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The Insulin-like Growth Factor-I–mTOR Signaling Pathway Induces the Mitochondrial Pyrimidine Nucleotide Carrier to Promote Cell Growth

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The insulin/insulin-like growth factor (IGF) signaling pathway to mTOR is essential for the survival and growth of normal cells and also contributes to the genesis and progression of cancer. This signaling pathway is linked with regulation of mitochondrial function, but how is incompletely understood. Here we show that IGF-I and insulin induce rapid transcription of the mitochondrial pyrimidine nucleotide carrier PNC1, which shares significant identity with the essential yeast mitochondrial carrier Rim2p. PNC1 expression is dependent on PI-3 kinase and mTOR activity and is higher in transformed fibroblasts, cancer cell lines, and primary prostate cancers than in normal tissues. Overexpression of PNC1 enhances cell size, whereas suppression of PNC1 expression causes reduced cell size and retarded cell cycle progression and proliferation. Cells with reduced PNC1 expression have reduced mitochondrial UTP levels, but while mitochondrial membrane potential and cellular ATP are not altered, cellular ROS levels are increased. Overall the data indicate that PNC1 is a target of the IGF-I/mTOR pathway that is essential for mitochondrial activity in regulating cell growth and proliferation.

INTRODUCTION

Insulin and insulin-like growth factor-I (IGF-I) regulate metabolism and cell survival, growth, and proliferation through the insulin or IGF-I receptors (IR or IGF-IR) and their downstream signaling pathways. Increased IGF-IR expression and activity have been associated with many human cancers (LeRoith and Roberts, 2003), and overexpression of the IGF-IR in murine tumor models promotes an invasive and metastatic phenotype (Lopez and Hanahan, 2002). Some of the most frequently altered tumor-suppressor genes or oncogenes in cancers encode proteins that directly affect the highly conserved signaling pathway from the IGF-IR via the Insulin Receptor Substrate (IRS) adapter proteins to the lipid kinase phosphoinositide 3-kinase (PI3-K), the serine threonine kinase Akt, and the serine threonine kinase mTOR (mTORC1 and mTORC2 complexes). PI3-kinase and Akt can both act as oncogenes, whereas tumor suppressors that regulate this pathway include the lipid phosphatase PTEN, the tuberous sclerosis complex (TSC1/TSC2), the LKB1 kinase, and the DNA damage–activated tumor suppressor p53 (Schmelzle and Hall, 2000; Altmare and Testa, 2005; Cully et al., 2006; Samuels and Ericson, 2006). Unsurprisingly, there is significant interest in targeting the IGF-IR and components of its signaling pathway for the treatment of cancer (Hofmann and Garcia-Echeverría, 2005).

The role of IGF-I signaling and Akt in regulating energy metabolism and glycolysis in tumor cells is receiving renewed attention. Tumor cells have long been recognized to have the ability to metabolize glucose and produce ATP rapidly through increased rates of glycolysis. This phenotype associated with increased production of lactic acid was described by Warburg in the 1920s (Warburg, 1924, 1930), and it can be detected using positron emission tomography (PET). Enhanced glycolysis is thought to confer cancer cells with a distinct competitive edge over normal cells by providing adequate ATP for rapid proliferation under hypoxic conditions and has also been proposed to protect cells from oxidative stress (Brand and Hermfisse, 1997). One of the ways in which the PI3-K and Akt pathway promotes cell survival and tumor growth is to stimulate glucose metabolism. Activated Akt increases levels of cell surface transporters for glucose (Plas et al., 2001; Edinger and Thompson, 2002; Plas and Thompson, 2005) and also regulates the expression and location of mitochondrial hexokinases, which
The expressed sequence tag clone of mouse pnc1 was obtained from the IMAP consortium. To generate full-length pnc1 for cloning in frame with green fluorescent protein (GFP) at the C terminus oligonucleotide primers for pnc1 were designed incorporating the restriction sites XhoI and ApaI. The sequence of these oligonucleotides is as follows: mpc1 forward primer 5'-GCGGCCAGGGCGCCCCAGCGCT3'; mpc1 reverse primer 3'-GCCG-CCAGTAAGACCGCTCT3'. The PCR products were ligated into the pEGFP-C1 plasmid that had been digested with XhoI and ApaI. The pcDNA3 vector encoding Ha-mPNC1 was generated by ligating the insert from pEGFP-C1- PNC1 into a modified version of the pcDNA3 plasmid encoding the Ha peptide. To generate the bacterial expression vector pRUN, the coding sequence for human pnc1 (mpc1) was amplified by PCR from testis cDNA, and the NdeI and HindIII restriction sites were introduced for ligation into pRUN.

The promoter sequence of the pnc1 gene encompassing a region of 3 kbp upstream of the transcription start site (+1) was extracted from the Ensembl database (Gene ID: ENSG00000171612). Putative transcription factor binding sites were identified in this sequence by analysis using the TSSSEARCH version 1.3 program (http://www.ncbi.nlm.nih.gov/Research/db/TSSSEARCH.html), which compared the sequence with a database of identified transcription factor binding sites (TRANSFAC database (Heinemeyer et al., 1998).

**Cell Culture, IGF-I/Insulin Stimulation, and Transfection**

MCF-7 breast carcinoma cells, R+ cells, R- cells, and DU145 and HeLa cells were Mamed in DMEM supplemented with 10% (vol/vol) fetal bovine serum (FBS), 10 mM l-glutamine, and antibiotic (all from Biowhittaker, Verviers, Belgium), which was designated complete medium (CM). HeLa cells were transiently transfected with pcDNA3 encoding Ha-PNC1 or empty pcDNA3 using LipofectAMINE 2000 (Invitrogen, Paisley, United Kingdom). To generate stable transfectants, MCF-7 cells were cultured in medium containing G418 (Calbiochem, Nottingham, United Kingdom; 1 mg/ml), and individual clones were selected and screened for expression of Ha-PNC1 by Western blotting. To analyze signaling response cells were starved from FBS before stimulation with IGF-I (100 ng/ml, PeproTech, Rocky Hill, NJ). To analyze pnc1 mRNA expression, cells were grown to a confluence of ~70%, serum-starved (for 4 h for R+ cells and for 12 h for MCF-7 and R- cells), and then stimulated with either IGF-I or insulin. In order to inhibit signaling pathways cells were incubated with 30 μM PD98059 (MAP kinase inhibitor), 20 μM LY294002 (PI-3 kinase inhibitor), or 100 nM rapamycin (mTOR inhibitor) for 30 min before stimulation with IGF-I. All inhibitors were from Calbiochem.

**Northern Blot Analysis**

Total RNA was isolated from R- and R+ cells using Trizol Reagent (Invitrogen) according to the manufacturer's instructions, separated on 1.5% (wt/vol) denaturing formaldehyde gels, and transferred to nylon membranes (Hybond-N, Amersham, Buckinghamshire, United Kingdom). A murine multiple tissue Northern blot was obtained from Clontech. α-32P-labeled pnc1 probes (1 × 10^6 cpm/ml) were generated by the random oligonucleotide primer method (NEBlot: New England Biolabs, Hertfordshire, United Kingdom). Prehybridization and hybridization were carried out at 42°C in 50% formamide, 5× SSC, 4× Denhardt's solution, 0.1% SDS, and salmon sperm DNA (100 μg/ml, Sigma, Dublin, Ireland) for 2 and 1.5 h, respectively, washed twice at 42°C using 2× SSC, 0.1% SDS for 5 min, and then twice at 42°C using 0.5× SSC and 0.1% SDS for 15 min, before being scanned for signal using a phosphorimager.

**Immunofluorescence and Flow Cytometry Assays**

For immunofluorescence, cells on cover slips were washed with phosphate-buffered saline (PBS) and placed in serum-free DMEM with 25 mM mitoTracker dye (Molecular Probes, Hamburg, Germany) for 30 min. Cells were fixed in 3.7% formaldehyde in PHEM buffer (60 mM Pipes, 25 mM HEPES, 10 mM KCl, 10 mM MgCl2, pH 6.9) for 10 min and permeabilized with 0.1% Triton X-100 in PHEM for 5 min. For staining with the anti-Ha antibodies, cells were first preblocked with 2.5% normal goat serum in PHEM for 30 min and then incubated with primary antibody, washed with PHEM, and incubated with Cy2-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Newmarket, United Kingdom). Cells were examined with a Nikon fluorescent microscope equipped with a Tripleview filter set for excitation and emission wavelengths of 510, 540, and 640 nm. Mitochondria mass was assessed by first fixing cells in PBS containing 2% formaldehyde and 2% glutaraldehyde for 30 min at 37°C, followed by incubation in PBS containing 25 nM MitoTracker dye (Molecular Probes) for 30 min. Cells were washed and analyzed by flow cytometry. Cellular ROS was assessed by incubating cells in PBS containing 10 μM H2DCF-DA fluorescent probe (Molecular Probes) for 15 min in the dark at 20°C.

For cell cycle analysis, cells were trypsinized, resuspended in cold PBS containing 200 μg/ml RNase A (Sigma), and kept on ice. Before analysis by flow cytometry, NP-40 and propidium iodide (Sigma) were added at a final concentration of 0.1% and 50 μg/ml, respectively. DNA content was measured in the FL2 channel of a flow cytometer using the CellQuest software (Becton Dickinson, Oxford, United Kingdom).

**Assays for Cell Proliferation and Cell Size**

Cells were cultured in CM at 3 × 10^4 cells per well in a 24-well plate. To measure growth, cells were reseeded at the indicated density, cultured until confluent, and then treated with IGF-I (100 ng/ml) for 30 min. R+ cells were resuspended in 100 μl of medium and counted using trypan blue exclusion.

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Cells in culture were stained with 1 μg/ml 3,3′-diaminobenzidine tetrachloride (DAB) for 10 min and analyzed by flow cytometry. Cellular ROS was determined by analyzing the red fluorescence intensity of DAB-stained cells using Cytofluor (Hycel) and FACS as described previously (Schieke et al., 2006).

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Cellular Protein Extracts and Western Blotting

Cellular protein extracts were prepared by lysing in buffer (Tris-HCL pH 7.4, 150 mM NaCl, 1% Nonidet P-40) plus the tyrosine phosphatase inhibitor Na2VO4 (1 mM) and the protease inhibitors phenylmethylsulfonyl fluoride (1 mM), pepstatin (1 μM), and aprotinin (1.5 μg/ml). After incubation at 4°C for 20 min, nuclear and cellular debris were removed by microcentrifugation at 20,000 × g for 15 min at 4°C. For Western blot analysis proteins were resolved by SDS-PAGE on 4–15% gradient gels and transferred to nitrocellulose membranes. Blots were incubated for 1 h at room temperature in tris-buffered saline containing 0.1% Tween 20 (TBS-T) and either 5% milk (wt/vol) or 2% bovine serum albumin (BSA). This was followed by primary antibody incubations overnight at 4°C. The anti-phospho p70 S6 Kinase Thr 389 and anti-phospho-p70 S6 Kinase, anti-phospho-p42/44 MAP kinase, anti-phospho p70 S6 Kinase Thr 421/Ser 424, anti-p70 S6 Kinase, anti-phospho-pho-4E-BP1, anti-phospho-Akt, anti-Akt and anti-phospho-p21/44 MAP kinase antibodies were all from Cell Signaling Technology (Beverly, MA). Anti-mitogen-activated protein kinase 2 (MAP2K) and anti-paxillin were from Upstate Biotechnology (Lake Placid, NY). The anti-Ha antibody 12CA5 was from Roche Molecular Biochemicals (East Sussex, United Kingdom) and anti-VDAC antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-actin mAb was from Sigma. Secondary antibodies conjugated with horseradish peroxidase (Dako, High Wycombe, United Kingdom) were used for detection with enhanced chemiluminescence (ECL, Amersham Biosciences, Little Chalfont, United Kingdom).

Small Interfering RNA Oligonucleotides and Transfection

Small interfering RNAs (siRNAs; Elbashir et al., 2001) oligonucleotides were obtained from MWG (Bergenham, Germany). An oligonucleotide complementary to both the human and mouse sequence of the pnc1 gene (aatgtggtggtgca; corresponding to nucleotides 311–332 in the human gene and nucleotides 304–325 in the mouse gene after the start codon) was used. Two other predevised antisense oligonucleotides specific for the human homolog of the pnc1 gene (Ambion, Huntington, United Kingdom; siRNA# ID no. 1236/2 and siRNA# ID no. 123673). A negative control siRNA (negative control no. 1) was also from Ambion. Transfection was carried out using Oligofectamine transfection reagent (Invitrogen) with concentrations of oligonucleotide ranging from 1 to 200 nM. All concentrations tested showed similar specific effects on suppressing protein expression and decreasing cell size. For most experiments 50 nM of oligonucleotide was used. Expression of the transfected Ha-PNC1 protein was assessed by Western blotting using the anti-Ha antibody. RNA levels were assessed using semiquantitative or quantitative RT-PCR 48–96 h after transfection.

Semiquantitative and Quantitative RT-PCR

Tissue extracts from prostate carcinomas and macrodissected adjacent normal prostate were isolated from radical prostatectomy specimens directly after surgery. All samples were used after informed consent of the patient and approval by the local Ethics Committee of the University of Würzburg, Germany. Total RNA was isolated using the Trizol method and cDNA synthesis and reverse transcription with equal amounts of RNA (2 μg) using a cDNA synthesis kit (Roche Molecular Biochemicals or Invitrogen). Equal amounts of cDNA were amplified and the quantity of reverse transcription reaction used for amplification was nonsaturating for the PCR products after the selected number of amplification cycles.

Quantitative PCR was carried out using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA; or in the case of prostate tissues by the LightCycler instrument; Roche Molecular Biochemicals) with the LightCycler SYBR Green technology (Roche Diagnostics, Crawley, West Sussex, United Kingdom). Primers used were as follows: pnc1: 5′-GCTGTGCGACTTCT- TATCACAAATTC-3′ and, 5′-AAACCTAAGCAGCACACTGGATGC-3′; gapdh: 5′-CCCCATTCCTGCTAGGGTGTAAG-3′ and 5′-TGCGTATCCTCTCCTCCAC- GATACC-3′; p38: 5′-ATTCGCGACAGGAATTGAAA-3′ and 5′-GCTGTAGC- CACATCTGCTGAAA-3′. Plates were heated for 15 min at 94°C, and 40 PCR cycles consisting of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C were applied. Samples were subsequently heated to 95°C. Results were expressed as DeltaDelta Ct (ΔΔCt) = [ΔCt(PNC1–CpNCPH)] – [ΔCt(gapdh–Cpgapdh)] and as relative amounts to negative control.

Purification of PNC1 from Escherichia coli

hpNC1 was expressed in inclusion bodies in E. coli BL21 (DE3) cells using procedures previously described for the bovine oxoglutarate carrier (Fierroton et al., 1993) and several other mitochondrial carriers (Palmieri et al., 2000). Control cultures containing the empty plasmid vector were processed in parallel. Inclusion bodies were purified on a sucrose density gradient (Fierroton et al., 1995) and washed with a Tris buffer (pH 7.0/0.1 M EDTA; buffers with 3% (wt/vol) Triton X-114, 1 mM EDTA, and 10 mM Pipes/NaOH, pH 7.0; and finally with buffer A. The proteins were solubilized in 1.8% (wt/vol) N-dodecylmaltoside (sarkosyl). Small residues were removed by centrifugation.

Reconstitution into Liposomes and Transport Measurements

The recombinant hpNC1 protein in sarkosyl was reconstituted into liposomes in both the presence and the absence of substrates, as described previously (Palmieri et al., 1995). The external substrate was removed from proteoliposomes on Sephadex G-75 columns pre-equilibrated with 50 mM NaCl and 10 mM PIPEs, pH 7.0. Transport at 25°C was started by adding the indicated labeled substrate and was stopped after 90 min by adding a mixture of 20 mM pyridoxal 5′-phosphate and 20 mM bathophenanthroline. In control samples the inhibitors were added at time zero according to the inhibitor stop method (Palmieri et al., 1995), the external radioactivity was removed, and the radioactivity in the liposomes was measured. The experimental values were corrected by subtracting the respective controls.

Mitochondrial Isolation

Cells were removed from plates by trypsinization followed by washing with PBS and centrifugation at 1000 × g for pellets. A volume of mitochondrial isolation buffer (10 mM Tris-Cl, pH 7.5, 210 mM sucrose, 70 mM KCl, 10 mM NaCl, 1 mM EDTA, 1.5 mM MgCl2) equivalent to three times the volume of the pellet was added. Cells were homogenized by fine needle (26-gauge) aspiration 10 times and the homogenates centrifuged at 1000 × g for 15 min at 4°C. The supernatants were removed to fresh tubes and centrifuged at 1000 × g to remove any residual cellular contaminations. The supernatants were again removed and centrifuged at 7000 × g for 15 min at 4°C. The pellet obtained represents the mitochondrial fraction. This was washed three times with PBS to remove any remaining cytosolic fraction contaminants.

Extraction and Analysis of Nucleotides

The cell or mitochondrial pellet was gently resuspended in an ice-cold 6% solution of trichloroacetic acid to precipitate protein. Samples were incubated for ice on 10 min and centrifuged at 20,800 × g for 10 min at 4°C. The protein pellet was discarded, and, to remove the acid, an equal volume of 70% (v/v) ethanol (in Freon) was added. After incubation at 4°C, the pellets were washed at 4°C first with buffer A (10 mM Tris-HCl, pH 7.0/0.1 M EDTA); twice with buffer A, then with buffer B, which was prepared by adding 30% methanol to buffer A. Buffers were filtered and degassed before use. Separation was achieved at 1 ml/min using the following gradient: 0–20% buffer B over 8 min, 20–70% B over 12 min, and then a decrease to 0% B over 5 min. Nucleotide standard solutions, prepared using a 5′-nucleotide and nucleoside kit from Sigma, were used to evaluate peak positions.

RESULTS

Expression of a Mitochondrial Carrier Is Enhanced in IGF-IR-Transfected Fibroblasts

Suppressive subtractive hybridization (SSH) was used to isolate cDNAs that were differentially expressed between the R+ and R− cell lines (Loughran et al., 2005b). The mRNA for one of the cDNAs was confirmed by Northern blotting to be more abundantly expressed in R+ cells than in R− cells (Figure 1A). This cDNA was used to prepare a multiple tissue blot, which showed high expression of mRNA in liver and testis, lower levels in heart and brain, but undetectable levels in spleen, lung, skeletal muscle, or kidney (Figure 1B).

A comparison of both the human and mouse sequences with those in the EMBL Nucleotide Sequence database showed that this cDNA codes for a member of the mitochondrial carrier family (Palmieri, 1994; Kunji, 2004). These carriers have three homologous repeats, each containing a signature motif, typically P-X-[DE]-X-[KR] (Figure 1C). The main structural fold is a six α-helical bundle with pseudo-threfoil symmetry as exemplified by the structure of the bovine ADP/ATP carrier (Peyba-Peyroula et al., 2003). This gene is located on human chromosome 1 (1p36.22), and its product (Q9BSK2) has another isoform (Q96CQ1) with 60% identity that is located on chromosome 3. It is also identical to the identity that is located on chromosome 3. It is also identical to the
Figure 1. Pnc1 mRNA is abundant in R+ cells compared with R- cells and encodes a mitochondrial carrier protein. (A) RNA was isolated from R+ and R- cells and used to generate Northern blots. This blot and a murine multiple tissue blot (B) were probed with radiolabeled probes derived from the murine pnc1 cDNA. Both were reprobed for actin expression to demonstrate RNA loading. (C) The predicted protein topology of the mitochondrial pyrimidine transporter PNC1 based on the homology to the bovine ADP/ATP carrier (Pebay-Peyroula et al., 2003), showing the six transmembrane α-helices, the threefold sequence repeats and the conserved signature motifs. Also shown is the alignment of PNC1 with the bovine ADP/ATP carrier ANT1, showing identical residues (*) and similar ones (± and =), the positions of the α-helices, and the signature motifs (box).
deviation in the signature motif of the second repeat, where the negatively charged amino acid has been replaced by a tryptophan (P-I-W-M-V-K), but the basic residue of the interacting salt bridge is retained. The e-electrons of the aromatic residues form a negatively charged face, which can interact electrostatically with positively charged residues (Dougherty, 1996) The human mitochondrial carrier is also closely related to three carriers from yeast—Rim2p or Pyt1p (Marobbio et al., 2006), Ndt1p, and Ndt2p (Todisco et al., 2006)—that share an identity of 29.5, 23.4, and 24.0%, respectively.

**pnc1 Expression Is Enhanced in Transformed Cells and Is Induced by IGF-I or Insulin**

*pnc1* mRNA is overexpressed in R+ cells compared with R− cells, so this suggested that its expression level and related transport activity are important determinants of its contribution to metabolism in R+ cells and other transformed cells.

To investigate this, we asked if *pnc1* expression was associated with cellular transformation in cells other than fibroblasts. *pnc1* mRNA was detected in the breast carcinoma cell line MCF-7, but not in the nontumorigenic breast myoepithelial cell line MCF10A (Figure 2A). Similarly *pnc1* mRNA was detected in the Jurkat T lymphocytic leukemia cell line, but not in primary T lymphocytes (Figure 2A). *pnc1* mRNA expression was then investigated in a panel of 11 primary prostate carcinomas compared with normal prostate tissue. Significantly higher *pnc1* expression was observed in the prostate tumors compared with normal tissues (Figure 2B).

Next we investigated whether *pnc1* expression was regulated by IGF-I or insulin. R+ cells were starved of serum, then stimulated with IGF-I, and analyzed by Northern blots for *pnc1* expression. In R+ cells *pnc1* mRNA was low in starved cells but was rapidly induced after 4 h of IGF-I stimulation (Figure 2C). In MCF-7 cells *pnc1* mRNA expression was induced by 4-h stimulation with IGF-I (Figure 2C), with a further increase after 24 h. R− cells were used to investigate whether *pnc1* transcription was responsive to insulin. *pnc1* mRNA expression was not detectable in starved R− cells but was induced by insulin after 2 and 4 h, and a further induction was observed after 8 and 12 h (Figure 2C). A similar pattern of *pnc1* induction was observed in the 3T3L1 preadipocyte cell line (data not shown).

Induction of *pnc1* mRNA by IGF-I in MCF-7 cells was found to be dependent on the activity of the PI-3 kinase and mTOR pathways, but it was repressed by the Erk MAPK pathway. This was determined by pharmacological inhibition of each of these three pathways with LY29004 (PI3 kinase inhibitor), rapamycin (mTOR inhibitor), and PD98059 (Mek inhibitor) before IGF-I stimulation (Figure 2D). Examination of the PNC1 promoter region revealed that in addition to the classical promoter components such as the TATA box, CBP/p300, and S8 ribosome unit binding sites a large number of putative binding sites for transcription factors responsive to the PI-3K, mTOR, MAPK, and PKA pathways were evident (Supplementary Figure S1).

Taken together these data indicate that *pnc1* expression is enhanced in transformed cells and primary tumors and is rapidly responsive to either insulin or IGF-I signaling through the PI-3 kinase/mTOR pathway.

**Overexpressed PNC1 Causes an Increase in Cell Size**

To investigate if overexpression of the PNC1 protein could be used to determine its contribution to IGF-I and insulin signaling in tumor cells, plasmids encoding PNC1 as either a GFP- or Ha-fusion protein were transiently transfected into MCF-7 or HeLa cells. Confocal microscopy showed that GFP-PNC1 colocalized with mitoTracker, which specifically labels mitochondria (Figure 3A). HeLa cells transiently expressing Ha-PNC1 were costained with an anti-Ha antibody and a human anti-mito antibody, which labels mitochondrial membranes. This demonstrated that Ha-PNC1 became localized to mitochondrial membranes. Overexpression of PNC1 had no discernible effect on mitochondrial membrane polarization as assessed with the JC1 fluorescent probe (data not shown), which indicates that it does not alter the integrity of the mitochondrial membrane.

The effects of PNC1 levels on cell size were assessed in clones of MCF-7 cells that stably overexpressed PNC1. MCF-7/He-PNC1 cells consistently exhibited an increased light scatter compared with Neo cells, which is indicative of in-
phenotype in complete medium. Van Dyck by the observation that Rim2p yeast mutants exhibit a petite overexpression of the protein. This hypothesis is supported by the observation that Rim2p yeast mutants exhibit a petite phenotype in complete medium (Van Dyck et al., 1995).

Several siRNA oligonucleotides directed against pnc1 were used to suppress its expression in MCF-7, MCF-7/HA-PNC1, DU145 prostate carcinoma, or HeLa cervical carcinoma cell lines. We could not generate rabbit antisera with sufficiently high affinity to detect endogenous PNC1 protein, so the HA antibody was used to detect overexpressed PNC1 protein and RT-PCR was used to detect endogenous pnc1 mRNA. HA-PNC1 protein expression was reduced at 2, 3, and 4 d in MCF-7/HA-PNC1 cells transfected with pnc1-specific siRNA compared with control (Figure 4A). RT-PCR analysis demonstrated that endogenous pnc1 mRNA was reduced in siRNA-treated MCF-7/Neo cells, but mRNAs for the folate and dicarboxylate mitochondrial carriers (Fiermonte et al., 1998; Titus and Moran, 2000) were not altered (shown in Figure 4B at 72 h after transfection).

Cell size was reduced in MCF-7/Neo, MCF-7/HA-PNC1 cultures at 48, 72, and 96 h after transfection with pnc1-specific siRNA compared with mock-transfected cells (Figure 4C). As a control, cells were treated with rapamycin, which causes decreased cell size through inhibition of mTOR. Rapamycin had a similar effect on cell size as the pnc1-specific siRNA in MCF-7/Neo cells and had a smaller effect than pnc1 siRNA on cell size in MCF-7/HA-PNC1 cells. Cell size was also reduced by siRNA-mediated reduction of PNC1 expression in DU145 cells and HeLa cells (Figure 4C).

Proliferation of MCF-7 cell cultures transfected with pnc1-specific siRNA was greatly decreased over 96 h compared with control siRNA-transfected cells (Figure 4D). Analysis of cell cycle progression in MCF-7 cells demonstrated that cells with PNC1 suppressed had more cells in the G1 phase of the cell cycle and fewer cells in the G2/M phases than control cells (Figure 4E). This indicates that the cell cycle is delayed in the G1 phase in cell cultures with reduced PNC1 expression, which may explain the reduced growth (size) and proliferation of these cells. Overall, these data demonstrate that reduced PNC1 expression causes retarded cell growth and proliferation.

**IGF-I–mediated Activation of mTOR Is Not Affected by PNC1 Expression**

Our data demonstrate that suppression of PNC1 reduces cell growth and proliferation. The effect on cell growth in MCF-7 cells was similar to the effects of the mTORC1 inhibitor rapamycin. Because both growth factors and nutrients are necessary for activation of the mTORC1 pathway, we hypothesized that PNC1 may contribute to a mitochondria-dependent signal required for mTOR activation. Therefore, we investigated the status of the mTOR pathway in cells with enhanced or suppressed PNC1 expression. As can be seen in Figure 5 IGF-I–mediated phosphorylation of Akt and the mTORC1 target proteins S6K1 and 4EBP1 was not altered in MCF-7 cells overexpressing PNC1. Moreover, suppression of PNC1 expression in MCF-7 or HeLa had no effect on IGF-I–mediated phosphorylation of these proteins (data not shown). Altogether these results indicate that the effect of PNC1 on cell size and proliferation is not due to altered IGF-I–mediated activation of the mTORC1 pathway.

**PNC1 Transports Pyrimidine Nucleotides with Preference for UTP**

Our data indicate that PNC1 is essential for cell growth, so we next sought to determine what substrate PNC1 transports into the mitochondria that might mediate its function in promoting cell growth. To investigate the substrate specificity of PNC1 transport assays were carried out after reconstitution of the purified recombinant protein into liposomes. The transport activity was tested in homo-exchange experiments, which have the same substrate on both sides of the membrane. The recombinant transporter catalyzed active transport of pyrimidine nucleotides, with UTP being the preferred substrate.

**Suppression of PNC1 by siRNA Causes Decreased Cell Size and Decreased Proliferation**

Because mitochondrial carrier proteins have low turnover rates and are present in the mitochondrial membrane at low levels (Palmieri, 1994), we hypothesized that suppression of PNC1 expression could have a greater impact on cells than overexpression of the protein. This hypothesis is supported by the observation that Rim2p yeast mutants exhibit a petite phenotype in complete medium (Van Dyck et al., 1995).

Several siRNA oligonucleotides directed against pnc1 were used to suppress its expression in MCF-7, MCF-7/HA-PNC1, DU145 prostate carcinoma, or HeLa cervical carcinoma cell lines. We could not generate rabbit antisera with sufficiently high affinity to detect endogenous PNC1 protein, so the HA antibody was used to detect overexpressed PNC1 protein and RT-PCR was used to detect endogenous pnc1 mRNA. HA-PNC1 protein expression was reduced at 2, 3, and 4 d in MCF-7/HA-PNC1 cells transfected with pnc1-specific siRNA compared with control (Figure 4A). RT-PCR analysis demonstrated that endogenous pnc1 mRNA was reduced in siRNA-treated MCF-7/Neo cells, but mRNAs for the folate and dicarboxylate mitochondrial carriers (Fiermonte et al., 1998; Titus and Moran, 2000) were not altered (shown in Figure 4B at 72 h after transfection).

Cell size was reduced in MCF-7/Neo, MCF-7/HA-PNC1 cultures at 48, 72, and 96 h after transfection with pnc1-specific siRNA compared with mock-transfected cells (Figure 4C). As a control, cells were treated with rapamycin, which causes decreased cell size through inhibition of mTOR. Rapamycin had a similar effect on cell size as the pnc1-specific siRNA in MCF-7/Neo cells and had a smaller effect than pnc1 siRNA on cell size in MCF-7/HA-PNC1 cells. Cell size was also reduced by siRNA-mediated reduction of PNC1 expression in DU145 cells and HeLa cells (Figure 4C).

Proliferation of MCF-7 cell cultures transfected with pnc1-specific siRNA was greatly decreased over 96 h compared with control siRNA-transfected cells (Figure 4D). Analysis of cell cycle progression in MCF-7 cells demonstrated that cells with PNC1 suppressed had more cells in the G1 phase of the cell cycle and fewer cells in the G2/M phases than control cells (Figure 4E). This indicates that the cell cycle is delayed in the G1 phase in cell cultures with reduced PNC1 expression, which may explain the reduced growth (size) and proliferation of these cells. Overall, these data demonstrate that reduced PNC1 expression causes retarded cell growth and proliferation.

**IGF-I–mediated Activation of mTOR Is Not Affected by PNC1 Expression**

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[3H]UTP/UTP exchange and to a lesser extent [3H]TTP/TTP and [3H]CTP/CTP exchange (Figure 6). Low or very low transport rates were observed for GTP, ATP, dATP, NAD, S-adenosylmethionine, citrate, and (not shown) several other nucleotides, amino acids, polyamines, and carboxylic acids. These data demonstrate that PNC1 is a pyrimidine nucleotide transporter of primarily UTP. This makes PNC1 different from its yeast counterpart Rim2p, which transports UTP, CTP, and TTP predominantly (Marobbio et al., 2006).

PNC1 Suppression Decreases Accumulation of UTP in Mitochondria

We next investigated potential changes in cellular nucleotide levels as a mechanism for the effects of PNC1 on cell growth and proliferation. Suppression of PNC1 expression would be expected to reduce mitochondrial UTP, but it could also affect the cellular ATP:ADP ratio due to mitochondrial dysfunction. To test this, total cellular nucleotides were extracted from pnc1 siRNA-transfected and control-transfected MCF-7 cells and quantified by HPLC analysis. The total amount of nucleotides extracted from pnc1 siRNA transfected-MCF-7 cells was always lower than control cells due to a decrease in proliferation. Therefore, the GTP peak was used as an internal standard, and the quantity of other nucleotides was expressed as a percentage of GTP levels. The levels of total cellular UTP were not significantly altered in cells where PNC1 expression was repressed by siRNA, compared with the controls (Figure 7A). The ATP and ADP pools were also not significantly different and the ratio of ADP/ATP appeared to be unaltered.

Nucleotide levels were assessed in mitochondrial fractions isolated from cells by differential centrifugation. UTP...
levels were significantly lower in pnc1 siRNA-transfected MCF-7 cells compared with controls, whereas ADP, ATP, and GTP levels were not significantly altered (Figure 7B). The ADP:ATP ratio, which is higher in isolated mitochondria than in total cell extracts, was not altered by suppression of PNC1. Expression of the mitochondrial marker VDAC and the cytoplasmic marker paxillin in the same amount of total protein demonstrated that the mitochondrial fraction was uncontaminated (Figure 7C). Mitochondrial mass was not significantly altered in cells with suppressed PNC1 compared with controls (Figure 7D).

Overall, the data indicate that suppression of PNC1 expression causes decreased accumulation of UTP in mitochondria. This suggests that the effects of PNC1 suppression on cell growth are due to its function as a UTP carrier and that mitochondrial UTP levels may regulate cell growth.

**PNC1 Expression Regulates Cellular ROS Levels**

We next asked if suppressed PNC1 expression may elicit a mitochondrial retrograde or stress signaling response, which is associated with increased cellular ROS and has been proposed as an important mechanism of communication between mitochondria and nucleus in response to physiological and pathological stimuli (Biswas et al., 1999; Amuthan et al., 2002; Butow and Avadhani, 2004). To do this, cellular ROS levels were measured in cells with either increased or decreased PNC1 expression. As can be seen in Figure 8A, MCF-7 cells overexpressing PNC1 had decreased basal cellular levels of ROS compared with Vector controls in normal culture conditions. MCF-7 cells with suppressed PNC1 had increased cellular ROS compared with controls (Figure 8B). HeLa cells with stably suppressed PNC1 expression also had increased ROS levels (data not shown). These data indicate that cellular ROS levels are strongly influenced by PNC1 expression and suggest that
PNC1 has a function in regulating mitochondrial retrograde signaling.

DISCUSSION

The contribution of mitochondria to the growth and proliferation of cancer cells and the integration of mitochondrial function with the IGF-I and mTOR signaling pathways, glycolysis, and energy metabolism is an important, but largely unexplored area of biology. Here, we have shown that insulin and IGF-I induce the expression of the mitochondrial pyrimidine nucleotide carrier PNC1. Suppression of PNC1 results in reduced mitochondrial UTP accumulation, reduced cell growth, and reduced cell proliferation. These findings suggest that enhanced expression of PNC1 is a mechanism by which mitochondrial activity contributes to the proliferation of transformed cells.

PNC1 expression is rapidly induced by both IGF-I and insulin, and this induction could be suppressed by inhibiting either PI-3 kinase or mTORC1 activity. The pnc1 promoter contains several response elements that are known targets of the PI-3 kinase and mTOR pathway (Heinemeyer et al., 1998). Several putative binding sites for transcription factors known to control the expression of genes encoding mitochondrial proteins (Sp1, NRF-2, and CREB; Scarpulla, 2002) were also observed in the promoter. Interestingly most of these are present in a small region near the transcription start site, which may indicate the presence of a proximal promoter. All of this suggests that PNC1 is a transcriptional target of the PI-3 kinase signaling pathway important for regulation of mitochondrial activity. Our data on enhanced expression of PNC1 in all transformed cell lines tested compared with nontransformed cells and in a panel of prostate carcinomas compared with normal prostate tissue suggest that PNC1 may be up-regulated to provide a growth advantage to cancer cells. This is in agreement with recent studies suggesting a central role for the mTORC1 signaling complex in oncosogenesis. By using a series of murine models that are deficient in specific Akt isoforms, it was demonstrated that the requirement for Akt signaling to promote oncogenesis and cell proliferation is exclusively dependent on the activity of the mTORC1 (mTOR-Raptor) complex (Skeen et al., 2006). Interestingly, we also observed increased PNC1 expression in tumor tissues of different origin and in prostate tissues exhibiting hyperplasia (data not shown). These observations suggest that increased expression of PNC1 may generally be associated with proliferating cells or may contribute to the early stages of malignant transformation.

Overexpression of PNC1 increased cell growth and suppression of PNC1 expression caused decreased cell growth. In MCF-7 cells this was accompanied by a decrease in the rate of progression through the cell cycle and overall decreased cell proliferation. The decreased cell growth may be a consequence of the retarded progression through the G1 phase of the cell cycle. However, although suppression of PNC1 caused decreased accumulation of UTP in the mitochondria, this was not accompanied by changes in the cellular ATP/ADP ratio or ATP levels. This suggests that the retarded cell cycle progression is not solely caused by altered cellular energy levels that could result from decreased ATP production by mitochondria and the consequent alterations in ADP and AMP levels.

The decreased size of cells with suppressed PNC1 expression prompted us to investigate the mTOR pathway as a target of the putative signal from mitochondria to the cell cycle. S6K1 and 4EBP1 have previously been shown to be dephosphorylated in response to disruption of mitochondrial function (Kim et al., 2002) and the mTOR-raptor complex to be regulated in a redox-sensitive manner (Sarbassov and Sabatini, 2005). However, in agreement with our observations on unchanged ATP levels and mitochondrial mass, we did not observe any significant effect on phosphorylation of S6K1 or 4EBP1 in cells with suppressed PNC1. It has been proposed that a homeostatic regulatory loop exists between mTORC1 and mitochondria (Schieke and Finkel, 2006). In addition to mTORC1 activity being regulated by mitochondrial signals, the activation and stability of the mTORC1 complex has been correlated with increased oxidative phosphorylation and oxidative capacity (Schieke et al., 2006). Overall, our data demonstrating induction of PNC1 in a mTORC1-dependent manner and the lack of effects of suppression of PNC1 on S6K1 and 4EBP1 phosphorylation support a role for mTORC1 as an upstream regulator of mitochondrial function.

Because there was no obvious effect on mitochondrial membrane potential or oxidative phosphorylation in cells with suppressed PNC1, we conclude that there was no gross mitochondrial dysfunction in these cells. However the effects of PNC1 suppression on cell cycle progression and cell size suggest that reduced PNC1 results in an inhibitory signal from mitochondria for cell growth and cell cycle progression. This signal may be a component of a mitochondrial retrograde signaling (mitochondrial stress signaling) response, which is thought to be an important mechanism of communication between mitochondria and nucleus in response to physiological and pathological stimuli (Biswa et al., 1999; Amuthan et al., 2002; Butow and Avadhani, 2004). Mitochondrial signaling has been associated with regulation of cell cycle progression (Boonstra and Post, 2004), with altered cytoplasmic calcium levels, production of ROS, altered stress kinase pathway activation, and altered nuclear gene transcription (Butow and Avadhani, 2004). Our data demonstrating decreased basal cellular ROS levels when PNC1 is overexpressed and increased ROS levels when PNC1 is suppressed suggest that PNC1 can regulate mitochondrial retrograde signaling. Increased cellular ROS levels have previously been shown to enhance or suppress cell cycle progression and signaling responses in cells (Boonstra and Post, 2004). In conditions where there is cell cycle arrest in G1, this may be associ-
ated with protection from oxidative damage and cell death (Rancourt et al., 2002). The precise mechanism of the effects of PNC1-regulated ROS levels on cell growth remain to be determined as do the consequences of this for transformed cells. However, it is apparently not dependent on p53 function because similar effects were observed in HeLa cells, which do not express functional p53, and in MCF-7 cells.

Mitochondria may propagate retrograde signals as a mechanism of promoting adaptations to physiological changes that are sensed in cells. This possibility is receiving significant attention as a causative factor in ageing and lifespan regulation in simple organisms (Biswas et al., 2005; Schieke and Finkel, 2006). Mitochondrial signals may also have a role in pathological adaptations such as in cancer progression. For example, in lung carcinoma cells mitochondrial retrograde signaling has been associated with induction of genes required for acquisition of an aggressive invasive phenotype (Biswas et al., 2005).

PNC1 is the first mammalian mitochondrial carrier identified to have selectivity for pyrimidine nucleotides. It displays a distinct preference for UTP over TTP and CTP unlike the closely related yeast carrier Rim2p, which transports UTP, CTP, and TTP (Marobbio et al., 2006). This suggests a more specialized function in mammalian cells than in yeast cells. However, it is likely that PNC1 is not solely responsible for transporting pyrimidine nucleotides into mitochondria, and there is compensatory activity by related isoforms or a carrier that can transport all purine and pyrimidine nucleotides without selectivity (Palermi, 2004). This might also explain the lack of gross mitochondrial dysfunction in cells when PNC1 was suppressed.

In summary, we have demonstrated that PNC1 is a target of the IGF-I and insulin signaling pathway that functions in integration of growth factor signaling and mitochondrial function with cell growth and proliferation. Increased expression of PNC1 in transformed cells suggests that PNC1 is important for the activity of mitochondria in the proliferation of cancer cells.

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