Mitochondrial Binding of Hexokinase II Inhibits Bax-induced Cytochrome c Release and Apoptosis*

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Proapoptotic proteins such as Bax, undergo translocation to the mitochondria during apoptosis, where they mediate the release of intermembrane space proteins including cytochrome c. Bax binds to the voltage-dependent anion channel (VDAC). VDAC is a β-barrel protein located in the outer mitochondrial membrane. In planar lipid bilayers, Bax and VDAC form a channel through which cytochrome c can pass. Hexokinase II (HXK II) also binds to VDAC. HXK II catalyzes the first step of glycolysis and is highly expressed in transformed cells, where over 70% of it is bound to the mitochondria. The present study demonstrates that HXK II interferes with the ability of Bax to bind to mitochondria and release cytochrome c. Detachment of HXK II from the mitochondria-enriched fraction isolated from HeLa cells promoted the binding of recombinant Bax-A19 and subsequent cytochrome c release. Similarly, the addition of recombinant HXK II to the mitochondria-enriched fraction isolated from hepatocytes, cells that do not express HXK II endogenously, prevented the ability of recombinant Bax-A19 to bind to the mitochondria and promote cytochrome c release. Similar results were found in intact cells, in which the detachment of mitochondrial bound HXK II or its overexpression potentiated and inhibited, respectively, Bax-induced mitochondrial dysfunction and cell death.

The major emphasis of apoptosis research was initially focused on the nucleus. This is understandable, given that the nucleus exhibits some of the most striking features of apoptosis, such as chromatin condensation and oligonucleosomal fragmentation of DNA. Recently, however, the involvement of mitochondria in apoptosis has come under close scrutiny. Mitochondria are the power plants of the cell, providing the bulk of ATP production for cellular metabolism. It has been demonstrated that cytochrome c, located in the intermembrane space (IMS) of the mitochondria, is released to the cytosol during apoptosis and helps trigger the activation of caspases, a family of enzymes that is integral to the breakup of apoptotic cells (1–5). Subsequently, a plethora of other proteins that are located in the mitochondrial IMS and are part of the apoptotic machinery have been discovered, including apoptosis-inducing factor, SMAC/DIABLO, caspases 9 and 8, and endonuclease G (6–8).

At present, there is considerable controversy over the mode by which IMS proteins escape from that compartment and enter the cytosol, where they become activated. Disruption of the outer mitochondrial membrane is one obvious mechanism. Due to the greater surface area of the inner mitochondrial membrane compared with the outer mitochondrial membrane, excessive swelling of the mitochondrial matrix results in rupture of the outer mitochondrial membrane and the release of intermembrane space proteins. Indeed, opening of the mitochondrial permeability transition pore with subsequent mitochondrial depolarization and outer mitochondrial membrane rupture does occur in some forms of apoptosis (9). The permeability transition pore is a large proteinaceous pore that spans both mitochondrial membranes (10). In some instances, inhibition of pore opening prevents many of the typical manifestations of apoptosis and preserves cell viability (11–13). However, in many cases of apoptosis, mitochondrial depolarization and swelling occur as late events, well after the release of cytochrome c and other IMS proteins has occurred (14, 15). In such cases, the release of IMS proteins appears to be more selective and not due to a wholesale disruption of the outer mitochondrial membrane. However, in both instances, the Bcl-2 family of proteins appears to play a key role in the control of outer membrane permeability.

The penultimate family member, Bcl-2, was discovered to be overexpressed in a B-cell lymphoma due to a t(11;14)(q23;32) chromosomal translocation (16). To date, there are 14 known human homologs of Bcl-2 family proteins that have been localized to the nucleus, endoplasmic reticulum, and mitochondria (17). At the mitochondria, Bcl-2 proteins can either inhibit or induce mitochondrial dysfunction. Bcl-2 and Bcl-XL are the best studied members of the family that inhibit cytochrome c release and apoptosis. The proteins share considerable homologies in four consensus regions known as BH 1–4. Bcl-XL has been demonstrated to interact through its BH 4 domain with the voltage-dependent anion channel (VDAC) (18). VDAC (porin) is a β-barrel protein that spans the outer mitochondrial membrane (19, 20). VDAC mediates ATP and ADP translocation across the outer mitochondrial membrane with the adenine nucleotide translocator (ANT) mediating transport across the inner membrane. Like Bcl-XL, Bax has been demonstrated to interact with VDAC (21). However, in contrast to Bcl-XL, Bax induces cytochrome c release and apoptosis. Bax translocates from the cytosol to the mitochondria following an apoptotic stimulus (22, 23). Like Bcl-XL, Bax possesses BH 1–3 regions. However, unlike Bcl-XL, reconstitut-

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1 The abbreviations used are: IMS, intermembrane space; CTZ, clotrimazole; HXX II, hexokinase II; G6P, glucose 6-phosphate; IND, indomethacin; HXX II, truncated hexokinase II; VDAC, voltage-dependent anion channel; HXX2VBD, hexokinase II VDAC binding domain; PBS, Ca2+/Mg2+-free phosphate-buffered saline; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; ANT, adenine nucleotide translocator.
tion experiments demonstrated that Bax forms a high conductance channel with VDAC in planar lipid bilayers, which allow the passage of cytchrome c.

For over 70 years, it has been known that tumor cells exhibit a high rate of glycolysis. The high glycolytic rate is now known to be due in part to the greatly increased expression of hexokinase II (HXK II) in transformed cells (24). In an ATP-dependent process, HXK II mediates the first step of glycolysis, phosphorylating glucose to produce glucose 6-phosphate. In transformed cells, up to 70% of the HXK II is bound to the mitochondria through an interaction with VDAC (25–27). At this localization, HXK II gains preferential access to mitochondria-produced ATP (28). The availability of mitochondria-generated ATP to VDAC-bound HXK II enables the cells to maintain a much greater rate of glycolysis (29). This state of cellular metabolism has been termed aerobic glycolysis. Because both Bax and HXK II bind to mitochondria through an interaction with VDAC, we wanted to determine whether the high levels of mitochondrial bound HXK II found in transformed cells had any effect on the ability of Bax to induce apoptosis.

In the present studies, we determined that the binding of HXK II to mitochondria prevents recombinant Bax-A19-induced cytchrome c release and induction of apoptosis. In particular, we demonstrate that dissociation of mitochondrial bound HXK II increases the binding of Bax-A19 to the mitochondria-enriched fraction and promotes cytosome c release. Furthermore, recombinant HXK II was found to inhibit Bax-A19 induced cytrome c release in the mitochondria-enriched fraction isolated from hepatocytes, a cell that does not express endogenous HXK II. In addition, we show that in intact cells, detachment of mitochondrial bound HXK II results in a potentiation of apoptosis by an agent that induces Bax translocation to the mitochondria and that overexpression of HXK II protects against the induction of Bax-dependent apoptosis.

**EXPERIMENTAL PROCEDURES**

**Tissue Culture—**HeLa cells (ATCC-CC-1; American Type Culture Collection) were maintained in 25-cm² flasks (Corning Costar Corp., Oneonta, NY) with Dulbecco’s modified Eagle’s medium containing 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 10% heat-inactivated fetal bovine serum, all incubated under an atmosphere of 95% air and 5% CO₂. Human embryonic kidney (HEK 293) cells were utilized for transfection with plasmids (pCDNA-LacZ in combination with pCDNA-Bax, pCDNA-HKII, and/or pCDNA-HXK II; kindly provided by Dr. John E. Wilson, Department of Biochemistry, Michigan State University). In all cases, the plasmids were transfected into the cells at a density of 54,000 cells/cm² in either 24-well plates used for the determination of cell viability or 25-ml flasks used for isolating the mitochondria-enriched fraction and analyzing DNA fragmentation. HeLa cells were washed once with PBS and then incubated with serum-free Dulbecco’s modified Eagle’s medium. The cells were then either left alone or pretreated for 30 min with chloroquine (10 μg/ml, Sigma) or 100 μM of cell-permeable HXK2VBD. Following preincubation, the cells were then either left alone or treated with 50 μM indomethacin (Sigma) for the time periods indicated in the figures.

**Measurement of Caspase-3 Activity—**For this assay, cells in 24-well plates at a final density of 1.0 × 10⁶ cells/well were used. The assay is based on the ability of the active enzyme to cleave the chromophore p-nitroanilide from the enzyme substrate Asp-Glu-Val-Asp-p-nitroanilide. Following treatment, the medium was removed and spun down to harvest any detached and dying cells. The remaining cells were scraped and combined with detached cells. Cell extracts were prepared and diluted 1:1 with 2× reaction buffer (10 mM Tris, pH 7.4, 1 mM dithiothreitol, 2 mM EDTA, 0.1% CHAPS, 1 mM phenylmethylsulfonyl fluoride, 10 μM/ml pepstatin, 10 μg/ml leupeptin). Asp-Glu-Val-Asp-p-nitroanilide was added to a final concentration of 50 μM, and the reaction was incubated for 1 h at 37 °C. The samples were then transferred to a 96-well plate, and the absorbance measurements were made in a 96-well plate reader at 405 nm.

**DNA Fragmentation Assay—**Treated and untreated cells in 25-ml flasks were collected in 1.5-ml microcentrifuge tubes and washed once with 1 ml of 0.2% EDTA in Hanks’ balanced salt solution without Ca²⁺ or Mg²⁺. A modified centrifugation for 5 min at 700 × g and then 40 μl of TE lysis buffer (10 mM Tris-HCl, 1 mM sodium-EDTA, pH 7.5) containing 0.25% Nonidet P-40 or Triton X-100 was added along with 5 μl of an RNase A solution (20 mg/ml) (Sigma). The mixture was suspended by gentle vortex mixing and incubated for 20 min at 37 °C. Then 5 μl of a proteinase K solution (20 mg/ml) (Sigma) was added to the sample and incubated an additional 20 min or until the solution cleared. A 6× loading buffer (5 μl of 0.025% bromphenol blue, 0.025% xylene cyanol FF, 30% glycerol) was added to 25 μl of the sample. The sample was then analyzed by electrophoresis on a 1.8% agarose minigel in TE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0) for 1–4 h at 36 V. Ethidium bromide-stained DNA was visualized by transillumination with UV light (300 nm) and photographed (5).

**Isolation of Cytosol and Mitochondria-enriched Fractions—**HeLa cells were plated in 25-cm² flasks and used at a final density of 1.35 × 10⁶ cells/flask. Following treatment, the cells were harvested by trypsinization and centrifuged at 600 × g for 10 min at 4 °C. The cell pellets were washed once in PBS and then resuspended in 3 volumes of isolation buffer (20 mM Hepes, pH 7.4, 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM dithiothreitol, and 10 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μM aprotinin) in 250 μl sucrose. After chilling on ice for 3 min, the cells were disrupted by 40 strokes of a glass homogenizer. The homogenate was centrifuged twice at 1500 × g for 30 min. The supernatant was removed and filtered through 0.2-μm and then 0.1-μm Ultrafree MC filters (Millipore Corp.) to give cytosolic protein. For determination of cytosome c release and Bax binding in intact cells, mitochondria-enriched fractions and cytosolic fractions were nor-
malized for protein content (25 μg/lane) and run on 12% SDS-PAGE gels followed by electrophoretically onto nitrocellulose membranes. The membranes were then stripped and reprobed with antibodies to cytochrome oxidase (1:500; Molecular Probes) and α-tubulin (1:500; Transduction Laboratories) to evaluate equal loading. Hepatocytes were isolated from rat liver. For hepatocytes, the mitochondria-enriched fraction was isolated following the same protocol used for isolation of the HeLa cell mitochondria-enriched fraction.

**Bax Antisense**—HeLa cells were transfected using Trans-IT transfection reagent with a 1 μM concentration of the antisense phosphorothioate oligonucleotide, 5'-gtgctggaccagctctgt-3', targeted against the first 15 nucleotides of the coding region of the 1.1 kb transcript of the human Bax. As a control, a 1 μM concentration of the corresponding sense oligonucleotides was transfected.

**Treatment of the Mitochondria-enriched Fraction**—The mitochondria-enriched fraction, at a final concentration of 0.5 mg/ml in a volume of 250 μl, was incubated in a KCl-based medium, respiratory buffer (150 mM KCl, 25 mM NaHCO₃, 1 mM MgCl₂, 3 mM KH₂PO₄, 20 mM Hepes, pH 7.4). Glutamate and malate (1 mM; Sigma) were added as respiratory substrates. To induce detachment of the mitochondrial bound HXK II, the mitochondria-enriched preincubation in media that contained either clotrimazole (20 μM), glucose 6-phosphate (1 mM) or a 100 μM concentration of a peptide corresponding to the 15 N-terminal amino acids of hexokinase II (HX2VBD). Following a 30-min preincubation, the mitochondria-enriched fraction was either left untreated or treated with Bax-Δ19 at a final concentration of 1 μM. For the determination of cytochrome c release, the mitochondria-enriched fraction was first pelleted at 12,000 × g for 30 min at 4 °C. The supernatant was removed and filtered through a 0.2- and 0.1-μm Ultrafree MC filter and concentrated in a Microcon concentrator (molecular mass cut-off of 30 kDa) to 100 μl. Mitochondrial pellets were normalized for protein content (25 μg/lane) and separated on 12% SDS-PAGE gels and electroblotted onto nitrocellulose membranes. Afterward, the gels were stripped and reprobed with an antibody (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) to the integral inner membrane protein cytochrome oxidase. 20 μl of the supernatant fraction was combined with SDS loading buffer and separated on 12% SDS-PAGE gels and electroblotted onto nitrocellulose membranes. Cytochrome c was detected with a monoclonal antibody to cytochrome c (Pharmingen, San Diego, CA). Bax-Δ19 binding was detected with a monoclonal antibody to Bax that binds to the N terminus (Santa Cruz Biotechnology). As a control, the pore-forming peptide alamethicin (final concentration of 60 μg/ml) was added to mitochondria to permeabilize the outer membrane and cause maximal release of cytochrome c. Hepatocytes express no hexokinase II. Therefore, the mitochondria-enriched fraction that was isolated from hepatocytes was utilized in experiments to determine the effect of adding recombinant hexokinase II (HXK II) and truncated hexokinase II (tHXK II) on Bax-Δ19 induced cytochrome c release. As above for HeLa cells, the hepatocyte mitochondria-enriched fraction was placed in a KCl-based medium with glutamate and malate as respiratory substrates. The hepatocyte mitochondria-enriched fraction (0.5 mg/ml) was then incubated with either HXK II or tHXK II in 250 μl of respiratory buffer. HXK II and thiol-tail (THK II) were added at a final concentration of 250 milliunits/mg of mitochondrial protein. The specific activity of HXK II and THK II used is 63 milliunits/mg of protein and 58 milliunits/mg of protein, respectively. After 30 min, the hepatocyte mitochondria were either left untreated or treated with a final concentration of 1 μM Bax-Δ19. The mitochondria-enriched fraction was pelleted, and the mitochondrial and supernatant fractions were treated as above with HeLa cell mitochondria for determination of cytochrome c release and Bax-Δ19 binding. Binding studies for HXK II and Bax-Δ19 were conducted as outlined above using varying concentrations of HXK II and a fixed concentration of Bax-Δ19 (1 μM). HXK II binding was quantitated enzymatically (a milliunit being the amount of enzyme that will convert 1 nmol of glucose to glucose 6-phosphate in 1 min), and Bax-Δ19 binding was assessed by densitometry of Western blots.

**Preparation of Purified Recombinant Bax and HXK II**—Recombinant Bax was isolated and purified as described previously (32). Full-length Bax is insoluble; therefore, PCR was used to generate a human Bax cDNA that lacked the coding region for the carboxyl-terminal 19 amino acids. The resulting cDNA was inserted into the EcoRI and Smal sites of pGEX-4T2 (Amersham Biosciences, Inc.) in frame with GST. The plasmid (pGEX-BaxΔ19) was used to transform the protease-deficient strain of E. coli, BL21. An overnight culture of bacteria was used to inoculate (1:10) 2 liters of LB medium containing 100 μg/ml of ampicillin and incubated at 37 °C. When the A₆₀₀ was between 0.7 and 1.0, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentra-
isolated from HeLa cells caused an almost complete release of HXK II from the mitochondrial fraction to the supernatant. The product of HXK II, glucose 6-phosphate (G6P), causes its dissociation from mitochondria (34). As expected, treatment of the HeLa cell mitochondria-enriched fraction with 1 mM of G6P also caused the release of mitochondrial bound HXK II (Fig. 1A, lane 3). The N-terminal 15 amino acids of HXK II are necessary and sufficient for it to bind to VDAC. Therefore, we synthesized a peptide comprising the N-terminal 15 amino acids of HXK II (hexokinase II VDAC binding domain; HXK2VBD). HXK2VBD was able to release mitochondrial bound HXK II. As demonstrated in Fig. 1A, lane 4, incubation with 100 μM of HXK2VBD resulted in an almost complete release of HXK II from the HeLa cell mitochondria.

We next wanted to determine whether detachment of HXK II from mitochondria modified their interaction with Bax. Recombinant Bax-Δ19 was added to the isolated HeLa cell mitochondria-enriched fraction. The mitochondrial fraction was then pelleted and washed. As demonstrated in Fig. 1B, lane 1, the addition of Bax-Δ19 at a final concentration of 1 μM resulted in little binding of Bax-Δ19 to the HeLa cell mitochondria-enriched fraction, with most of it remaining in the supernatant. Conversely, when the mitochondrial-enriched fraction was pretreated with CTZ, G6P, or HXK2VBD, agents that release mitochondrial bound HXK II, significant binding of Bax-Δ19 to the HeLa cell mitochondria-enriched fraction occurred (Fig. 1B, lanes 2–4). We next determined whether detaching HXK II from mitochondria had any effects on the ability of Bax-Δ19 to release cytochrome c from the intermembrane space. Fig. 2 shows that the addition of Bax-Δ19 to a final concentration of 1 μM resulted in no release of cytochrome c from the control mitochondria-enriched fraction. Next, Bax-Δ19 was added to mitochondria-enriched fractions that were pretreated with CTZ, G6P, and HXK2VBD. As shown in Fig. 2, CTZ, G6P, and HXK2VBD alone resulted in little or no cytochrome c release. However, when 1 μM of Bax-Δ19 was added to mitochondria-enriched fractions preincubated with CTZ, G6P, or HXK2VBD, there was an almost complete release of cytochrome c from the mitochondria to the supernatant. These results demonstrate that detachment of HXK II from mitochondria leads to a potentiation of Bax-Δ19-induced cytochrome c release associated with an increased binding of Bax-Δ19 to the mitochondria. Only at a 10-fold greater concentration of Bax-Δ19 (10 μM), was binding of Bax-Δ19 and cytochrome c release detectable in untreated HeLa cell mitochondria-enriched fractions (results not shown).

Hexokinase II Inhibits Bax-induced Cytochrome c Release from the Hepatocyte Mitochondria-enriched Fraction—Hepatocytes express little HXK II (35). Therefore, hepatocyte mitochondria are ideal to test the effects of exogenously added HXK II on the ability of Bax-Δ19 to bind to the mitochondria-enriched fraction and release cytochrome c. As demonstrated in Fig. 3A, mitochondrial binding sites become saturated at 250 milliunits of HXK II added per mg of mitochondrial protein, and this is accompanied by an inhibition of binding of Bax-Δ19 (1 μM concentration). As shown in Fig. 3B, lane 1, in contrast to the mitochondria-enriched fraction isolated from HeLa cells, the addition of 1 μM Bax-Δ19 to the hepatocyte mitochondria-enriched fraction resulted in a significant binding of Bax and the release of cytochrome c. Next, recombinant HXK II (250 milliunits/mg of mitochondrial protein) was incubated with the hepatocyte mitochondria-enriched fraction for 30 min to allow binding, followed by the addition of Bax-Δ19. As demonstrated in Fig. 3, lane 2, the addition of Bax-Δ19 to the hepatocyte mitochondria-enriched fraction and Bax-Δ19-induced cytochrome c release. As mentioned above, the N-terminal 15 amino acids of HXK II are required for it to bind to mitochondria. Therefore, we expressed a version of HXK II in which the first 15 amino acids were truncated (tHXK II). As with full-length HXK II, the hepatocyte mitochondria-enriched fraction was preincubated with tHXK II (250 milliunits/mg of mitochondrial protein) prior to the addition of Bax-Δ19. As demonstrated in Fig. 3, lane 3, unlike full-length HXK II, tHXK was unable to prevent the binding of Bax to the hepatocyte mitochondrial fraction or its induction of cytochrome c release. Even at 1000 milliunits/mg of mitochondrial protein, tHXK II exhibited no ability to inhibit Bax-Δ19 binding or cytochrome c release (data not shown). These results indicate that HXK II binding to mitochondria is necessary for it to inhibit the binding of Bax-Δ19 and the resulting induction of cytochrome c release.

Detachment of HXK II from Mitochondria Potentiates Bax-dependent Apoptosis—As demonstrated above, a peptide comprising the N-terminal 15 amino acids of HXK II (HXK2VBD) was able to detach HXK II from isolated HeLa cell mitochondria. For intact cell experiments, we synthesized a peptide containing the N-terminal 15 amino acids of HXK II fused to an antennapedia sequence that facilitates peptide internalization.
As demonstrated in Fig. 4, lane 2, incubation of HeLa cells with 100 μM cell-permeable HXK2VBD resulted in the detachment and translocation of HXK II from the mitochondria to the cytosol. As mentioned above, clotrimazole has been demonstrated to cause the detachment of HXK II from mitochondria in B16 melanoma cells. As shown in Fig. 4, lane 3, treatment of HeLa cells with 20 μM CTZ resulted in the translocation of HXK II from the mitochondria to the cytosolic fraction. Having validated their ability to detach mitochondrial bound HXK II in intact cells, we next determined the effects of these agents on Bax-induced cytochrome c release and apoptosis.

Nonsteroidal anti-inflammatory agents, such as indomethacin, have been demonstrated to bring about apoptosis that is dependent on Bax (36). Indomethacin at a dose of 50 μM produced only an 8% loss of cell viability in HeLa cells after 24 h of exposure. As demonstrated in Fig. 5A, incubation of HeLa cells with either 50 μM indomethacin or 20 μM clotrimazole separately produced little cytotoxicity. However, when the two agents were combined, there was a marked increase in cytotoxicity. By 4 h, 75% of the HeLa cells lost viability, with 83% of the cells dead by 6 h. As with CTZ, incubation of HeLa cells with 100 μM cell-permeable HXK2VBD produced little cytotoxicity over 6 h. However, when cell-permeable HXK2VBD was combined with 50 μM indomethacin, there was a marked loss of cell viability, with over 85% of the cells dead by 6 h (Fig. 5B).

Importantly, the cytotoxicity induced by indomethacin in combination with CTZ or HXK2VBD was dependent on Bax. As demonstrated in Fig. 6 and Table I, an antisense oligonucleotide directed against Bax decreased Bax expression in HeLa cells and prevented indomethacin-induced cell killing in the presence of CTZ or HXK2VBD.

The loss of cell viability caused by the combinations of IND and CTZ or IND and cell-permeable HXK2VBD was due to apoptosis. As shown in Fig. 5, C and D, treatment of HeLa cells with IND and CTZ or IND and cell-permeable HXK2VBD produced a progressive increase in caspase 3 activity starting at 30 min and reaching a maximum of 6-fold above control levels at 2 h. Preincubation of the cells with a 50 μM concentration of the broad-spectrum caspase inhibitor benzylxycarbonyl-Val-Ala-Asp-fluoromethyl ketone or with 50 μM of the caspase 8-specific inhibitor Asp-Glu-Val-Ala-Asp-fluoromethyl ketone prevented the loss of cell viability caused by IND plus CTZ or IND plus cell-permeable HXK2VBD. A hallmark of apoptotic cell killing is the cleavage of nuclear DNA to oligonucleosome-sized fragments. As shown in Fig. 7, IND plus CTZ or IND plus cell-permeable HXK2VBD produced DNA laddering after 3 h of treatment. By contrast, none of the agents alone produced DNA fragmentation.

Importantly, the cell killing brought about by IND in the
presence of agents that detach mitochondrial bound HXK II was associated with increased binding of Bax to the mitochondria-enriched fraction and cytochrome c release. As demonstrated in Fig. 8, lane 2, treatment of HeLa cells with 50 μM indomethacin produced a small amount of Bax binding to the mitochondria-enriched fraction with no release of cytochrome c to the cytosol. By contrast, in cells preincubated for 30 min with either CTZ or cell-permeable HXK2VBD and then treated with 50 μM indomethacin, there was a marked increase in the binding of Bax to the mitochondria (Fig. 8, lanes 3 and 4).

Expression of HXK II Inhibits Bax-induced Cell Death—
Mitochondrial Bound HXK II Inhibits Bax-induced Apoptosis

Human embryonic kidney (HEK 293) cells were utilized in transfection studies. HEK 293 cells were cotransfected with pCDNA-LacZ and pCDNA-Bax in combination with either empty vector or pCDNA-HXK II. Importantly, at 16 h post-transfection, 63% of the cells transfected with pCDNA-Bax that did stain for β-galactosidase displayed apoptotic features (Table II). By contrast, when pCDNA-HXK II was cotransfected with pCDNA-Bax, only 7% of β-galactosidase-positive cells displayed apoptotic features at 16 h.

FIG. 7. Indomethacin induces DNA fragmentation in HeLa cells upon detachment of mitochondrial bound HXK II. HeLa cells were either left untreated or incubated with CTZ (20 μM) or cell-permeable HXK2VBD (100 μM) for 30 min. The cells were then either left alone or treated with 50 μM of indomethacin. After 3 h, nuclear DNA was extracted as described under “Experimental Procedures.” Equal amounts of DNA were run out on a 1.8% agarose gel and visualized with ethidium bromide staining using the Kodak 440 digital imager.

FIG. 8. Detachment of mitochondrial bound HXK II by CTZ or cell-permeable HXK2VBD promotes binding of Bax to the mitochondria-enriched fraction and the release of cytochrome c from the mitochondria in intact HeLa cells. HeLa cells were either left untreated or incubated with CTZ (20 μM) or cell-permeable HXK2VBD (100 μM) for 30 min. The HeLa cells were then left alone or treated with 50 μM IND. Mitochondrial and cytosolic fractions were isolated as described under “Experimental Procedures.” Samples were normalized for protein content. 25 μg of protein/lane was run in a 12% SDS-polyacrylamide gel and electroblotted onto nitrocellulose. Bax was detected using an anti-Bax monoclonal antibody and a secondary anti-mouse horseradish peroxidase-labeled antibody. Cytochrome c was detected using an anti-cytochrome c monoclonal antibody and a secondary anti-mouse horseradish peroxidase-labeled antibody. The blots were visualized using enhanced chemiluminescence and imaged on a Kodak 440 digital imager. The results are typical of three independent experiments.

Table II

| Apoptotic cells                  | %  |
|----------------------------------|----|
| LacZ + empty vector              | 7 ± 3 |
| LacZ + Bax                       | 62 ± 11 |
| LacZ + Bax + HXK II              | 5 ± 2 |

By binding to VDAC, HXK II gains preferential access to mitochondrial generated ATP. HXK II catalyzes the first and a rate-controlling step in glycolysis. By having preferential access to mitochondrial ATP production, the mitochondrial bound HXK II can greatly increase the rate of glycolysis (27, 39). Since the high glycolytic rate mediated by HXK II in transformed cells is dependent on mitochondrial ATP production, it has been termed aerobic glycolysis. In contrast to anaerobic glycolysis, where the main products are ATP and lactate, aerobic glycolysis provides a number of metabolic intermediates used for biosynthetic reactions. This is important for transformed cells that divide rapidly and require large amounts of building blocks for fatty acid synthesis, DNA replication, and protein production. In addition, the increased levels of G6P are utilized in the pentose phosphate pathway to produce NADPH. NADPH is a cofactor required for many biosynthetic reactions and utilized in the glutathione reductase pathway for antioxidant defense (40).
VDAC (porin) is an integral 30-kDa β barrel protein that forms a voltage-regulated channel in the outer mitochondrial membrane (19, 41, 42). The open channel displays a 2:1 preference for CI over K+. VDAC closes to a lower conducting state when either positive or negative potentials are applied. VDAC has been found to be associated with the ANT at contact sites between the outer and inner mitochondrial membranes (43). VDAC and the ANT are putative components of the permeability transition pore complex. Also found to associate with VDAC and the ANT is the peripheral benzodiazepine receptor located in the outer mitochondrial membrane (44, 45). Agonistic ligands of the benzodiazepine receptor promote opening of the permeability transition pore in isolated mitochondria and can induce apoptosis in intact cells (46, 47).

Bax is a proapoptotic protein that translocates to the mitochondria in some forms of apoptosis. Bax has been demonstrated to interact with both VDAC and the ANT in in vitro reconstitution experiments (21, 48). VDAC and Bax were found to create a large pore with conductance levels 10-fold greater than those of VDAC and Bax individually (49). Cytochrome c was shown to pass through a single VDAC-Bax channel in a planar lipid bilayer. In contrast to Bax, the antiapoptotic protein, Bcl-XL, closed VDAC. Antibodies directed against VDAC exhibited properties similar to that which HXK II displayed in the present study (50). Anti-VDAC antibodies prevented Bax-induced cytochrome c release in isolated mitochondria. In addition, microinjection of anti-VDAC antibodies into HeLa cells prevented apoptosis induced by Bax overexpression and potentiated apoptosis induced by etoposide, paclitaxel, and staurosporine. However, unlike HXK II, the anti-VDAC antibodies did not inhibit binding of Bax to mitochondria. The ability of the anti-VDAC antibodies to prevent Bax-induced cytochrome c release was attributed to the antibodies inducing VDAC to assume a closed state. Conversely, our results demonstrate that HXK II prevents Bax from binding to mitochondria, although the exact mechanism is unknown. Upon in vitro incubation with purified VDAC, HXK II binds to VDAC and forms tetramers. Such a result suggests that HXK II may prevent the binding of Bax to VDAC through steric hindrance. Alternatively, HXK II may induce VDAC to assume a conformational state in which it is unable to bind Bax.

When expressed in NIH3T3 and Rat1a cell lines, HXK II was found to increase cell proliferation and inhibit apoptosis (51, 52). The binding of HXK II to mitochondria may be controlled by a conformational state in which it is unable to bind Bax. When expressed in NIH3T3 and Rat1a cell lines, HXK II was found to increase cell proliferation and inhibit apoptosis (51, 52). The binding of HXK II to mitochondria may be controlled by a conformational state in which it is unable to bind Bax. When expressed in NIH3T3 and Rat1a cell lines, HXK II was found to increase cell proliferation and inhibit apoptosis (51, 52). The binding of HXK II to mitochondria may be controlled by a conformational state in which it is unable to bind Bax.
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