschwind, J. F. and Ko, Y. H. (2002) Mitochondrial bound type II hexokinase: a key player in the growth and survival of many cancers and an ideal prospect for therapeutic intervention. Biochim. Biophys. Acta **1555**, 14–20
3 Rempel, A., Bannasch, P. and Mayer, D. (1994) Differences in expression and intracellular distribution of hexokinase isoenzymes in rat liver cells of different transformation stages. Biochim. Biophys. Acta **1219**, 660–668
4 Sebastian, S. and Kenkare, U. W. (1997) Insulin like growth factor I induces tumor hexokinase RNA expression in cancer cells. Biochem. Biophys. Res. Commun. **235**, 389–393
5 Rose, I. A., Warms, J. V. and Kosow, D. P. (1974) Specificity for the glucose-6-P inhibition site of hexokinase. Arch. Biochem. Biophys. **164**, 729–735
6 Gottlob, K., Majewski, N., Kennedy, S., Kandel, E., Robey, R. B. and Hay, N. (2001) Inhibition of early apoptotic events by Akt/PKB is dependent on the first committed step of glycolysis and mitochondrial hexokinase. Genes Dev. **1** 1406–1418
7 Bryson, J. M., Coy, P. E., Gottlob, K., Hay, N. and Robey, R. (2002) Increased hexokinase activity, of either ectopic or endogenous origin, protects renal epithelial cells against acute oxidant-induced cell death. J. Biol. Chem. **277**, 11392–11400
8 Pastorino, J. G., Shulga, N. and Hoek, J. B. (2002) Mitochondrial binding of hexokinase II inhibits Bax-induced cytochrome *c* release and apoptosis. J. Biol. Chem. **277**, 7610–7618
9 Shinohara, Y., Ishida, T., Hino, M., Yamazaki, N., Baba, Y. and Terada, H. (2000) Characterization of porin isoforms expressed in tumor cells. Eur. J. Biochem. **267**, 6067–6073
10 Benz, R. (1994) Permeation of hydrophilic solutes through mitochondrial outer membranes: review on mitochondrial porins. Biochim. Biophys. Acta **1197**, 167–196
11 Colombini, M. (1994) Anion channel in the mitochondrial outer membrane. Curr. Top. Membr. **42**, 73–101
12 Gincel, D., Zaid, H. and Shoshan-Barmatz, V. (2001) Calcium binding and translocation by the voltage-dependent anion channel: a possible regulatory mechanism in mitochondrial function. Biochem. J. **358**, 147–155
13 Rostovtseva, T. and Colombini, M. (1997) VDAC channels mediate and gate the flow of ATP: implications for the regulation of mitochondrial function. Biophys. J. **72**, 1954–1962
14 Hodge, T. and Colombini, M. (1997) Regulation of metabolite flux through voltage-gating of VDAC channels. J. Membr. Biol. **157**, 271–279
15 Zoratti, M. and Szabo, I. (1995) The mitochondrial permeability transition. Biochim. Biophys. Acta **1241**, 139–176
16 Crompton, M., Virji, S., Doyle, V., Johnson, N. and Ward, J. M. (1999) The mitochondrial permeability transition pore. Biochem. Soc. Symp. **66**, 167–79
17 Zamzami, N. and Kroemer, G. (2003) Apoptosis: mitochondrial membrane permeabilization – the (w)hole story? Curr. Biol. **13**, 71–73

18 Crompton, M. (1999) The mitochondrial permeability transition pore and its role in cell death. Biochem. J. **341**, 233–249
19 Lemasters, J. J., Qian, T., Bradham, C. A., Brenner, D. A., Cascio, W. E., Trost, L. C., Nishimura, Y., Nieminen, A. L. and Herman, B. (1999) Mitochondrial dysfunction in the pathogenesis of necrotic and apoptotic cell death. J. Bioenerg. Biomembr. **31**, 305–319
20 Wilson, J. E. (1995) Hexokinases. Rev. Physiol. Biochem. Pharmacol. **126**, 65–198
21 Xie, G. and Wilson, J. E. (1988) Rat brain hexokinase: Hydrophobic amino-terminus of the mitochondrially bound enzyme is inserted in the lipid bilayer. Arch. Biochem. Biophys. **267**, 803–810
22 Beutner, G., Ruck, A., Riede, B. and Brdiczka, D. (1998) Complexes between porin, hexokinase, mitochondrial creatine kinase and adenylate translocator display properties of the permeability transition pore. Implication for regulation of permeability transition by the kinases. Biochim. Biophys. Acta **1368**, 7–18
23 Vyssokikh, M. Y. and Brdiczka, D. (2003) The function of complexes between the outer mitochondrial membrane pore (VDAC) and the adenine nucleotide translocase in regulation of energy metabolism and apoptosis. Acta Biochim. Pol. **50**, 389–404
24 Linden, M., Gellerfors, P. and Nelson, B. D. (1982) Pore protein and the hexokinase-binding protein from the outer membrane of rat liver mitochondria are identical. FEBS Lett. **141**, 189–192
25 Babel, D., Walter, G., Gotz, H., Thinnes, F. P., Jurgens, L., Konig, U. and Hilschmann, N. (1991) Studies on human porin. VI. Production and characterization of eight monoclonal mouse antibodies against the human VDAC "Porin 31HL" and their application for histotopological studies in human skeletal muscle. Biol. Chem. Hoppe Seyler **372**, 1027–1034
26 Johnson, D. L. H. (1967) Rat liver mitochondria preparation. Methods Enzymol. **10**, 94–96
27 Wilson, J. E. (1989) Rapid purification of mitochondrial hexokinase from rat brain by a single affinity chromatography step on Affi-Gel blue. Prep. Biochem. **19**, 13–21
28 Azoulay-Zohar, H. and Aflalo, C. (2000) Binding of rat brain hexokinase to recombinant yeast mitochondria: identification of necessary physico-chemical determinants. Eur. J. Biochem. **267**, 2973–2980
29 Gornall, A. G., Bardawill, C. J. and David, M. M. (1949) Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. **177**, 751–766
30 Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248–254
31 Gincel, D., Silberberg, S. D. and Shoshan-Barmatz, V. (2000) Modulation of the voltage-dependent anion channel (VDAC) by glutamate. J. Bioenerg. Biomembr. **32**, 571–583
32 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature (London) **227**, 680–685
33 Shoshan-Barmatz, V. and Gincel, D. (2003) The voltage-dependent anion channel: characterization, modulation and role in mitochondrial function in cell life and death. Cell. Biochem. Biophys. **39**, in the press
34 Tsujimoto, Y. and Shimizu, S. (2002) The voltage-dependent anion channel: an essential player in apoptosis. Biochimie **84**, 187–193
35 Halestrap, A. P., Doran, E., Gillespie, J. P. and O'Toole, A. (2000) Mitochondria and cell death. Biochem. Soc. Trans. **28**, 170–177
36 Zamzami, N. and Kroemer, G. (2001) The mitochondrion in apoptosis: how Pandora's box opens. Nat. Rev. Mol. Cell. Biol. **2**, 67–71
37 Xie, G. and Wilson, J. E. (1990) Tetrameric structure of mitochondrially bound rat brain hexokinase: a crosslinking study. Arch. Biochem. Biophys. **276**, 285–293
38 Aflalo, C. and Azoulay, H. (1998) Binding of rat brain hexokinase to recombinant yeast mitochondria: effect of environmental factors and the source of porin. J. Bioenerg. Biomembr. **30**, 245–255
39 Vieira, H. L., Haouzi, D., El Hamel, C., Jacotot, E., Belzacq, A. S., Brenner, C. and Kroemer, G. (2000) Permeabilization of the mitochondrial inner membrane during apoptosis: impact of the adenine nucleotide translocator. Cell Death Differ. **7**, 1146–1154
40 Haworth, R. A. and Hunter, D. R. (1980) Allosteric inhibition of the Ca^{2+}-activated hydrophilic channel of the mitochondrial inner membrane by nucleotides. J. Membr. Biol. **54**, 231–236
41 Novgorodov, S. A., Gudz, T. I., Jung, D. W. and Brierley, G. P. (1991) The nonspecific inner membrane pore of liver mitochondria: modulation of cyclosporin sensitivity by ADP at carboxyatractyloside-sensitive and insensitive sites. Biochem. Biophys. Res. Commun. **180**, 33–38
42 Shimizu, S., Matsuoka, Y., Shinohara, Y., Yoneda, Y. and Tsujimoto, Y. (2001) Essential role of voltage-dependent anion channel in various forms of apoptosis in mammalian cells. J. Cell Biol. **152**, 237–250
43 Adams, J. M. and Cory, S. (1998) The Bcl-2 protein family: arbiters of cell survival. Science **281**, 1322–1326
44 Ichas, F. and Mazat, J. P. (1998) From calcium signaling to cell death: two conformations for the mitochondrial permeability transition pore. Switching from low- to high-conductance state. Biochim. Biophys. Acta **1366**, 33–50
45 Liu, X., Kim, C. N., Yang, J., Jemmerson, R. and Wang, X. (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. Cell **86**, 147–157
46 Brdiczka, D., Beutner, G., Ruck, A., Dolder, M. and Wallimann, T. (1998) The molecular structure of mitochondrial contact sites. Their role in regulation of energy metabolism and permeability transition. Biofactors **8**, 235–242
47 Fanciulli, M., Paggi, M. G., Bruno, T., Del Carlo, C., Bonetto, F., Gentile, F. P. and Floridi, A. (1994) Glycolysis and growth rate in normal and in hexokinase-transfected NIH-3T3 cells. Oncol. Res. **6**, 405–409
48 Oltvai, Z. N., Milliman, C. L. and Korsmeyer, S. J. (1993) Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. Cell **74**, 609–619
49 Hashimoto, M. and Wilson, J. E. (2000) Membrane potential-dependent conformational changes in mitochondrially bound hexokinase of brain. Arch. Biochem. Biophys. **384**, 163–173
50 Bustamante, E., Morris, H. P. and Pedersen, P. L. (1981) Energy metabolism of tumor cells. Requirement for a form of hexokinase with a propensity for mitochondrial binding. J. Biol. Chem. **256**, 8699–8704

Received 24 September 2003/15 October 2003; accepted 16 October 2003
Published as BJ Immediate Publication 16 October 2003, DOI 10.1042/BJ20031465