

Pancreatic Expression and Mitochondrial Localization of the Progestin-AdipoQ Receptor PAQR10

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Steroid hormones induce changes in gene expression by binding to intracellular receptors that then translocate to the nucleus. Steroids have also been shown to rapidly modify cell function by binding to surface membrane receptors. We identified a candidate steroid membrane receptor, the progestin and adipoQ receptor (PAQR) 10, a member of the PAQR family, in a screen for genes differentially expressed in mouse pancreatic β -cells. *PAQR10* gene expression was tissue restricted compared with other PAQRs. In the mouse embryonic pancreas, *PAQR10* expression mirrored development of the endocrine lineage, with PAQR10 protein expression confined to endocrine islet-duct structures in the late embryo and neonate. In the adult mouse pancreas, PAQR10 was expressed exclusively in islet cells except for its reappearance in ducts of maternal islets during pregnancy. PAQR10 has a predicted molecular mass of 29 kDa, comprises seven transmembrane domains, and, like other PAQRs, is predicted to have an intracellular N-terminus and an extracellular C-terminus. *In silico* analysis indicated that three members of the PAQR family, PAQRs 9, 10, and 11, have a candidate mitochondrial localization signal (MLS) at the N-terminus. We showed that PAQR10 has a functional N-terminal MLS and that the native protein localizes to mitochondria. PAQR10 is structurally related to some bacterial hemolysins, pore-forming virulence factors that target mitochondria and regulate apoptosis. We propose that PAQR10 may act at the level of the mitochondrion to regulate pancreatic endocrine cell development/survival.

Online address: <http://www.molmed.org>

doi: 10.2119/2008-00072.Gonez

INTRODUCTION

Steroid hormones signal through intracellular receptors that translocate to the nucleus and function as transcriptional regulators of target genes. Other steroid-induced events are rapidly triggered independent of transcription via signaling pathways classically associated with cell membrane receptors, including ion channels, second messengers, and protein kinase cascades (1). These so-called nongenomic effects of steroids include estrogen-induced proliferation of breast cancer cells (2), estrogen-induced vasodilation (3), and the progesterone-initiated

acrosomal reaction in sperm (4). The mechanisms underlying this rapid signaling by steroids are poorly defined. Studies have identified membrane-associated, steroid-binding proteins in different tissues and species, and there is evidence that some nongenomic steroid effects are mediated by classical G protein-coupled receptors (GPCRs) or involve cytoplasmic activation of the nuclear steroid receptors [reviewed in (5)].

Membrane progestin receptors (mPRs) have recently been identified (6) that belong to a new family of seven-transmembrane proteins termed prog-

estins and adipoQ receptors (PAQRs), present in all species except those in the Archae (7,8). This family includes 11 mammalian genes (*PAQR1-11*), *YOL002c* and related genes from *Saccharomyces cerevisiae* (9), and the gene for *hemolysin III* from *Bacillus cereus* (10) as well as hemolysin-related bacterial genes. Phylogenetic analysis (8) allows the mammalian PAQR family to be divided into three main subgroups: the adiponectin-related receptors, which include PAQR 1 (adipoR1), PAQR 2 (adipoR2), PAQR 3, and PAQR 4; the mPRs, which include PAQR 5 (mPR γ), PAQR 6, PAQR 7 (mPR α), PAQR 8 (mPR β), and PAQR 9; and the hemolysin III-related receptors, PAQR 10 and PAQR 11.

Conflicting evidence exists regarding the membrane topology and subcellular localization of PAQR family members, as well as the mechanisms by which they bind ligands and transduce signals. The mPRs PAQR5, 7, and 8 are thought to have extracellular N-termini similar to classic GPCRs (11), whereas the adiponectin-type receptors PAQR1 and

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2 have intracellular N-termini (12). Tang *et al.* (8) predicted a common type I membrane topology for all members of the PAQR family, with an intracellular N-terminus and extracellular C-terminal domain, but experimental confirmation of these structural predictions is lacking for most PAQR family members. Indeed, more recent studies (13,14) reveal that PAQR7 has both intracellular N- and C-termini and localizes predominantly to the endoplasmic reticulum. The mPRs are proposed to signal as GPCRs (11), whereas adiponectin receptor signaling is not coupled to G proteins but involves activation of AMP kinase and the peroxisome proliferator-activated receptor (PPAR)- α (12). Here we describe the structure, tissue, and subcellular localization of PAQR10, following its cloning from mouse pancreatic islet β -cells.

MATERIALS AND METHODS

cDNA Cloning and Sequencing

The BA12 clone encoding PAQR10 (15) was isolated from a β TC3 cDNA library constructed in the lambdaZAP Express vector (Stratagene, La Jolla, CA, USA). Total RNA was extracted from β TC3 cells using RNazolB reagent (Tel-Test, Inc., Friendswood, TX, USA). Poly A⁺ RNA was prepared from total RNA using Poly A Tract mRNA Isolation System (Promega Corp., Madison, WI, USA). Double-stranded cDNA was then synthesized by AMV reverse transcriptase using a RiboClone cDNA synthesis Systems (Promega) and ligated into lambdaZAP Express phagemid vector (Stratagene). The library was packaged using a GIGAPACK II system (Stratagene) and had a titer of 6×10^8 pfu/mL. Screening by hybridization was performed with the β -cell-specific BA12 DNA originally identified by PCR-based representational difference analysis (15), ³²P-labeled with the Megaprime DNA labeling system (Amersham-Pharmacia Biotech, Uppsala, Sweden). After three rounds of screening, insert-containing pBK-CMV plasmids were excised from

phage clones and the resulting pBK-BA12 sequenced.

Plasmids were sequenced by the dideoxy-terminator method using a 320A sequenator (Applied Biosystems, Foster City, CA, USA). The BLAST algorithm was used to scan databases for protein and DNA homologies.

Northern Blot Analysis

A mouse multiple tissue Northern blot (BD Biosciences Clontech, Palo Alto, CA, USA) was probed with a ³²P-labeled cDNA probe corresponding to full-length BA12. Hybridization was performed for 2 h at 68°C in ExpressHyb Hybridization Solution (Clontech). Filters were washed in 2 \times saline-sodium citrate (SSC) solution, 0.05% SDS, for 30 min at room temperature followed by 0.1 \times SSC, 0.1% SDS for 30 min at 50°C, and exposed to Hyperfilm MP (Amersham Pharmacia Biotech) for 24 h at -70°C.

Tissue Screening by RT-PCR

Mouse tissues were dissected from 6- to 8-wk-old C57Bl/6 mice and washed in ice-cold phosphate buffered saline (PBS); RNA was extracted using RNazol B reagent. DNase I-treated RNA was reverse transcribed with 200 units MMLV RT (Life Technologies, Invitrogen Corp., Carlsbad, CA, USA) in the presence of 0.5 μ M random hexanucleotides (Bresatec, GeneWorks Pty. Ltd., Thebarton, SA, Australia) and 200 μ M dNTPs. One-tenth volumes of the first-strand synthesis reactions were amplified by PCR in PCR buffer (Perkin Elmer Inc., Shelton, CT, USA) containing 200 μ M dNTPs, 1 unit *Taq* polymerase, and 1 μ M each of sense and antisense oligonucleotide primers specific for PAQR10 (forward primer 5'-CGCGGCGATGTTCACTCTGCCAG-3' and reverse primer 5'-CAGGCAGATCTTGGCACAGTTCAC-3'), PAQR1 (forward primer 5'-GGCAATGGGGCTCCTTCTGGTAACA-3' and reverse primer 5'-GAACGAAGCTCCCCA TAATCAGTAG-3'), PAQR7 (forward primer 5'-CACTGGTGGAGGGAA AAGAA-3' and reverse primer 5'-AGCTG GAAACAGTGTGCAAGA-3'), PAQR9

(forward primer 5'-CGGGTCTCTT CGACATCATT-3' and reverse primer 5'-TTACTGCAGAAATTCGGTGCTG-3'), PAQR11 (forward primer 5'-GATCA ATGCGGTTACAGGAAT-3' and reverse primer 5'-TCTCCCAGCAGTCATCAGAC A-3') and β -actin (forward primer 5'-GTGGGCCCGCCCTAGGCACCA-3' and reverse primer 5'-CTCTTTGATG TCACGCACGATTTC-3'). PCR reactions were performed for 35 cycles (95°C/30 s; 56°C/1 min; 72°C/1 min), and amplified products were analyzed on 1.5% agarose gels.

Expression Constructs

Plasmid constructs were generated to produce versions of PAQR10 protein tagged either at the N-terminus, the C-terminus, or both. To construct a version of PAQR10 FLAG-tagged at the N-terminus (FLAG-PAQR10), full-length sequences were amplified from pBK-BA12 with a specific forward primer (5'-CCCGAAGCGGATCCTGGTGTT-3') to introduce a *Bam*HI restriction enzyme site and a reverse primer (5'-GACCCCTCGAGTCTGGGCCAC-3') to introduce a *Xho*I site. The PCR product was digested with *Bam*HI and *Xho*I and ligated into *Bg*III/*Xho*I-digested pCMV-Tag1 vector (Stratagene).

PAQR10 Myc-tagged at the C-terminus (PAQR10-Myc) was constructed with the same forward primer and a reverse primer (5'-GGCCACTCACTCGAGCACCTTGGT-3') to introduce a *Xho*I site. The PCR product was digested with *Bam*HI and *Xho*I and ligated into pCMV-Tag1 vector similarly digested with *Bam*HI and *Xho*I. The same PCR product digested with *Bam*HI and *Xho*I was ligated into *Bg*III/*Xho*I-digested pCMV-Tag1 vector to generate double-tagged PAQR10 (FLAG-PAQR10-Myc).

PAQR10 tagged with enhanced green fluorescent protein (EGFP) at the N-terminus (GFP-PAQR10) was constructed using the same forward primer and a reverse primer (5'-TCACTGCAGATGTTGCTTTGA-3') to introduce a *Pst*I site. The PCR product was digested with *Bam*HI and *Pst*I and ligated into

*Bgl*III/*Pst*I-digested pEGFP-C2 (Clontech). A different reverse primer (5'-CACTC ATCTGCAGACCTTGGT-3'), introducing a *Pst*I site, was used to construct PAQR10 EGFP-tagged at the C-terminus (PAQR10-GFP). The PCR product was digested with *Bam*HI and *Pst*I and ligated into *Bgl*III/*Pst*I-digested pEGFP-C2 (Clontech).

Cell Culture and Transfection

All culture media were from Invitrogen-Gibco (Carlsbad, CA, USA). SV40-transformed mouse cell lines β TC3 and α TC1 (16) that secrete the hormones insulin and glucagon, respectively, were kindly provided by Dr. Doug Hanahan (University of California, San Francisco, CA, USA). Both cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose, 10% FCS, and antibiotics under a 10% CO₂ atmosphere at 37°C. For transfection, β TC3 cells were seeded at 80% confluency in 6-well plates and transfected with 5 μ g DNA/well using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

CHO-K1 cells plated at a density of 1×10^6 per well were transfected with 1 μ g DNA using Lipofectamine 2000 within 24 h of plating.

Antibodies

FLAG-tagged proteins were detected using an anti-FLAG M2 monoclonal antibody (Sigma-Aldrich, Castle Hill, NSW, Australia). Myc-tagged proteins were detected using anti-Myc-Tag monoclonal antibody (clone 9B11; Cell Signaling Technology, Inc., Beverly, MA, USA). GFP fusion proteins were detected by direct fluorescence in the FITC channel. A rabbit polyclonal anti-prohibitin antibody (RB-292; NeoMarkers, Fremont, CA, USA) was used to determine enrichment in mitochondrial localization assays. Rabbit and rat antisera were generated against a peptide corresponding to amino acid residues 20–36 (NDRVPAH KRYQPTEYEH) of PAQR10. Peptides were synthesized with an extra cysteine residue at the N-terminus and then cou-

pled to diphtheria toxoid (DT) via a maleimidocapoyl-*N*-hydroxysuccinimide (MCS) linker. Free and coupled peptides were purchased from Mimotopes Pty. Ltd. (Clayton, Victoria, Australia). DT-coupled peptides (0.5 mg) were emulsified with Freund's complete adjuvant and injected at multiple subcutaneous sites into two rabbits each. Following two booster immunizations 6 and 8 wks later, rabbits were bled and sera stored at –20°C. Rabbit immunoglobulins (IgGs) were purified from antisera by protein G-Sepharose (Amersham-Pharmacia Biotech) affinity chromatography. To produce rat antisera, two Wistar rats were injected intraperitoneally with 50 μ g DT-coupled peptide in Freund's complete adjuvant; after two booster immunizations 4 and 8 wks later, rats were killed and their blood collected and sera stored at –20°C.

Immunohistochemistry

Adult C57BL/6 mice were housed under standard 12-h light/dark conditions and provided with food and water *ad libitum*. All experiments were approved by the Animal Research Ethics Committee, Melbourne Health. For the collection of fetal tissue, timed pregnant mice were killed by CO₂ narcosis at gestational times e10.5, e12.5, e13.5, e15.5, and e17.5 d; tissue was also collected from newborn mice. Pancreata dissected from embryos and adult mice were fixed for 2–4 h in 4% paraformaldehyde (PFA) in PBS, dehydrated, and embedded in paraffin. Tissue sections (4 μ m) were placed on uncoated glass slides, deparaffinized in xylene, and rehydrated in graded alcohols. Endogenous peroxidase was blocked by immersion in 0.03% hydrogen peroxide for 15 min. Rat anti-PAQR10 sera were diluted 1:200 in PBS, added to the slides, and incubated for 60 min at room temperature in a humidified chamber, followed by incubation with HRP goat anti-rat secondary antibody (Silenus, Hawthorn, Victoria, Australia) diluted 1:300 in PBS for 30 min at room temperature. The sections were then visualized using 3,3'-diaminobenzidine as

chromogen and counterstained with Mayer's hematoxylin. To confirm the specificity of staining, anti-PAQR10 serum was preincubated for 16 h at 4°C with 20 μ g/mL of the immunizing peptide before addition to the slides. Digital images were captured with an Axiocam camera from an Axioplan2 compound microscope (Carl Zeiss, Göttingen, Germany).

Mitochondrial Localization

Mitochondria were enriched from α TC1 and β TC3 cells by selective permeabilization with low concentrations of digitonin (17). Briefly, cells were harvested by trypsinization, resuspended in ice-cold PBS, collected by centrifugation (800g for 5 min) in microfuge tubes at 1×10^6 cells/sample; cell pellets were resuspended in 100 μ L ice-cold lysis buffer (80 mM KCl, 250 mM sucrose, 200 μ g/mL digitonin in PBS). After 5 min on ice, samples were centrifuged at 10,000g for 5 min; the supernatants containing mainly cytoplasmic proteins and mitochondria-enriched pellets were recovered. Samples were solubilized in 4 \times SDS sample buffer before SDS-PAGE in 10% to 20% Tris-glycine Novex gels (Invitrogen) and Western blotting.

Confocal Microscopy

Transfected CHO-K1 cells were grown to 50% confluency on sterile 18 \times 18-mm microscope glass cover slips (Chance Propper Ltd., Smethwick, Warley, England) in 6-well culture plates. Twenty-four hours after transfection, Mitotracker red (Molecular Probes, Inc. Eugene, OR, USA) at a 0.25 nM final concentration was added to the medium for 30 min at 37°C. The cover slips were washed twice in PBS, fixed in 4% paraformaldehyde, and incubated with mouse anti-FLAG and anti-Myc primary antibodies for 60 min at room temperature, followed by sheep anti-mouse IgG conjugated to FITC (Silenus-AMRAD Biotech, Boronia, Victoria, Australia). Confocal images were obtained with a Leica TCS4 SP2 spectral confocal scanner and a Leica DMIRE2 microscope (Leica Microsys-

tems, Gladesville, NSW, Australia) equipped with a 100× oil immersion objective.

RESULTS

We previously reported candidate genes specific for pancreatic β-cells identified by PCR-based representational difference analysis of the mouse pancreatic insulin-producing βTC3 and glucagon-producing αTC1 cell lines (15). One of the PCR-generated sequences (BA12) was used to isolate and sequence a cDNA clone from a βTC3 cDNA library. The isolated BA12 clone contained a single open reading frame encoding a protein of 247 amino acids. A BLAST search of the deduced BA12 protein sequence showed it to be identical to monocyte to macrophage differentiation 2 (Mmd2) factor, which had been identified in a screen for mouse gonad-specific genes (18), and to progesterin adipoQ receptor 10 (PAQR 10) annotated in GenBank (accession no. AY424299) as a member of the PAQR family. Initial analysis of the BA12 amino acid sequence using the Predict-Protein program (<http://cubic.bioc.columbia.edu/predictprotein/>) revealed a protein with a predicted molecular mass of 29 kDa, comprising seven transmembrane domains oriented with an intracellular N-terminus and an extracellular C-terminus. This topology was consistent with that predicted by Tang *et al.* (8) for all members of the PAQR family. The protein also contained a mitochondrial targeting sequence (MTS) at the N-terminus and a predicted cleavage site between Arg at position 28 and Tyr at position 29. Comparison of the mouse and human PAQR10 cDNAs in the UCSC Genome Bioinformatics databases (<http://genome.ucsc.edu/>) revealed that mouse and human PAQR10 genes are encoded by seven exons with similar splicing sites. Mouse PAQR10 maps to chromosome 5qG2 and the human gene to chromosome 7p22.1. These mouse and human chromosomal regions show significant syntenic homology.

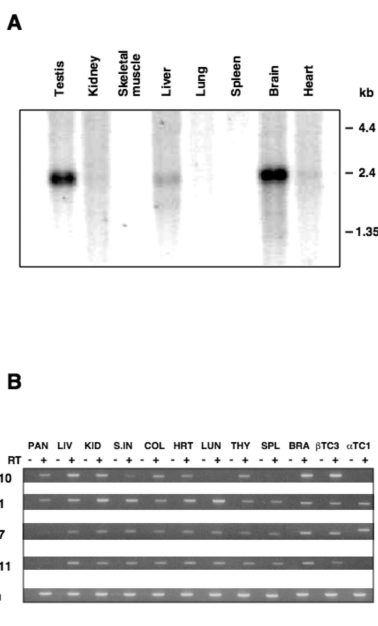


Figure 1. Tissue expression of PAQR10 mRNA. (A) Northern blotting. A mouse multiple tissue Northern blot (Clontech) was hybridized with a PAQR10-specific cDNA probe as described in Materials and Methods. (B) RT-PCR. Total RNA extracted from different tissues was (+) or was not (-) reverse transcribed and subjected to PCR with specific oligonucleotides for PAQR10, PAQR1, PAQR7, PAQR11, and β-actin (internal control). Amplified products were analyzed on 1.5% agarose gels. PAN, pancreas; LIV, liver; KID, kidney; S.IN, small intestine; COL, colon; HRT, heart; LUN, lung; THY, thymus; SPL, spleen; BRA, brain.

Northern blot analysis (Figure 1A) revealed a 2.3-kb PAQR10 transcript strongly expressed in testis and brain and weakly in liver, heart, and kidney. By RT-PCR, we examined expression of PAQR10 in different mouse tissues in comparison to PAQR1, PAQR7, and PAQR11 by RT-PCR (Figure 1B). PAQR1, PAQR7, and PAQR11 were selected as being representative, respectively, of the adiponectin receptor, mPR, and hemolysin III subtypes of PAQR family members. PAQR10 was expressed in pancreas, liver, kidney, small intestine, colon, heart, thymus, and brain, and in βTC3 but not αTC1 cells. In contrast, PAQR1, PAQR7, and PAQR11 were expressed in all tissues, except the pan-

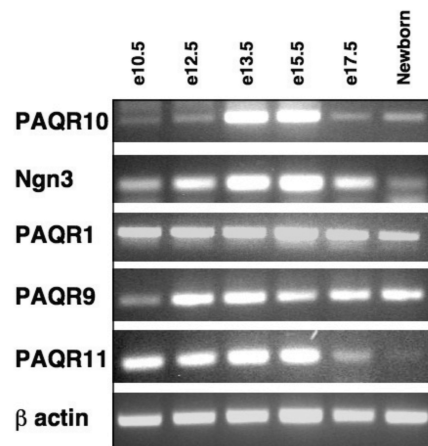


Figure 2. Developmental expression of the PAQR10 gene. Total RNA was extracted from mouse embryos at e10.5 and e12.5 or from dissected pancreas at e13.5, e15.5, e17.5, and newborn stages. RNA was (+) or was not (-) reverse transcribed and subjected to PCR with specific oligonucleotides for PAQR10, Ngn3, PAQR1, PAQR9, PAQR11, and β-actin (internal control). Amplified products were analyzed on 1.5% agarose gels.

creas, where PAQR7 and PAQR11 expression was negligible or absent. Expression of PAQR7 and PAQR11 was, however, consistently detected in the βTC3 cell line. In the αTC1 cell line, a single, slightly larger PCR product was observed for PAQR7, consistent with mRNA processing by alternative splicing.

In investigating the expression of PAQR10 in the pancreas during mouse embryonic development, we took advantage of the fact that Ngn3, a basic helix-loop-helix transcription factor, marks the pancreatic endocrine lineage (19). Moreover, gene profiling in e13.5 and e15.5 Ngn3-deficient mice indicated that expression of PAQR10 is dependent on that of Ngn3 (20). PAQR10 expression was mapped in relation to that of Ngn3 and PAQR family members representative of the three subtypes (Figure 2). PAQR10 expression was low in whole embryos at e10.5 and e12.5. In the pancreas, PAQR10 expression was highest at e13.5 and e15.5, when the pancreatic epithelium is undergoing branching morphogenesis

and differentiation toward the endocrine lineage, and then progressively decreased in older embryonic (e17.5) and newborn pancreas, when it is presumably restricted to β -cells. This pattern of *PAQR10* expression mirrors that of *Ngn3*. Of the other PAQR family members, *PAQR1* and *PAQR9* showed consistent expression throughout pancreas development, and *PAQR11* was expressed during embryogenesis but was undetectable in the newborn pancreas (Figure 2).

In the embryonic pancreas, expression of PAQR10 protein was restricted to branching epithelial structures at e15.5, being absent from parenchyma (Figure 3A). At e17.5, expression was detected in endocrine islet structures and in the ducts (Figure 3B), a pattern maintained in the newborn (Figure 3C). In the adult pancreas, PAQR10 expression was restricted to islet cells, presumably β -cells, being absent in ducts (Figure 3D). Notably, however, prominent expression was observed in both islets and ducts of the maternal pancreas during pregnancy (e9.5) (Figure 3E). The punctate pattern of cytoplasmic staining of PAQR10 (Figure 3F) suggested localization to mitochondria. We therefore determined if the MLS at the N-terminus of PAQR10 directed the subcellular localization of the protein.

Bioinformatic analysis (Table 1) indicated that of the 11 family members only PAQR 9, 10, and 11 were predicted to localize to mitochondria. For PAQR10, TargetP predicted a mitochondrial targeting sequence of similar length in mouse (29 amino acids) and human (28 amino acids), with an identical cleavage site. To determine if the MTS of PAQR10 was functional, we examined the subcellular localization of endogenous PAQR10 in β TC3 cells and of tagged versions of PAQR10 after overexpression in CHO cells. A 29-kDa band corresponding to PAQR10 was detected by Western blotting in total cell lysates and in enriched mitochondrial fractions of β TC3 but not α TC1 cells (Figure 4A, left panel). Mitochondrial enrichment was confirmed by blotting

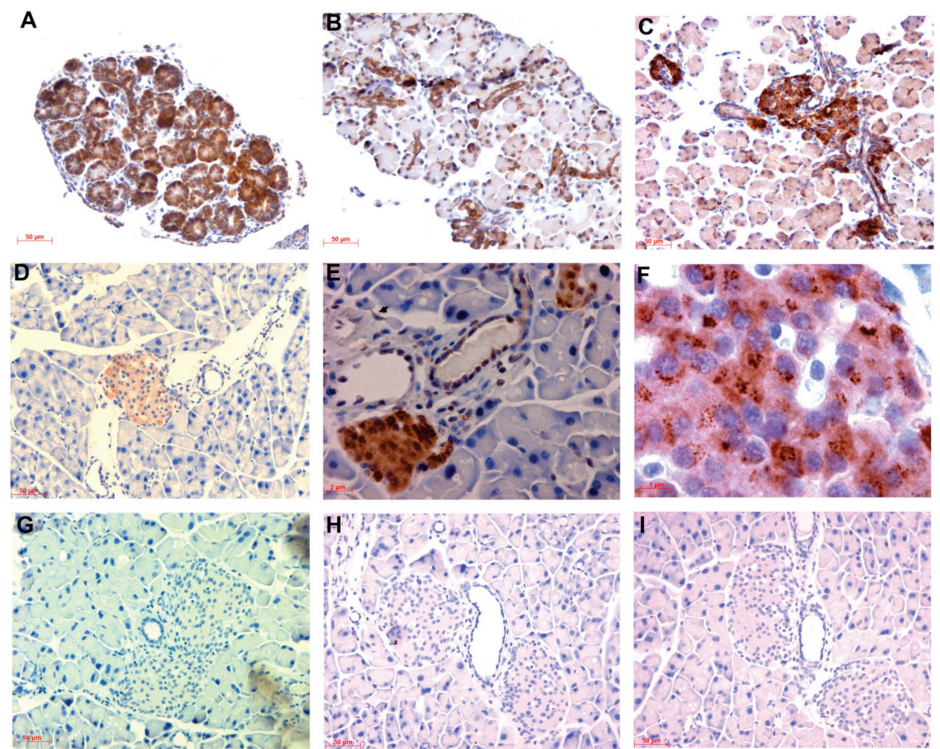


Figure 3. Expression of PAQR10 in the developing and adult pancreas. Immunohistochemistry with rat anti-PAQR10 serum was performed on PFA-fixed sections of mouse pancreas, as described in Materials and Methods. Shown are sections of pancreas from e15.5 (10 \times) (A) and e17.5 (20 \times) (B) embryos, newborn female mouse (20 \times) (C), adult female mouse (20 \times) (D), and e9.5 maternal mouse pancreas at 40 \times (E) and 100 \times (F). For controls, maternal mouse pancreas at e9.5 was stained in the absence of primary antibody (G), with rat anti-PAQR10 serum blocked with immunizing peptide (H), or with normal rat serum (I) (all 20 \times).

Table 1. Prediction of mitochondrial localization of mammalian PAQR family members.

Protein	TargetP	Predotar	iPSort	MitoProt	Overall Prediction
PAQR1	0.214 (-)	0.00 (-)	-	0.0091 (-)	-
PAQR2	0.206 (-)	0.00 (-)	-	0.0082 (-)	-
PAQR3	0.091 (-)	0.02 (-)	-	0.0943 (-)	-
PAQR4	0.470 (M)	0.03 (-)	-	0.4352 (PM)	PM
PAQR5	0.270 (-)	0.38 (PM)	-	0.0254 (-)	-
PAQR6	0.388 (M)	0.55 (M)	-	0.3055 (-)	PM
PAQR7	0.142 (-)	0.23 (PM)	-	0.2474 (-)	-
PAQR8	0.465 (-)	0.01 (-)	-	0.6927 (M)	-
PAQR9	0.931 (M)	0.37 (PM)	M	0.7932 (M)	M
PAQR10	0.616 (M)	0.39 (PM)	M	0.8140 (M) ^a	M
PAQR11	0.645 (M)	0.48 (M)	M	0.9670 (M)	M

Mitochondrial localization prediction programs: TargetP (36), <http://www.cbs.dtu.dk/services/TargetP/>; Predotar (37), <http://urgi.infbioingen.fr/predotar/>; iPSort (38), <http://biocaml.org/ipsort/iPSORT/>; MitoProtII (39), <http://ihg.gsf.de/ihg/mitoprot.html>. M, mitochondrial localization; PM, possible mitochondrial localization; -, no mitochondrial localization predicted.

^aHuman sequence prediction, value for mouse PAQR10 is 0.1452 (-).

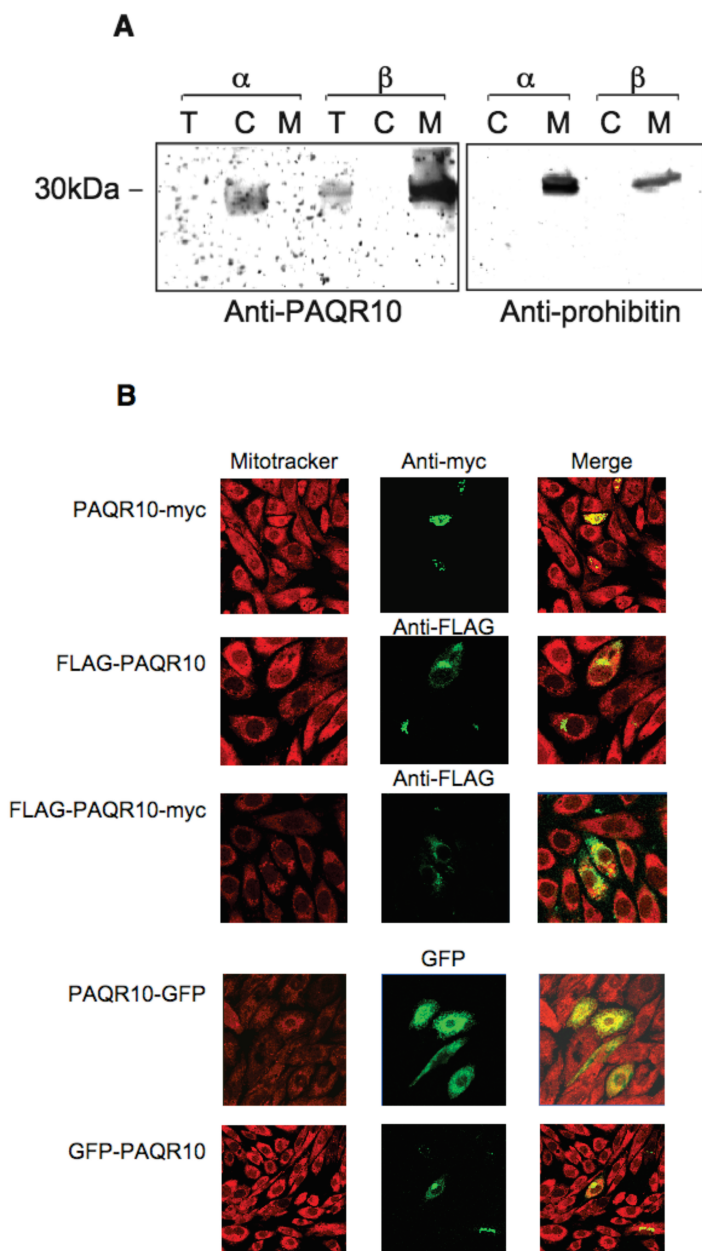


Figure 4. Mitochondrial localization of PAQR10. (A) Mitochondrial localization assay. Total (T), cytoplasmic (C), or mitochondria-enriched (M) α TC1 (α) and β TC3 (β) lysates were analyzed for PAQR10 expression by Western blot (left panel). Mitochondrial enrichment was confirmed by Western blot analysis of prohibitin expression in the same C and M lysates (right panel). (B) Confocal microscopy. CHO cells were transfected with plasmid constructs encoding various versions of PAQR10 tagged at the C-terminus with myc (PAQR10-myc) or enhanced green fluorescent protein (PAQR10-GFP), at the N-terminus with flag peptide (Flag-PAQR10) or GFP (GFP-PAQR10), or at both ends Flag-PAQR10-myc. Mitochondria were detected by red fluorescence after staining with Mitotracker Red (left panels). PAQR10 or protein tags were detected with the indicated specific antibodies and FITC-labeled secondary antibodies. GFP was visualized under FITC channels (center panels), and the images were merged after confocal microscopy (right panels). These data demonstrate that PAQR10 localizes to mitochondria and that N-terminal tagging of PAQR10 prevents localization and changes its intracellular distribution to perinuclear sites.

the cytoplasmic and mitochondrial fractions from these cells with an antibody against the mitochondrial protein, prohibitin. A 30-kDa band corresponding to prohibitin was detected only in mitochondrial fractions (Figure 4A, right panel). The localization of tagged versions of PAQR10 transfected into CHO cells was then investigated by immunofluorescence and confocal microscopy (Figure 4B). Mitochondria were labeled by exposure of transfected CHO cells to Mitotracker dye. The C-terminal tagged versions of the protein, either PAQR-myc or PAQR-GFP, localized to mitochondria. Mitochondrial localization was absent if the protein was N-terminally tagged with FLAG or GFP, after which it was observed in perinuclear sites. These results indicated that the MTS at the N-terminus of PAQR10 directs its localization to mitochondria.

DISCUSSION

We identified *PAQR10*, a member of the highly conserved PAQR gene family, in a screen for genes differentially expressed in pancreatic β -cell versus α -cell lines. Compared with other PAQR genes, the expression of *PAQR10* was more restricted and in adult mouse pancreas was confined to islets. This was confirmed by staining for PAQR10 protein, which was localized to pancreatic ducts and endocrine tissue in the embryo, neonate, and pregnant adult, but to islets only and not ducts in the nonpregnant female. Consistent with this endocrine localization, expression of *PAQR10* in the embryo appeared to mirror that of *Ngn3*, a transcription factor that marks the endocrine lineage. Petri *et al.* (20) reported that *PAQR10* was one of many genes not expressed in *Ngn3*-knockout mice, which fail to develop an endocrine pancreas. Its expression pattern infers a role for PAQR10 in endocrine pancreas development and hyperplasia in pregnancy.

The potential function of PAQR10 is suggested by its localization to the mitochondrion, which has recently been recognized as a primary site of action of steroid and thyroid hormones (21). Mito-

chondrial localization is predicted for PAQR9 and PAQR11. PAQR9 is a putative mPR, whereas PAQR10 and 11 are closely related to bacterial hemolysins. Hemolysin III functions as a pore-forming membrane protein (10), and bacterial virulence factors have recently been shown to target mitochondria and up- or downregulate apoptosis in target cells (22). In eukaryotes, pro- and anti-apoptotic members of the Bcl-2 family regulate the mitochondrial pathway of apoptosis by controlling permeability of the outer mitochondrial membrane (23). Because PAQR10 targets mitochondria, and similar targeting is predicted for PAQR11, it will be of interest to determine if these PAQRs, structurally related to hemolysins, have pore-forming, apoptosis-regulating properties, and their relationship to the function of Bcl-2 family members. We suggest that PAQR10 may promote growth-survival of the endocrine pancreas via an effect on the mitochondrial pathway of apoptosis. The ligand for PAQR10 is unknown, but the receptor could act as a signal transducer that translocates to mitochondria upon ligand binding. There are precedents for translocation of signaling molecules between the cell surface and mitochondria: the C1q receptor, a globular protein structurally related to adiponectin, contains a functional MTS at its N-terminus and traffics between the cell surface and mitochondria (24); the receptor for stanoic acid, a hormone which regulates calcium and phosphate excretion in the kidney and gut, resides in both the plasma membrane and mitochondria (25).

In pregnancy, in response to an increased demand for insulin, pancreatic β -cells undergo major changes to compensate for systemic insulin resistance. These include increases in cAMP metabolism and glucose oxidation, gap junction coupling between β -cells, glucose-stimulated insulin release, insulin synthesis, and β -cell proliferation (26–28). In rodents, maternal β -cell proliferation in pregnancy is induced by the lactogenic hormones, prolactin (PRL) and placental lactogen (PL), and counter-

regulated by the steroid hormone progesterone (29). Mice with deletion of the classic progesterone receptor have increased insulin secretion and glucose clearance associated with an increase in β -cell proliferation and mass (30), consistent with an inhibitory effect of progesterone on β -cell proliferation and function. The function of mPRs in the pancreas is unknown, but progesterone has been shown to inhibit insulin secretion directly, by a cell membrane-initiated, nongenomic effect that decreases Ca^{2+} influx (31). Three PAQR family members, PAQR5, 7, and 8, specifically bind progesterins (6,11), and because of sequence similarities, PAQR6 and 9 are also putative mPRs (8). It would be of interest therefore to determine the contribution of PAQR family mPRs to the inhibitory effect of progesterone on β -cell proliferation and function. In regard to PAQR10, however, there is no evidence currently that the ligand for this receptor is a progesterin, and our findings suggest that it may not be. Thus, PAQR10 expression mirrored pancreatic endocrine development in the late embryo-neonate and was detected not only in pancreatic islets but also ducts, from which β -cells are known to derive, in pregnancy. These findings are not in keeping with the inhibitory effect of progesterone on β -cell proliferation and function, but rather with promotion of β -cell development and survival.

Finally, the localization of *PAQR10* expression to β -cells raises the possibility that it may be a diabetes susceptibility gene. Susceptibility to type 2 diabetes maps to chromosomal regions containing genes for the adiponectin receptors PAQR1 and PAQR2 (32), and family studies show significant associations between *adiponectin* (33) and *PAQR1* (34) polymorphisms and type 2 diabetes. A genome-wide scan for type 2 diabetes genes in Japanese sib pairs (35) identified a region of strong linkage at 7p21–22, with a maximum LOD score at marker D7S517, which is just 0.5 Mb away from *PAQR10*. We conclude that further studies are likely to establish a key role for PAQR10 in pancreatic β -cell biology.

ACKNOWLEDGMENTS

This work was supported by a partnership Program Grant from the Juvenile Diabetes Research Foundation (JDRF) and the National Health and Medical Research Foundation of Australia (NHMRC). L.C.H. is a Senior Principal Research Fellow of the NHMRC. L.J.G. was supported in part by a grant from Diabetes Australia Research Trust. The authors thank Catherine McLean for secretarial assistance.

DISCLOSURE

The authors declare that they have no financial or other conflict of interest relating to the work described in this manuscript.

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