SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Materials

[^{14}C]Formate (53 mCi/mmol), [8-^{14}C]adenine (52 mCi/mmol), [8-^{14}C]hypoxanthine (56 mCi/mmol), and [1-^{14}C] (53 mCi/mmol)and [6-^{14}C]glucose (55 mCi/mmol) were from Moravek Radiochemicals; [γ-^{32}P]ATP and $^{32}PO_4$ were from Perkin-Elmer. AG50 resin was from Bio-Rad, and protease inhibitors were from Pierce Biotechnology. Insulin, halt phosphatase inhibitor cocktail, and LY294002 were from Calbiochem-EMD Chemicals, and MK2206 was from Selleck Chemicals; LY294002 and MK2206 were dissolved in DMSO, with the final DMSO concentration in culture medium not exceeding 0.1%. Torin 1 was from Tocris Bioscience and NBD peptide was from Enzo Life Science. Oligomycin A, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), rotenone, antimycin A, and alkaline phosphatase bound to agarose beads were from Sigma-Aldrich. Purified hypoxanthine phosphoribosyltransferase was from NovoCIB SAS. Antibodies against Akt, phospho-Akt (Thr^{308}), phospho-Akt (Ser^{473}), phospho-p70 S6 Kinase (Thr^{389}), p70 S6 Kinase-α, and phospho-GSK-3β (Ser^{9}) were from Cell Signaling Technology. Anti-TKT antibody and HRP-conjugated β-actin were from Santa Cruz Biotechnology. Anti-GSK-3β antibody was from BD Bioscience. Flag-tagged constitutively active (CA) Akt and kinase dead dominant negative (DN) Akt plasmids were kindly provided by J.R. Woodgett; the CA-Akt contains a Src myristoylation signal on the N-terminus, and the dominant negative Akt contains several mutations in the catalytic domain rendering it inactive (Ramaswamy, et al, 1999). Lipofectamine-2000 was from Life Technologies, and Dharmafect I, and Raptor and rictor siRNAs were from Dharmacon, Inc.

Measurement of Cellular Metabolites

Cells were washed twice with ice-cold phosphate-buffered saline (PBS), harvested quickly by scraping with a rubber policeman, and centrifuged at 12,000 g for 20 sec at 4 °C. The cell pellet was flash frozen in liquid nitrogen, lyophilized, and sent to the University of California,

Davis West Coast Metabolomics Center where it was extracted. The extracts were analyzed by high performance liquid chromatography mass spectrometry (Budczies et al, 2013).

Measurement of Glucose 6-phosphate Dehydrogenase Activity

Cells were harvested and extracted as described for measuring TKT activity, and glucose 6-phosphate dehydrogenase activity was measured in the extracts following NADP reduction in the presence of glucose 6-phosphate as described previously (Boss and Pilz, 1985). An extract blank was included that had < 10% of absorbance change of the full system.

Cloning of Flag-tagged Wild Type and Mutant TKT

Total mRNA was extracted from HeLa cells using TRI reagent (Molecular Research). Full length cDNA was synthesized from mRNA using superscript III reverse transcriptase (Life Technologies). TKT with a C-terminus Flag-tag was generated by PCR using Phusion high fidelity DNA polymerase (New England Biolabs), with the forward primer containing an EcoR1 restriction site followed by the TKT 5' sequence, and the reverse primer containing a Not1 site, a stop codon, the Flag sequence, and the TKT 3' sequence respectively. The PCR product was purified by agarose gel electrophoresis, restriction digested, and treated with calf intestinal phosphatase; it was then ligated with pcDNA3.1(+) empty vector using rapid DNA ligation kit (Roche Applied Science,) and transformed into DH5α *E. coli*. TKT threonine 382 to alanine (T382A) and serine 387 to alanine (S387A) mutants were generated using the Quick-change mutagenesis kit. Primer sequences were: Wild type forward—ATATTATCCG GAATTCACCATGGAGAGCTACCACAAGCCTGACCAGCAGAAGCTGCAGGCCTTG; wild type reverse—AAGGAAAAAAGCGGCCGCTCACTTGTCGTCATCGTCCTTGTAGTCGGCCTTGG TGATGAGGCCCCTCACAGCTTGTGCAATG; T382A forward—5CCACCCG CAACAGGGCG GTGCCCTTCTGC3; T382A reverse—5GCAGAAGGGCACCGCCCTGTTGCGGGTGG3; S387A forward—5CAGGACGGTGCCCTTCTGCGCCACTTTTGCAGCCTTCTTC3; S387A reverse—5GAAGAAGGCTGCAAAAGTGGCGCAGAAGGGCACCGTCCTG3.

Purification of Recombinant TKT from *E.coli*

Modified pET vector containing human TKT with a C-terminal poly-histidine tag was transformed into BL21BL21-CodonPlus® (DE3)-RP *E. coli* (Stratagene, USA) as the expression host under ampicillin and chloramphenicol selection. Six one liter cultures of Terrific Broth supplemented with 5% glycerol were grown to an OD_{595} between 0.6-0.8 at which time cells were induced with 0.5 mM IPTG for 5 h. Cells were harvested and lysed using an EmulsiFlex C-3 cell disruptor (Avestin, Canada) in binding buffer (100 mM Hepes, 300 mM NaCl, 25 mM imidazole, 1 mM $CaCl_2$, 0.1 mM TPP at pH 8.0). The lysate was clarified by centrifugation at 20,000 × *g* for 20 min at 4 °C. The clear supernatant was incubated with nickel-NTA agarose beads (Qiagen, USA), and washed with binding buffer. Bound protein was eluted in 100 mM Hepes, 300 mM NaCl, 500 mM imidazole, 1 mM $CaCl_2$, 0.1 mM TPP, pH 8.0 and fractions were analyzed by SDS-PAGE. TKT-containing fractions were pooled, concentrated by ultrafiltration, and applied to a Superdex™ 200 size exclusion column (Amersham Pharmacia, USA) eluted with PBS buffer. Protein purity was assessed by SDS-PAGE and fractions containing >95% purity were pooled. His-tagged TKT wild type, and T382A and S387A mutants were produced and purified from bacteria (Mitschke et al., 2010).

Immunoprecipitation of Proteins

Cells were harvested in ice-cold PBS, and lysed in 150 mM Tris-HCl (pH 7.4), 75 mM NaCl, 0.5% NP40, 10% glycerol, and protease and phosphatase inhibitors. The lysates were incubated with anti-Flag M2 affinity gel (Sigma Aldrich) at 4^{o} C for 2 h, or with Akt antibody (Cell Signaling Technology) overnight at 4^{o} C, followed by incubation at 4^{o} C for 1 h with protein G-agarose beads. The immunoprecipitated proteins were extracted in a sodium dodecyl sulfate (SDS)-based sample buffer and analyzed by immunoblotting.

Immunoblotting of Proteins

Cells were extracted in hot SDS sample buffer *in situ* and applied to 7.5-9% polyacrylamide gels. Resolved proteins were transferred to polyvinyl difluoride membranes,

incubated with the primary antibody followed by a horse radish peroxidase-tagged secondary antibody, and detected using a chemiluminescence kit (Pierce Biotechnology).

***In vitro* Phosphorylation Experiments**

Flag-tagged TKT and Flag-tagged CA-Akt were immunoprecipitated from separate batches of HeLa cells and eluted from the affinity resin with Flag peptide. In some experiments, the TKT was pre-incubated with alkaline phosphatase-bound agarose beads or protein-G bound agarose beads, and in other experiments His-tagged TKT purified from bacteria (as described above) was used. Samples were incubated at 25^{o} C for 30 min with 2 μCi of [γ-^{32}P]ATP (final ATP concentration 10 μM) in the presence or absence of the CA-Akt; 1 μM MK2206 was present in some samples and in some experiments purified wild type Akt was used. Post-incubation, samples were subjected to SDS-PAGE followed by autoradiography and immunoblotting.

***In vivo* Phosphorylation Experiments**

Cells transfected with the indicated plasmids were incubated for 1 h in phosphate-free DMEM, followed by 3 h in phosphate-free medium with or without lysine and containing 150 μCi $^{32}PO_4$ (final concentration 50 μM). In some cases, 1 μM MK2206 was present during the 3 h incubation period. Flag-tagged TKT was isolated by immunoprecipitation, subjected to SDS-PAGE analysis, and analyzed by autoradiography and immunoblotting.

Mass Spectrometry Analysis of TKT from HeLa Cells

About 200 mg of wet cell pellet was lysed in M-PER® mammalian extraction reagent (Thermo Scientific) containing protease and phosphatase inhibitors. Cell debris was removed by centrifugation, and the supernatant was concentrated by ultracentrifugation. The sample was applied to a 16/60 Superdex™ 200 size exclusion column (Amersham Pharmacia), and TKT-containing fractions were identified by immunoblotting and placed over a 5/50 GL MonoQ™ column (Amersham Pharmacia). Fractions containing TKT were placed over two 4-20%

Criterion™ TGX™ SDS-PAGE gels (BioRad): one gel was used to identify TKT by immunoblotting, and the other was stained with Coomassie Blue. The ~ 70 kDa band from the latter gel was excised, destained, alkylated with 55 mM iodoacetamide, and digested overnight with trypsin. The peptides were analyzed by nano-LC/MS/MS, and the resulting data were compared to Mascot's NCBInr Homo sapiens database and human TKT (NCBI Reference Sequence: NP_001055.1). Phosphorylation of serine and threonine residues were confirmed by manual data analysis.

SUPPLEMENTAL REFERENCES

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