

Targeting of hexokinase 1 to liver and hepatoma mitochondria

(hepatoma cell line/reporter gene construct/chloramphenicol acetyltransferase/*N,N'*-dicyclohexylcarbodiimide)

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ABSTRACT The proportion of hexokinase (HK; EC 2.7.1.1) isozyme 1 (HK1) that is bound to the outer mitochondrial membrane is tissue specific and developmentally regulated. HK activity is known to be markedly elevated in many cancer cells and a significant fraction is mitochondrial bound. This study examined the role of the 15-amino acid N-terminal domain of HK1 in binding to liver and hepatoma mitochondria. A chimeric reporter construct, pCMVHKCAT, encoding this HK1 domain coupled to the chloramphenicol acetyltransferase (CAT) gene was electroporated into mouse Hepa 1-6 hepatoma cells. After digitonin treatment, cell fractions were assayed for HK, lactate dehydrogenase, and CAT activities. Digitonin (75 µg/mg of protein) caused cytosolic leak but 70% of HK remained with the pellet. HKCAT, like HK, remained predominantly with the pellet; CAT from the control, pCMVCAT, remained mostly unbound. Binding of membrane-free cell extracts to rat liver mitochondria *in vitro* showed 91% of the HKCAT bound, whereas only 12% of CAT bound. Specificity of HKCAT binding to mitochondria was demonstrated by competition of HK1 for HKCAT binding sites on rat liver mitochondria as well as by blockage of HKCAT binding by *N,N'*-dicyclohexylcarbodiimide, which covalently binds to porin and blocks HK1 binding. Deletional mutant constructs of HKCAT showed reduced binding with increasing deletion size. In summary, these studies demonstrate that the 15-amino acid N-terminal domain of HK1 is necessary and sufficient to confer mitochondrial binding properties to CAT and that there is specificity for this binding to the mitochondria.

Hexokinase (HK; EC 2.7.1.1) catalyzes the phosphorylation of glucose to glucose 6-phosphate, an ATP-dependent reaction that serves as the entry point for glucose into glycolysis and the hexose-monophosphate shunt. Four isozymes of HK are present in mammalian tissues. HK1, like HK2 and HK3, contains a catalytic domain in the carboxyl half of the protein and a regulatory domain in the amino half. Sequence data for HK1 cDNA from several species demonstrate substantial homology between the two halves as well as an additional 15-amino acid domain at the N terminus that is completely conserved between rat brain, human kidney, and bovine heart HK1 (1–3).

HK1 exhibits ambiquitous behavior[¶] within the cell, being found either free in the cytosol or bound to the outside of the outer mitochondrial membrane (4). The proportion of HK1 bound to the mitochondria is tissue specific, developmentally regulated, and varies with the metabolic state of the cell (5–7). It is notable that in hepatoma cells, which have markedly elevated HK activity, a significant proportion is mitochondrial bound (8, 9). It has been suggested that this HK1 binding is metabolically advantageous for HK1 and the cell. Binding of HK1 to the outer mitochondrial membrane is thought to induce a conformational change that renders the

enzyme less sensitive to inhibition by its product and therefore increases glucose phosphorylation (10). In addition, it has been postulated that the mitochondrial-bound HK1 has preferential access to ATP generated in the mitochondrial matrix, which serves to coordinately stimulate glucose utilization and oxidative phosphorylation (7). The site of HK1 binding is at or near the pore protein, mitochondrial porin, which resides in the outer mitochondrial membrane (11). The N-terminal domain of HK1 is known to participate in mitochondrial binding, since removal of 9 of the 15 amino acid residues from the N terminus of HK1 by a limited chymotryptic digestion eliminates binding to mitochondria (12).

This study sought to pursue the role of the 15-amino acid N-terminal domain of HK1 in mitochondrial binding and in the specificity of this HK1 binding. We generated a chimeric reporter construct containing the first 15 amino acids of HK1 coupled to chloramphenicol acetyltransferase (CAT). Our results demonstrate that these N-terminal amino acids of HK1 confer mitochondrial binding properties to CAT, which otherwise does not have affinity for this organelle. In addition, we show that HKCAT binds specifically to mitochondrial HK1 binding sites since HK1 competes with HKCAT for these binding sites and HKCAT binding is blocked by *N,N'*-dicyclohexylcarbodiimide (DCCD), a chemical that binds covalently to porin (13) and prevents HK1 binding to porin (14).

MATERIALS AND METHODS

Expression Vectors. pCMVBGal (15) was digested with *Not* I and *Eco*RV and pCMV was isolated. The HKCAT insert was obtained by PCR amplification (16) using a 68-base-pair (bp) oligonucleotide 5' primer containing the 5'-terminal 45 bp of the rat brain HK1 cDNA coding region (1) upstream to 21 nucleotides of the 5' end of the CAT gene beginning at codon 2. The first codon (ATG) of the CAT gene was excluded to prevent alternative initiation during transcription. The 3' primer was a 20-bp oligonucleotide from the 3' end of CAT. PCR was performed using pSVOCAT DNA as template and the following conditions: 1 cycle with 30 sec of denaturation (94°C), 1 sec of annealing (65°C), and 1 min of extension (72°C) followed by 30 cycles with 30 sec of denaturation (94°C) and 90 sec of annealing/extension (72°C). The product was tailed with phosphorylated *Not* I linkers (New England Biolabs) using T4 ligase at 15°C in a cooling bath. Unincorporated *Not* I linkers were removed and a *Not* I digestion was performed. The HKCAT DNA was ligated into the vector with T4 ligase and competent DH5α *Escherichia*

Abbreviations: CAT, chloramphenicol acetyltransferase; DCCD, *N,N'*-dicyclohexylcarbodiimide; HK, hexokinase; mU, milliunit(s); LDH, lactate dehydrogenase; CMV, cytomegalovirus; SV40, simian virus 40.

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[¶]"Ambiquitous" is a term coined by Wilson (4) and refers to the property of being in both places in contrast to ubiquitous, in all places.

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coli were transformed with the plasmid. pCMVHKCAT was identified by colony hybridization with CAT DNA labeled with [³²P]dCTP by the random hexamer method (17), and correct insert orientation was verified by digestion with *Eco*RI. The 5' end of this insert was sequenced directly after asymmetric PCR (18) by the dideoxy method. The host vector consisted of the cytomegalovirus (CMV) immediate early gene promoter and enhancer, the simian virus 40 (SV40) late viral protein gene 16S/19S splice donor and acceptor signals, and the SV40 late gene poly(A) signal on a pUC19 backbone (Fig. 1). pCMVCAT, which comprises only CAT inserted into the same plasmid construct, was cloned in a similar fashion.

A series of mutant constructs deleted for the codons encoding HK1 amino acids 2–5, 2–9, and 2–13 were cloned and are referred to as pCMVHKCATdel 4, 8, and 12, respectively (Fig. 2). The mutant HKCAT sequences were generated by PCR. A common 3' primer corresponding to the 3' end of the HKCAT sequence was used in the generation of all three mutants. Three additional 5' primers were synthesized, which included the initial ATG codon and the final 33, 21, and 9 bp of the HK1 binding domain, respectively. PCR was performed using HKCAT DNA as template and the following conditions: 1 cycle with 30 sec of denaturation (94°C), 30 sec of annealing (60°C), and 90 sec of extension (72°C) followed by 30 cycles with 30 sec of denaturation (94°C) and 2 min of annealing/extension (72°C). The remainder of the cloning was as before.

Cell Culture and Transfection. The mouse hepatoma cell line Hepa 1-6 was utilized for transient expression studies. The Hepa 1-6 cell line is a subclone derived from the original Hepa-1 cells described by Darlington *et al.* (19). The cells were grown to near confluence in 5% CO₂ in air at 37°C in medium consisting of 3:1 minimum essential medium/Weymouth medium supplemented with glutamine, 10% (vol/vol) fetal bovine serum, and antibiotics and then harvested by trypsinization. Electroporation was performed with 0.5 ml of cells at 1 × 10⁷ cells per ml and 25 μg of pCMVHKCAT, pCMVCAT, or a pCMVHKCATdel plasmid that had been purified twice by equilibrium centrifugation in CsCl/ethidium bromide. A Bio-Rad GenePulser was used with 360 V and 250 μF of capacitance. Time constants ranged from 4.5 to 5.0

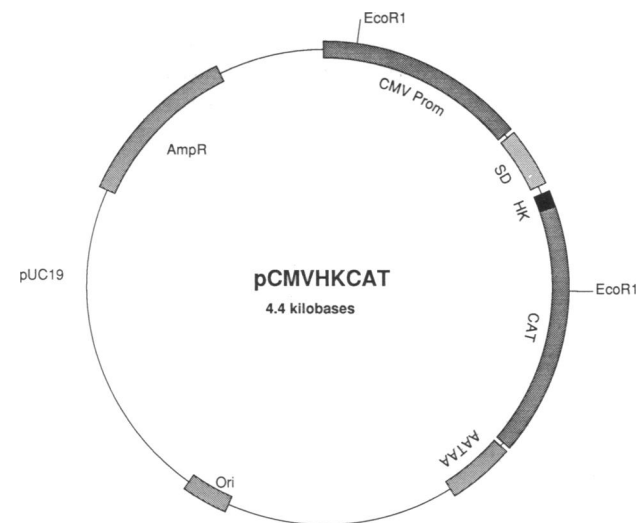


FIG. 1. pCMVHKCAT. HKCAT DNA was inserted into the mammalian expression vector pCMVB (10), consisting of the CMV immediate early gene promoter and enhancer (CMV Prom), the SV40 late viral protein gene 16S/19S splice donor and acceptor signals (SD), and the SV40 late gene poly(A) signal (AATAA) on a pUC19 backbone. The ampicillin-resistance gene (AmpR), origin of replication (Ori), and *Eco*RI sites are indicated.

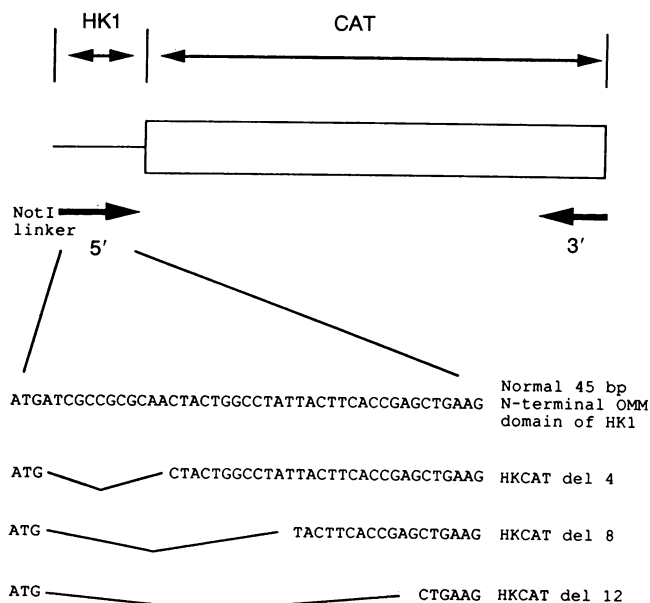


FIG. 2. pCMVHKCAT deletion constructs. The portions of the HK1 sequence incorporated into the 5' PCR primers used to generate the deletion constructs are shown. The first sequence is the normal 45-bp HK1 sequence encoding the 15-amino acid N-terminal domain necessary for outer mitochondrial membrane (OMM) binding. Subsequent sequences contain increasingly larger deletions from the 5' end of that HK1 sequence with conservation of the initial ATG codon.

sec⁻¹. Electroporated cells were replated in 15 ml of fresh medium and incubated for 48–72 hr to near confluence. The cells were reharvested in phosphate-buffered saline and then centrifuged at 100 rpm for 5 min. The supernatant was removed and the cells were resuspended in Krebs/Henseleit solution (118 mM NaCl/6 mM Na₂HPO₄/2 mM NaH₂PO₄/26 mM NaHCO₃, pH 7.5). After removal of an aliquot for protein determination, 0.1 volume of 20% (wt/vol) bovine serum albumin was added. Protein content was determined by the method of Bradford after three freeze-thaw cycles (20).

Cell Fractionation. Digitonin (Sigma) was used to fractionate the Hepa 1-6 cells into cytoplasmic and retained particulated pools (21). Cells were exposed to digitonin at concentrations of 0, 75, and 1000 μg/mg of cell protein for 30 sec at room temperature. Cell fractions were separated by centrifugation in a Microfuge at 8160 relative centrifugal force for 1 min. Pellet fractions were resuspended in Krebs/Henseleit solution and 5% Triton X-100.

Enzyme Activities. Lactate dehydrogenase (LDH), which served as a cytoplasmic marker, and HK activities were assayed spectrophotometrically by monitoring NADPH appearance or disappearance, respectively, at OD₃₆₆ (22). Total Hepa 1-6 HK activity was expressed in milliunits (mU)/mg of cell protein.

CAT assays were performed on the cell fractions by incubating with [¹⁴C]chloramphenicol (New England Nuclear, 2 GBq/mmol) and acetyl-CoA at 37°C for 30 and 60 min (23, 24). Acetylated and non-acetylated chloramphenicol was separated by thin-layer chromatography. Autoradiography was performed at room temperature for 16–24 hr. Acetylation fractions were obtained by scintillation counting of the appropriate portions of the thin-layer chromatography plates containing the acetylated and unacetylated chloramphenicol.

Mitochondrial Binding of HK1, HKCAT, and CAT. Rat liver mitochondria were isolated after homogenization by differential centrifugation (25) in medium containing 250 mM sucrose/10 mM Hepes, pH 7.4, supplemented with 1 mM

EDTA. The final mitochondrial resuspension solution did not include EDTA. Mitochondrial protein content was assayed by the method of Bradford. Aliquots of 0, 20, 50, 100, 200, and 300 mU of isolated rat brain HK1 (26) were incubated with mitochondria (1 mg of protein) in the presence of 10 mM MgCl₂ for 30 min at 0°C. After separating the mitochondria from the incubation solutions, HK activities were measured in both fractions as described. Mitochondrial HK activity was expressed as mU/mg of protein. Membrane-free Hepa 1-6 cell extracts containing either HKCAT or CAT were incubated with the rat liver mitochondria under identical conditions. After separating the mitochondria from the incubation solutions, all samples were heated to 65°C for 10 min to quench the deacetylase activity present in the mitochondrial preparations. CAT assays of the fractions were performed as before.

Competition and DCCD Studies. Isolated rat liver mitochondria were preincubated with 0, 50, or 250 mU of isolated rat brain HK1 with mitochondria (1 mg of protein) for 30 min at 4°C. Subsequently HKCAT was added and incubated for an additional 1 hr at 4°C. The mitochondria were pelleted in a Microfuge at 10,000 relative centrifugal force for 3 min, and the pellet was washed with 100 μ l of sucrose/Hepes medium and resuspended. CAT assays were performed on the resuspended pellet and the supernatant fractions.

Isolated rat liver mitochondria were incubated with DCCD (Sigma) at concentrations of 0–60 nmol/mg of protein for 30 min at room temperature in a glass vial (14). DCCD was dissolved in ethanol and serial dilutions were made in order to maintain a constant volume for incubation. An equal volume of ethanol was added to an aliquot of the mitochondria for the 0 DCCD datum. After incubation with DCCD, the mitochondria were washed three times with 250 mM sucrose/10 mM Hepes, pH 7.4. Bovine serum albumin (1 mg/ml) was also included in the first wash. Binding of rat brain HK1 was performed and assayed as before. HKCAT was incubated with mitochondria treated with 0 or 20 nmol DCCD per mg of protein and assayed as before.

RESULTS

Hepa 1-6 Transfection Studies. The total HK activity of the Hepa 1-6 cells was 14 mU/mg of cell protein. Cell fractionation experiments on native Hepa 1-6 cells showed that after exposure to a digitonin concentration of 75 μ g/mg of cell protein, >95% of the LDH activity was found in the supernatant, whereas \approx 70% of the HK activity remained with the pellet. After exposure to digitonin at 1000 μ g/mg of cell protein, nearly 100% of LDH and HK activities were found in the supernatant. The effects of digitonin treatment upon electroporated cells were identical to those observed in Hepa 1-6 cells that had not been electroporated.

The plasmid constructs pCMVHKCAT and pCMVCAT were transfected into the Hepa 1-6 cells by electroporation. CAT assays were performed on the supernatant and pellet fractions after treatment with digitonin (75 μ g/mg of cell protein). In the cells expressing HKCAT, the majority of the CAT activity remained with the pellet after digitonin treatment, whereas in the cells expressing CAT, the CAT activity was predominantly in the supernatant (Fig. 3). Some artifactual HKCAT and CAT leakage occurred in the absence of exposure to digitonin and was proportional to the centrifugal force with which the cells were pelleted. These studies established that HKCAT, like HK, is primarily membrane bound in Hepa 1-6 cells.

Rat Liver Mitochondrial Binding Studies. To demonstrate that HKCAT, like HK1, could bind to mitochondria, mitochondrial binding was performed *in vitro*. Isolated rat liver mitochondria were incubated with rat brain HK1 in the presence of 10 mM MgCl₂ and binding saturation was ob-

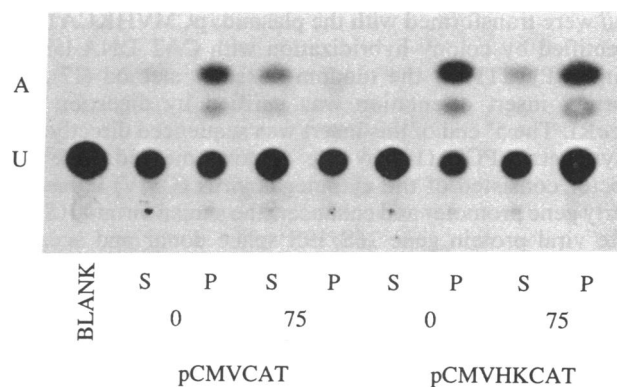


FIG. 3. CAT assays of Hepa 1-6 cells transfected with pCMV-CAT or pCMVHKCAT. Hepa 1-6 cells were exposed to 0 or 75 μ g of digitonin per mg of cell protein for 30 sec. Autoradiography of CAT assays performed upon the supernatant (S) and pellet (P) fractions revealed conversion of unacetylated (U) to acetylated (A) chloramphenicol by CAT or HKCAT. Without digitonin, pellets from Hepa 1-6 cells transfected with either pCMVCAT or pCMVHKCAT displayed high levels of CAT activity. After exposure to digitonin, CAT activity in the Hepa 1-6 cells transfected with pCMVHKCAT was detected predominantly in the supernatant; in cells transfected with pCMVHKCAT, CAT activity was largely retained by the pellet.

served at 30 mU/mg of mitochondrial protein. The baseline HK activity of the mitochondria was 3 mU/mg of protein. Membrane-free Hepa 1-6 cell extracts containing equivalent HKCAT or CAT activities were incubated with the mitochondria under the same conditions. The results of the CAT assays indicated that 91% of the HKCAT bound to the mitochondria, whereas only 12% of the CAT bound. These experiments establish that the HK1 domain in HKCAT is sufficient for mitochondrial binding.

Competition and DCCD Studies. Receptor competition and DCCD blocking studies were performed to determine whether the binding of HKCAT to hepatic mitochondria had properties similar to those observed with HK1 binding. Rat liver mitochondria were preincubated with 0, 50, and 250 mU of rat brain HK1, and HKCAT was subsequently added to the incubation mixture. With increasing HK1 concentration, HKCAT activity was observed to decrease in the mitochondrial pellet and to increase in the supernatant (Fig. 4). The addition of 250 mU of HK1 per mg of mitochondrial protein (which is beyond that required to saturate the mitochondria,

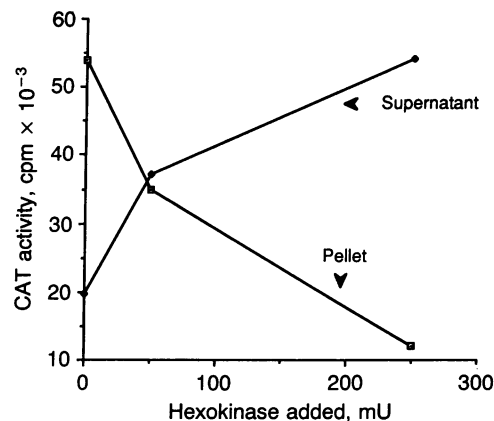


FIG. 4. HKCAT binding to mitochondria preincubated with increasing amounts of HK1. After preincubation with 0, 50, or 250 mU of HK1, isolated rat liver mitochondria were incubated with HKCAT. Mitochondrial pellet and supernatant fractions were assayed for CAT activity. HKCAT activity decreases in the pellet and increases in the supernatant with increasing HK1.

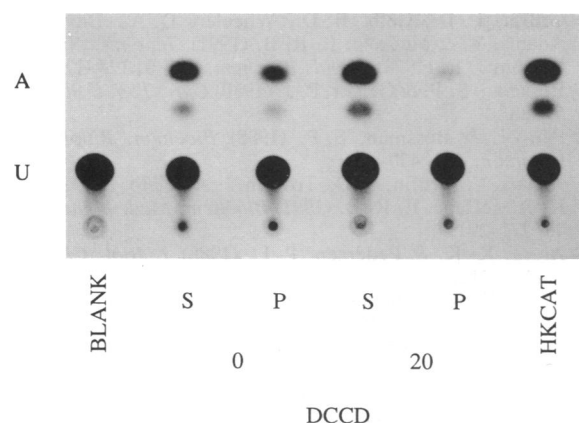


FIG. 5. CAT assays of HKCAT binding to mitochondria after DCCD treatment. Isolated rat liver mitochondria were treated with either 0 or 20 nmol of DCCD per mg of mitochondrial protein. After incubation with HKCAT and separation of the mitochondrial pellet (P) from the supernatant (S), CAT assays were performed on both fractions. Autoradiography shows binding of HKCAT to untreated mitochondria but substantially reduced binding to DCCD-treated mitochondria. U and A, unacetylated and acetylated chloramphenicol, respectively.

30 mU/mg of protein) reduces the binding of HKCAT by 80% and the balance is recovered in the supernatant.

Rat liver mitochondria were incubated with DCCD, a markedly hydrophobic compound that blocks HK1 binding (14). DCCD alone did not inhibit CAT activity. HK1 binding to DCCD-treated mitochondria was reduced by 60% at a DCCD concentration of 20 nmol/mg of protein and a saturating concentration of rat brain HK1; this concentration of DCCD was used with HKCAT. After incubation of HKCAT with DCCD-treated mitochondria, negligible CAT activity was demonstrable in the mitochondrial pellet (Fig. 5).

Hence, the mitochondrial binding of the HK1 domain in HKCAT is a specific interaction that is blocked when the mitochondrial HK1 binding sites are not available either through occupation by saturating HK1 or through the action of DCCD.

Deletional Mutation Studies. Hepa 1-6 cells were transfected with pCMVHKCATdel 4, 8, and 12 by electroporation, fractionated with digitonin (75 μ g/mg of protein), and assayed for CAT activity. pCMVHKCAT and pCMVCAT were used as controls. CAT assays revealed that retention of the HKCATdel proteins in the pellets decreased with increasing deletion size approaching the level of CAT control (Fig. 6). The low proportion of bound CAT activity with pCMVHKCATdel 8 and 12 is consistent with the previous observation that HK1, which is missing the N-terminal nine amino acids, does not bind to mitochondria (12).

DISCUSSION

These experiments demonstrate that the 15-amino acid N-terminal portion of HK1 is necessary and sufficient to confer mitochondrial binding properties to CAT, an enzyme that otherwise does not exhibit these properties. The results show that HKCAT, like native HK, is primarily membrane bound in Hepa 1-6 cells. The competition and blocking studies, using HK1 and DCCD, respectively, establish that HKCAT interacts in a specific manner with the mitochondria. Specificity is also inferred from the results of the HKCAT deletion studies. Removal of HK1 binding domain amino acids 2-5 reduces binding to 50% (62% of HKCAT binding), whereas larger deletions (amino acids 2-9 and 2-13) reduce binding to <30% (35% of HKCAT binding), nearly to the level of the CAT control. It is unclear whether the specific amino acid

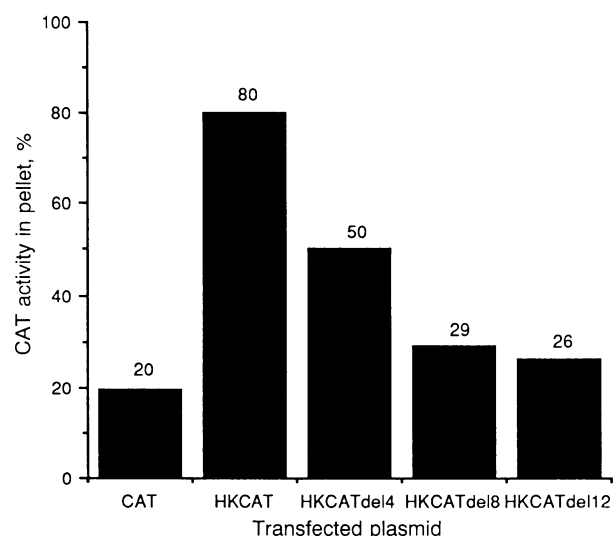


FIG. 6. Comparison of CAT activity in cell pellets with HKCAT deletion constructs. Hepa 1-6 cells transfected with pCMVCAT, pCMVHKCAT, or one of the three pCMVHKCAT deletion constructs were exposed to either 0 or 75 μ g of digitonin per mg of cell protein. Supernatant and pellet fractions were separated and assayed for CAT activity. The fraction of total cellular CAT activity retained by the pellet after digitonin treatment, expressed as a percentage, is compared among the five constructs. Numbers above the bars indicate those percentages.

sequence is critical or if overall hydrophobicity is more important, such that elimination of a certain proportion of these residues might weaken the interaction with the mitochondrial binding site sufficiently to prevent adequate anchoring of the HKCAT protein with the receptor.

HK exists in a wide variety of adult tissues but differs significantly in isozymic distribution and the proportion of the enzyme bound to mitochondria. Brain, renal medulla, and many neoplasias are similar in exhibiting relatively high HK1 activities and high proportions of bound enzyme (7-9). These tissues also share a heavy reliance upon glucose for metabolism, and it has been suggested that the binding of HK1 to mitochondria occupies an important role in metabolic regulation in these tissues. The Hepa 1-6 cells examined in this study exhibited high total HK activity with \approx 70% bound.

The principal advantage attributed to HK1 binding to porin is preferred access to mitochondrial ATP for the bound enzyme. Although porin is randomly distributed on the outer mitochondrial membrane, HK1 binds preferentially to porin at contact sites between the inner and outer mitochondrial membranes (12, 27). The adenine nucleotide translocator is associated with the inner mitochondrial membrane and is the putative inner mitochondrial membrane transit site for ATP that subsequently traverses porin (7). The adenine nucleotide translocator is believed to interact with porin either directly or indirectly via other proteins at the mitochondrial contact sites. The binding of HK to porin serves dual purposes in providing direct access to mitochondrial ATP for the enzyme and facilitating phosphorylated adenine nucleotide traffic to and from the mitochondrial matrix.

The regulation of HK1 binding appears to involve HK1 directly as well as its mitochondrial target. Wilson (28) has shown that the binding of glucose 6-phosphate to HK1 induces a conformational change in the N-terminal domain involved in mitochondrial binding. This conformational change presumably alters the presentation of the hydrophobic N terminus, making hydrophobic binding less favored. Porin has been noted to be a ubiquitous protein in the outer mitochondrial membrane (29). The observation that HK1 does not randomly bind to porin but shows a preference for

porin at contact sites (30) suggests that porin and/or an associated protein at the contact sites contributes to the regulation of HK1 binding. The existence of genetically distinct porins and of multiple isoforms has been proposed (31). Porin at mitochondrial contact sites switches ion selectivity after a soluble protein induces a conformational change (32). Ion selectivity of the pore also depends on the inner mitochondrial membrane potential (33). These and related factors may influence the association of HK1 with the mitochondrion by altering the environment at the site of HK1 binding.

HK1 is not the only porin-bound kinase with preferential access to mitochondrial ATP. In addition to HK2 (34), glycerol kinase also shares these properties (7). Like HK1, the proportion of glycerol kinase bound to porin varies with tissue type, metabolic state, and developmental stage. Glycerol 3-phosphate can cause debinding of porin-bound glycerol kinase. Neither protein nor DNA sequence data are available for vertebrate glycerol kinase, so a comparison of the porin binding domains of HK1 and glycerol kinase is not possible at present. The sequence of HK2 has been reported recently (35), and the N-terminal domain of HK2 shows 53% sequence identity with HK1.

Two models for the porin-HK1 interaction have been proposed (36, 37). One model suggests that the hydrophobic N-terminal domain of HK1 inserts into the outer mitochondrial membrane lipid with porin interacting specifically with another domain of the HK1 molecule. An alternative model suggests that there exists a direct and specific interaction between the N-terminal domain of HK1 and porin. Conclusive differentiation between these models and detailed elucidation of the interaction between HK1 and its binding site on the outer mitochondrial membrane will require isolation and investigation of the ligand-receptor complex. In this report we describe a construct, HKCAT, that shares only the N-terminal 15 amino acids with HK1 and is approximately one-third the size of HK1. Since this construct binds to rat liver and hepatoma mitochondria with properties quite similar to those exhibited by native HK1, it will be useful in defining this ligand-receptor complex. This refinement of the HK1-porin model is important in extending our understanding of the organization of mitochondrial contact sites. Knowledge of the HK1 binding domain, which could be compared in the future with similar investigations utilizing the recently published HK2 sequence, may provide a better understanding of the variation in HK binding patterns that exist across tissue type and developmental stage.

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