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Localization and Regulation of Mouse Pantothenate Kinase 2

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Abstract

Coenzyme A (CoA) biosynthesis is initiated by pantothenate kinase (PanK) and CoA levels are controlled through differential expression and feedback regulation of PanK isoforms. PanK2 is a mitochondrial protein in humans, but comparative genomics revealed that acquisition of a mitochondrial targeting signal was limited to primates. Human and mouse PanK2 possessed similar biochemical properties, with inhibition by acetyl-CoA and activation by palmitoylcarnitine. Mouse PanK2 localized in the cytosol, and the expression of PanK2 was higher in human brain compared to mouse brain. Differences in expression and subcellular localization should be considered in developing a mouse model for human PanK2 deficiency.

Keywords

Coenzyme A; pantothenate; pantothenate kinase; mitochondria; pantothenate kinase-associated neurodegeneration; palmitoylcarnitine

1. Introduction

CoA [1] is an essential cofactor that plays a central metabolic role as the predominant acyl group carrier in living organisms [1]. Regulation of the cofactor concentration is achieved through feedback inhibition of pantothenate kinase (PanK), the first enzyme in the biosynthetic pathway [2-4] catalyzing the ATP-dependent phosphorylation of pantothenate. Mammals express four catalytically active isoforms that have different sensitivities to inhibition by free CoA or CoA thioesters [5-7]. PanKs share a >80% identical catalytic core [5;6;8] attached to unrelated N-terminal extensions that range from a few residues in the case of PanK1β and PanK3 to ∼100 −200 residues in the case of PanK1α and PanK2. The N-terminus of human PanK2 (hPanK2) directs it to the mitochondria where hPanK2 is sequentially processed twice to yield a 48 kDa mature protein [8-10]. A catalytically active hPanK2 inside the mitochondria was irreconcilable with the high organelle CoA concentration [11;12] until the discovery of palmitoylcarnitine as a positive regulator of hPanK2 [13]. Palmitoylcarnitine releases the acetyl-CoA inhibition by competing for its binding site, and this regulatory mechanism activates hPanK2 when the mitochondrial demand for CoA is high for β-oxidation.

Pantothenate kinase-associated neurodegeneration (PKAN) is a rare autosomal recessive disease caused by mutations in the *PANK2* gene [14;15]. PKAN patients exhibit pathological accumulation of iron in the brain, movement and speech defects [14-16]. This disease suggests

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an important role for mitochondrial PanK2 in mammalian physiology; however, the *Pank2* knockout mice do not recapitulate the neurological abnormalities found in humans [17]. This study shows that although the biochemical regulatory properties of the two enzymes are indistinguishable, there are significant differences in gene structure, subcellular localization of the proteins and relative abundance of PanK2 expression in human and murine tissues.

2. Materials and Methods

2.1. Cloning of the murine Pank2 and generation of cell lines

A 1.6 kb fragment encoding the long form of mPanK2 [8] was assembled from 3 ESTs (NCBI accession numbers BG091057, CB247865 and BQ715345) and ligated into pcDNA3.1+ between EcoRI and XbaI restriction sites to generate pPJ256. For biochemical assays, HEK293T cells (gift from Suzanne Baker, St. Jude Children's Research Hospital) were transiently transfected for 48 h with either pPJ256 or pKM56 (a pcDNA3.1-derived vector encoding the mature hPanK2 (residues 141−570) [7]). For immunofluorescence analysis, HEK293 cells (ATCC) were stably transfected with either pPJ256 or pKM4 (a pcDNA3.1-derived vector encoding the mitochondrial precursor hPanK2 (residues 1−570) [7]). Clones were selected in growth medium containing 600 μg/ml of G418 and screened for expression.

2.2. Real time qRT-PCR analysis

Total RNA was either purchased (mouse and human brain sections, human liver, brain and testes) or isolated from whole brain and caudate nucleus region, whole liver and testes of 2−3 mice, using the TRIZOL reagent. Synthesis of first-strand cDNA was obtained by reverse transcription using SuperScript™ II RNase H$^-$ reverse transcriptase, the RNA templates and random primers. Quantitative real-time PCR was performed in triplicate using the ABI Prism® 7700 Sequence Detection System with the primers and probes listed in Table S1. The Taqman human and rodent GAPDH (Applied Biosystems) were used as controls. All of the values were compared using the C_T method [18], and the amount of cDNA ($2^{-\Delta CT}$) was reported relative to glyceraldehyde-3-phoshate dehydrogenase (GAPDH) mRNA.

2.3. Western blot, immunofluorescence and mitochondria isolation

Peptides corresponding to protein stretches unique to mPanK2 (GESADSEARRRDPLRRR) and hPanK2 (EGRRQEPLRRRASSASV) were synthesized and coupled to keyhole limpet hemocyanin and polyclonal antisera raised in rabbits. Affinity purification was performed as previously described [5;19]. Horseradish peroxidase-conjugated protein A was used as the secondary antibody at a dilution of 1:5,000 to detect proteins in the western blots. The membranes were then stripped using a recycling kit (Alpha Diagnostic International) and reprobed with antibodies against the mitochondrial marker pyruvate dehydrogenase or the cytosolic marker β-actin used at concentrations of 2.5 μg/ml and 5 μg/ml, respectively. The immuno complexes were detected using the ECL kit (GE Healthcare).

The localization of mPanK2 and hPanK2 was determined by confocal microscopy using a Zeiss LSM 510 META multiphoton microscope with a Plan-Neofluor 100x/1.3 oil immersion objective. HEK293 cell lines grown in chamber slides were incubated with 250 nM MitoTracker red CMXRos to stain the mitochondria. Cells were then fixed with 3.7% formaldehyde, permeabilized with 0.2% Triton X-100, and incubated with the mPanK2 and hPanK2 antibodies. Anti-rabbit IgG conjugated to Alexa Fluor 488 was used to visualize the proteins. The slides were mounted with ProLong Gold antifade reagent plus DAPI.

Heavy mitochondria were isolated from wild type and *Pank2* knockout mouse brain as described by Graham [20].

2.5. Pantothenate kinase assay

The lysates of transfected HEK293T cells were obtained for measuring the pantothenate kinase activity as described previously [7]. Briefly, the reaction mixtures contained 45 μM D-[1-^{14}C] pantothenate (specific activity 27.5 mCi/mmol), 250 μM ATP pH 7.0, 10 mM $MgCl_2$, 0.1 M Tris-HCl pH 7.5, 5 μg of protein and varying concentrations of acetyl-CoA or palmitoylcarnitine.

2.6. Bioinformatic analysis

Genomic sequences for human (chromosome 20, NT_011387.8), chimpanzee (chromosome 20, NW_001230484), cow (chromosome 13, NW_001493148.1), dog (chromosome 24 NW_876277.1), cat (no chromosome information, AANGO1231943.1) and mouse *Pank2* (chromosome 2, NW_000178.1) were retrieved from NCBI. The macaque genomic sequence was retrieved from the sequencing center's website (http://www.hgsc.bcm.tmc.edu/projects/rmacaque/). Sequence comparisons were performed using the VISTA tools for comparative genomics (http://genome.lbl.gov/vista/index.shtml) [21].

2.7. Materials

Materials were purchased from the following suppliers: oligonucleotides and probes from the Hartwell Center at the St. Jude Children's Research Hospital; Poly(A)$^+$ RNAs from mouse and human brain sections, human liver, brain and testes from Clontech; molecular biology reagents from Qiagen; restriction enzymes and T4 DNA ligase from Promega; SuperScript™ II RNase H$^-$ reverse transcriptase, TRIZOL reagent, and pcDNA3.1 vectors from Invitrogen; Real-time PCR mix from Applied Biosystems; Bradford dye-binding protein assay solution from Bio-Rad; D-[1-^{14}C]pantothenate (specific activity, 55 mCi/mmol) from American Radiolabeled Chemicals. PanK2 knockout mice were kindly provided by Jane Gitschier [17]. All other reagents were of analytical grade or better and were obtained from Sigma Chemical Co.

3. Results and Discussion

The amino-terminus of hPanK2 contains a mitochondrial targeting sequence (residues 1−140) that is encoded by exon 1 [8]. The nucleotide sequences from six organisms were compared using the VISTA tools for comparative genomics (Fig. 1). This analysis revealed that while the macaque sequence encoded an intact mitochondrial targeting sequence, the aligned nucleotide regions from mouse, cat, dog and cow were not predicted to be translated. The predicted ATG start codon in these organisms corresponded to residue 123 of hPanK2 and excluded the mitochondrial targeting sequence. The targeting sequence was found in the predicted chimpanzee PanK2 protein (NCBI accession number XP_001163366) but the encoding gene was not included in the VISTA analysis due to the poor quality of the downstream DNA data. These data indicated that emergence of a mitochondrial targeting sequence occurred following the divergence of primates and suggested that non-primate PanK2 may have distinctly different properties than the human isoform.

We compared the mature hPanK2 to full length mPanK2 to determine if there were differences in their regulatory properties. The two enzymes were similarly inhibited by acetyl-CoA and de-inhibited by palmitoylcarnitine (Fig. 2). The acetyl-CoA IC_{50}s were estimated at 125 nM for hPanK2 and 62.5 nM for mPanK2, and the concentration of pamitoylcarnitine that maximally restored the activity was 16 μM in both cases (Figs. 2A and 2B). Thus, the regulation of PanK2 in both species was similar. The difference in the acetyl-CoA IC_{50} values between mPanK2 and hPanK2 (0.06 μM vs. 0.12 μM) is not considered to be significant in comparison with the IC_{50} values determined for the other PanK isoforms which are 10-fold and 100-fold higher, e.g., PanK3, 1 μM; PanK1β, 10 μM [6].

Next, the mRNA expression levels of the murine and human PanK isoforms in brain, testes and liver were quantified (Fig. 3). Expression of PanK4 was not taken into account because it does not exhibit PanK activity [22]. *PANK1* transcripts (*PANK1α* + *PANK1β*) dominated human liver, whereas *Pank1* transcripts (*Pank1α* + *Pank1β*) in mouse liver were equivalent to *Pank3* transcripts (Fig. 3) and lower than in human, relative to the expression of glyceraldehyde-3-phosphate dehydrogenase mRNA, a metabolic housekeeping gene which was quantified as a measure of the total mRNA pool. *PANK2* transcripts, on the other hand, were relatively higher than *PANK1* and equivalent to *PANK3* in human brain, whereas *Pank2* transcripts were low in mouse brain and *Pank3* expression was highest. *Pank2* transcripts were extremely high in mouse testis accounting for 46% of the expressed isoforms, but were considerably less abundant in human testis. Human *PANK2* transcripts were highest in brain, and represented 45% of the *PANK* transcripts. A detailed analysis of specific areas of the brain revealed a high expression of human *PANK2* transcripts in the cerebellum, which controls motor coordination and movement, and, as previously reported, in the caudate nucleus [8] (Fig. 3D). The overall abundance of the murine *Pank* transcripts was lower than what was measured in the corresponding human brain fractions (Fig. 3C), but the level of *Pank2* was higher in the cerebellum and cerebral cortex compared to whole brain. These data revealed significant differences in the tissue-specific distribution of PanK isoforms in mice and humans.

The localization of hPanK2 and mPanK2 was investigated *in situ* by confocal immunofluorescence microscopy using mPanK2- and hPanK2-specific antibodies (Fig. 4). Unlike the mitochondrial hPanK2, mPanK2 was found in the cytosol. These results were the same in either the human cell line, HEK293 (Fig. 4), or the mouse cell line, NIH3T3 (data not shown). The cytosolic location of mPanK2 was confirmed by western blotting analysis of cytosolic and mitochondrial fractions obtained from the brains of wild-type and PanK2 knockout mice (Fig. 5). mPanK2 was found exclusively in wild type cytosol and not in mitochondria. The size of the endogenous PanK2 protein matched the size of mPanK2 predicted from genomic analysis (48.6 kDa) indicating that mPanK2 was not processed. The faint band appearing in the knockout cytosolic fraction most likely represents non-specific binding of the antibody to an unrelated protein. It is larger than the recombinant mPanK2 protein shown in the far right lane and can be seen faintly in the WT cytosol as well.

4. Conclusions

The comparison of the mouse and human PanK2 proteins reveal significant differences in their expression pattern and subcellular localization. Perhaps the symptoms of PKAN disease in humans arise in part from an overall lack of sufficient PanK activity in the brain. The fact that PanK2 comprises a higher percentage of the total PanK enzyme in human brain compared to mouse brain may contribute to the inability to demonstrate a neurological phenotype in *Pank2* knockout mice [17]. However, PanK2 transcripts are high in the testis, the organ system primarily affected in the PanK2 knockout mice [17]. The most surprising result is the finding that the evolution of a mitochondrial targeting sequence occurred following the divergence of the primate lineage. Our data are not in agreement with the previous conclusion [9] that both mouse and human PanK2 proteins are associated with mitochondria which was based on the immunolocalization of the proteins in transiently transfected COS7 cells and tissue sections. However, our examination of the same data suggests that there is a difference between the localization of the two proteins [9;17]. The biochemical regulatory properties are essentially the same between the two proteins, indicating that they perform the same function of monitoring the status of mitochondrial β-oxidation via palmitoylcarnitine and that mitochondrial localization is not required. However, the location of hPanK2 in the mitochondria places the protein in the same cellular compartment where palmitoylcarnitine is generated, thus providing greater sensitivity to changes in the concentration of the ligand. The lower contribution of PanK2 to the total PanK activity in mouse brain compared to humans

and its localization in the cytosol rather than the mitochondria are two factors that need to be considered in the understanding of the role of PanK isoforms in CoA homeostasis and in the development of a mouse model that recapitulates the molecular aspects of human PKAN disease. A recent report [23] suggests that pantothenate-deficiency in wild-type mice leads to a movement disorder that may provide a phenocopy for human PKAN disease, whereas pantothenate-deficiency in Pank2 knockout mice causes precipitous death, similar to the global mouse knockout of all pantothenate kinase isoforms with a chemical inhibitor [22].

Acknowledgments

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

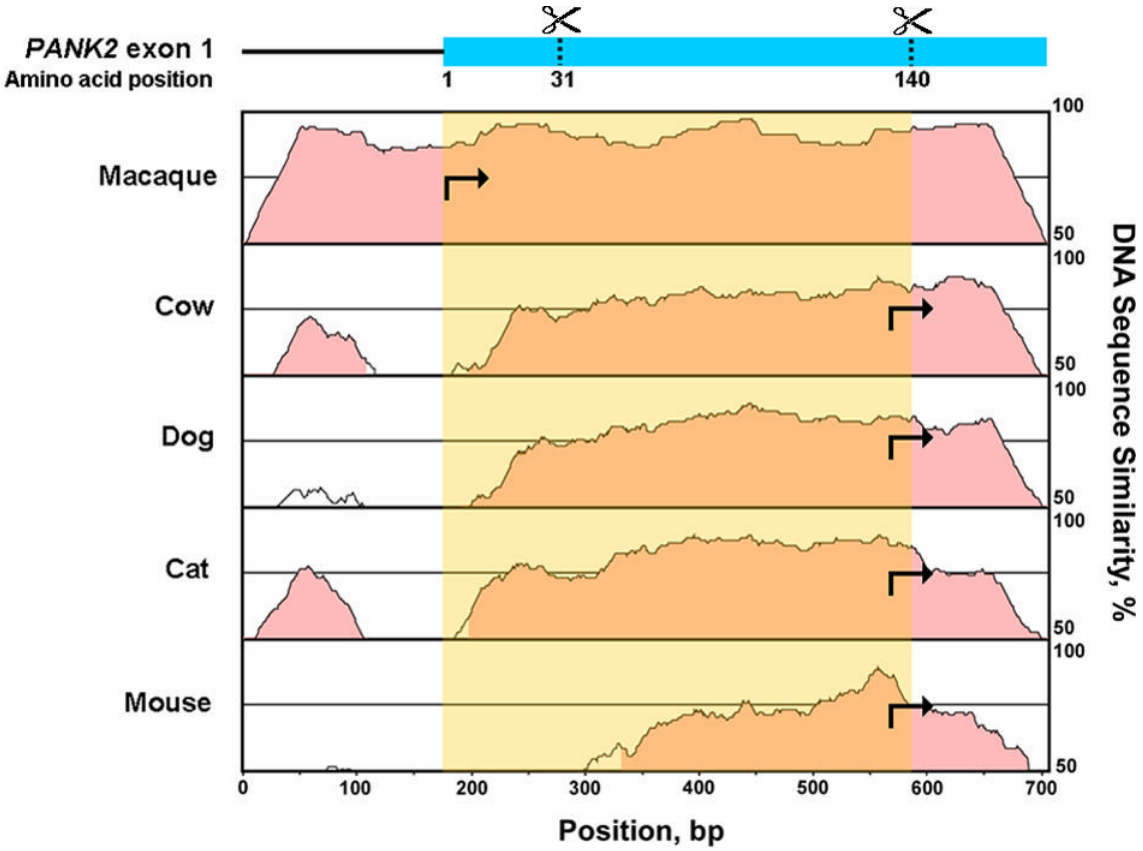

Fig 1.

DNA level sequence similarity of the 5′-untranslated regions and exon 1 of six mammalian PanKs. Genomic DNA sequences were aligned using the VISTA set of tools for comparative genomics with human *PANK2* as the reference sequence. Black arrows indicate the positions of the predicted start codons in the corresponding organisms. The region corresponding to the human mitochondrial targeting signal has been shadowed and the positions of the mitochondrial processing peptidase cleavage sites [10] are indicated by the scissor symbol at the top.

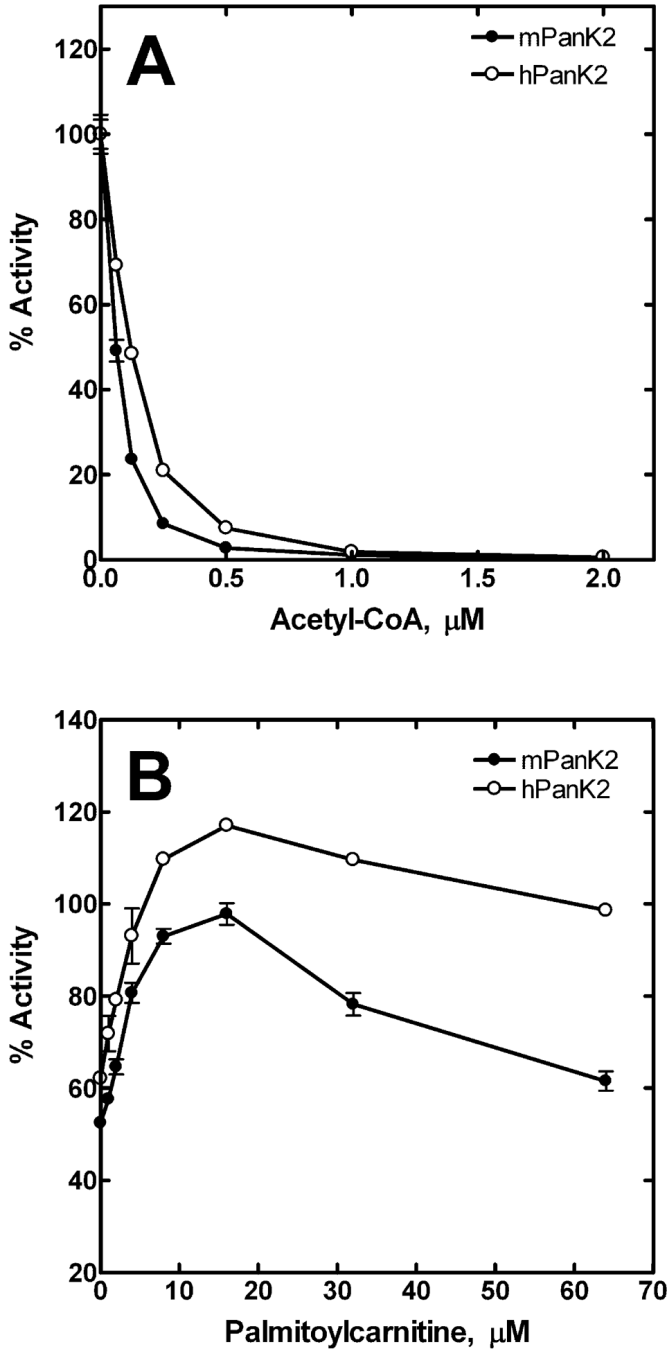

Fig 2.

Regulatory properties of mPanK2 and hPank2. (A) Acetyl-CoA inhibited both mPanK2 (●) and the mature form of hPanK2 [7] (○) with similar IC_{50}s of 62.5 and 125 nM, respectively. (B) Inhibition by acetyl-CoA was released by the addition of palmitoylcarnitine. The enzymes were incubated with fixed concentrations of acetyl-CoA corresponding to the respective IC_{50}s and with increasing amounts of the activator. The data represent the average of two independent experiments ± the standard error.

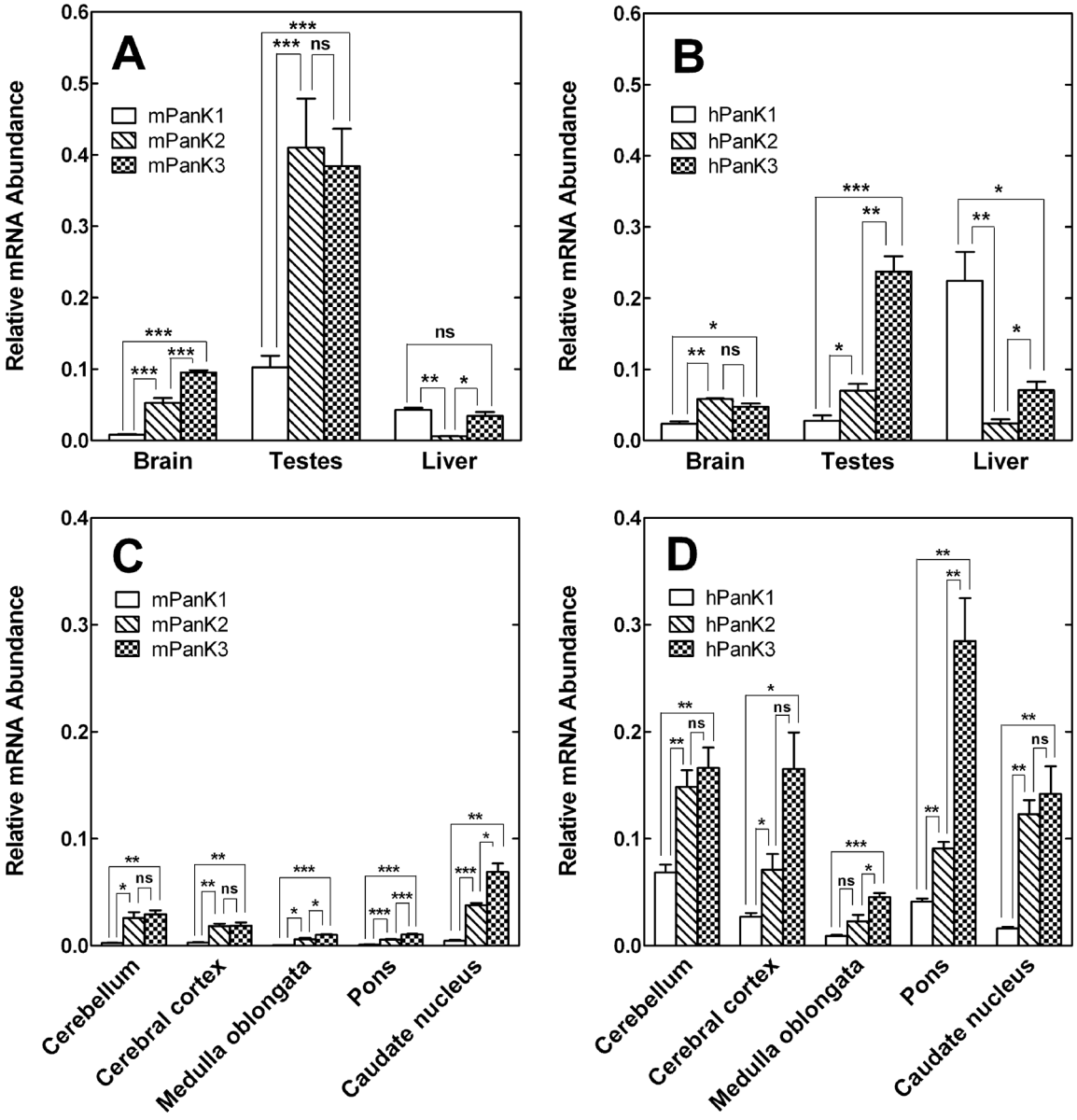

Fig 3.

PanK isozyme expression in murine and human tissues. (A and B) The abundance of murine (A) or human (B) PanK isoform mRNAs in brain, testes and liver. (C and D) The same analysis was conducted on mRNA from specific regions of the murine (C) and human (D) brain. Individual samples were analyzed in triplicate, averaged and plotted relative to mouse or human glyceraldehyde-3-phosphate dehydrogenase mRNA. All data are plotted as mean ± the standard error, and statistical significance was assessed by the students *t* test: *, $0.01<p<0.05$; **, $0.001<p<0.01$; ***, $p<0.0001$; NS, not significant. Analysis of variance by the ANOVA test between mouse and human PanK isoforms in each brain region (panels C and D) resulted in significance values of $p\leq0.0001$ for the difference in expression levels between the two species.

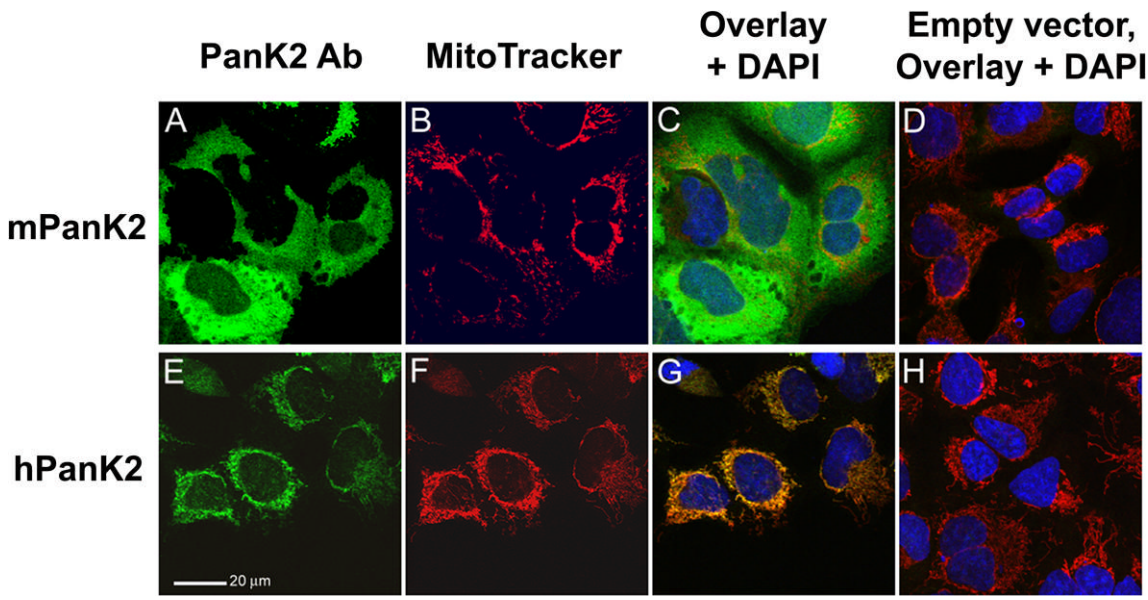

Fig 4.

Subcellular localization of mPanK2 and hPanK2. (A-C) Stably transfected HEK293 cells expressing mPanK2, (E-G) the mitochondrial precursor form of hPanK2 [7] or containing (D and H) the empty vector were cultured and prepared for immunofluorescence as described under 'Materials and Methods'. (B and F) Cells were incubated with MitoTracker red CMXRos and with specific antibodies against (A) mPanK2 or (E) hPanK2, followed by incubation with anti-rabbit IgG conjugated to Alexa Fluor 488 green; nuclei were stained with DAPI (blue). Panels C and D show the merged images for mPanK2 and empty vector, respectively; panels G and H show the merged images for hPanK2 and empty vector, respectively. hPanK2 localized to the mitochondria, while mPanK2 was in the cytosol.

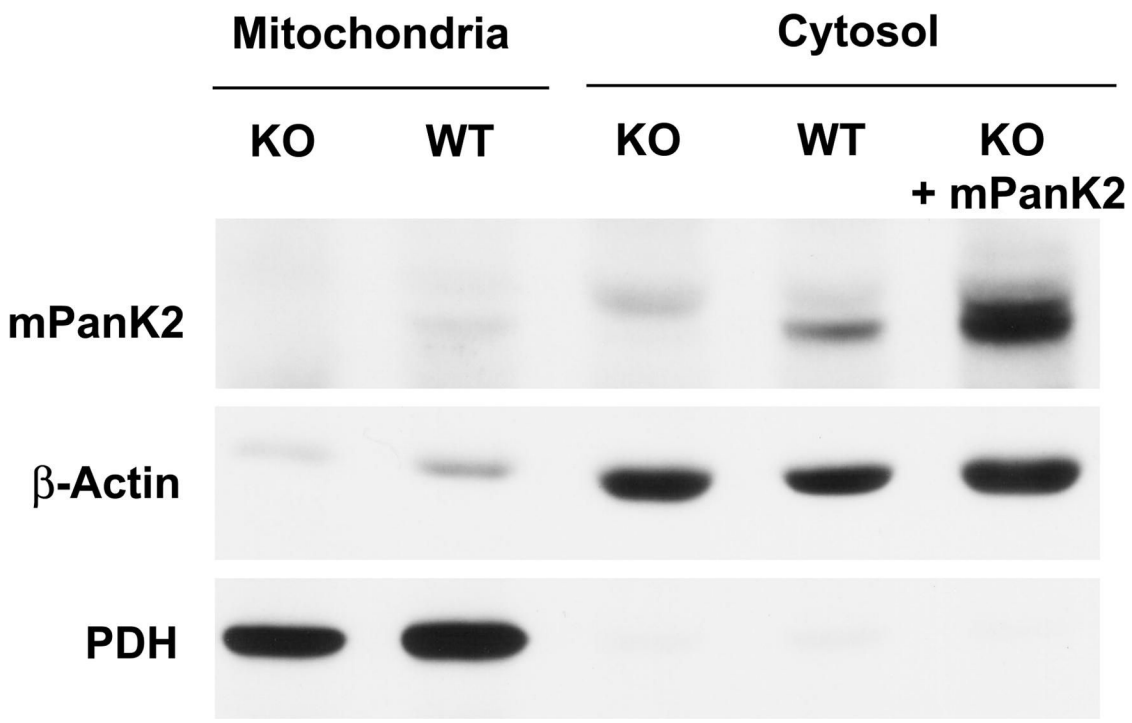

Fig 5.

Cytosolic localization of mPanK2 in mouse brain. The western blot was performed using 200 μg of brain mitochondrial and cytosolic fraction protein from wild-type and *Pank2* knockout mice and the specific mPanK2 antibody. 195 μg of cytosolic protein from knockout mice were mixed with 5 μg of lysate protein from stably transfected HEK293 cells expressing mPanK2 and used as a control. The blot was then stripped and reprobed with anti-β-actin as a marker protein for cytosol, and then with anti-pyruvate dehydrogenase (PDH), a mitochondrial marker protein.