Proteomic and Metabolic Analyses of S49 Lymphoma Cells Reveal Novel Regulation of Mitochondria by cAMP and Protein Kinase A

Cyclic AMP (cAMP), acting via protein kinase A (PKA), regulates many cellular responses, but the role of mitochondria in such responses is poorly understood. To define such roles, we used quantitative proteomic analysis of mitochondria-enriched fractions and performed functional and morphologic studies of wild-type (WT) and kinase-null (kin−) murine S49 lymphoma cells. Basally, 75 proteins significantly differed in abundance between WT and kin− S49 cells. WT, but not kin−, S49 cells incubated with the cAMP analog 8-(4-chlorophenylthio)adenosine cAMP (CPT-cAMP) for 16 h have (a) increased expression of mitochondria-related genes and proteins, including ones in pathways of branched-chain amino acid and fatty acid metabolism and (b) increased maximal capacity of respiration on branched-chain keto acids and fatty acids. CPT-cAMP also regulates the cellular rate of increased maximal capacity of respiration on branched-chain keto acids and fatty acids. CPT-cAMP may derive from effects on mitochondrial proteins.

The second messenger cAMP is found in virtually every eukaryotic cell. Through compartmentalized signaling and activation of protein kinase A (PKA) and other effectors, cAMP regulates functional activities at discrete cellular locations (1, 2). PKA activation can be pro-apoptotic in lymphoid cells through activation of a mitochondria-dependent apoptotic pathway (3). Mitochondrial proteins involved in oxidative phosphorylation, ketogenesis, the TCA cycle, and fatty acid oxidation have consensus sequences for phosphorylation by PKA and can be phosphorylated (4). However, the role of PKA in regulating mitochondrial metabolism remains controversial due to difficulty in distinguishing the effects of PKA that occur outside or inside mitochondria, opposing theories regarding the source of cAMP within the mitochondrial matrix, and the poorly understood trafficking of PKA to mitochondria (5). Moreover, even though PKA is a key mechanism for cAMP-mediated regulation of cells, knowledge is limited regarding the mitochondrial proteins that are regulated by PKA.

Murine S49 T lymphoma (CD4+/8+) cells are a useful system to assess cAMP/PKA signaling (6–10). Selective pressure by cAMP analogs has been used to generate clonal variants of WT S49 cells (6, 10, 11). Incubation with cAMP analogs or agents that raise endogenous cAMP induces G1-phase cell-cycle arrest and apoptosis of WT S49 cells (6, 7, 12). The S49 variants that resist cAMP-induced apoptosis have helped reveal the functional role of components in the cAMP/PKA signaling pathway (3, 6, 8, 10).

kin− S49 cells are a clonal S49 variant that lack active PKA. The PKA catalytic (C) subunit in kin− cells has improper cis-phosphorylation at Ser338 during translation, rendering the C subunit insoluble.

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ub; accordingly, kin^− S49 cells have no PKA activity (13) yet grow robustly and resist cAMP-induced growth arrest and apoptosis (7, 8, 11). kin^− cells thus provide a PKA-null cell system to identify cAMP/PKA-dependent responses.

We previously found (14, 15) that cAMP acts via PKA to alter the expression of many mRNAs in WT, but not kin^− S49 cells, indicating that PKA produces substantial transcriptional regulation (14). Changes in mRNA may not, however, predict effects on protein expression as mRNA and protein expression may not highly correlate (16, 17). Due to the role of mitochondria in cAMP/PKA-mediated death of S49 cells (6, 7), here we used proteomic analysis to identify cAMP/PKA-mediated changes in protein expression in a mitochondria-enriched fraction of S49 cells and conducted additional studies to assess cAMP/PKA-regulated control of mitochondrial proteins and mitochondrial function.

**Experimental Procedures**

**Growth of S49 Cells and Treatment with a cAMP Analog—**

WT and kin^− S49 cells were grown in suspension culture in a humidified atmosphere containing 10% CO₂ at 37 °C in DMEM with 4.5 g/liter glucose supplemented with 10% heat-inactivated horse serum, 1 mM sodium pyruvate, and 10 mM HEPES (pH 7.4). Cells were incubated for the indicated times with 100 μM CPT-cAMP (Sigma), a cAMP analog that activates PKA (18). Cultures were initiated at a density of 2 × 10⁶ cells/ml; cells were maintained at a density of 1 × 10⁶–2 × 10⁶ cells/ml. Cell viability was determined using a Coulter Z2 Particle analyzer (Beckman Coulter).

**Mitochondria Preparation—**

Mitochondria were prepared according to an adaptation of the method described by Kristián et al. (19). Briefly, 2 × 10⁶ cells were harvested by centrifugation (1000 × g, 5 min, 4 °C), washed with ice-cold PBS, centrifuged again, and resuspended in MSHE (0.21 M mannitol, 0.07 M sucrose, 10 mM HEPES (pH 7.4), 1 mM EGTA) with a protease inhibitor mixture (MSHE-P) (Sigma). The cells were disrupted by 1400 p.s.i. for 10 min by nitrogen cavitation (Parr). The homogenate was centrifuged at 625 × g for 10 min to remove nuclei and unbroken cells. The supernatant was then centrifuged at 15,000 × g for 10 min. The supernatant (containing the endoplasmic reticulum) was removed. The pellet, the mitochondria-enriched fraction, was washed twice by resuspension in MSHE-P with centrifugation at 15,000 × g for 10 min followed by resuspension in MSHE-P.

**Proteomic Analysis—**

Equal (100 μg) aliquots of proteins from WT and kin^− S49 cells (0, 6, and 16 h CPT-cAMP treatment) were prepared for isobaric tagging and analyzed by mass spectrometry (MS) as previously described (15) with the following modification; the peptides were labeled with different 4-plex isobaric tagging for relative and absolute quantitation (iTRAQ) reagents (20). Spectrum Mill v3.03 was used to analyze the MS data as described (15) using 3 biological replicates to calculate protein iTRAQ reporter ion intensities. Proteins with five or more unique peptides were selected for quantitative analysis. A minimal total iTRAQ reporter ion intensity (sum of 4 channels compared) of 100 was used to filter out low intensity spectra. Conclusions regarding a change in protein abundance required the following criteria to be fulfilled. 1) The protein had to be quantified in at least two replicates. 2) If the protein was quantified in all three replicates, its abundance ratios had to be ≤0.67 or ≥1.5 in all three replicates. 3) If the protein was quantified in only two datasets, both had to yield abundance ratios of ≤0.67 or ≥1.5. We opted not to use a t test for iTRAQ quantification because that test can be too stringent for identifying proteins with -fold differences that are biologically significant (21). The DAVID 6.7 Bioinformatics tool (david.abcc.ncifcrf.gov) (22) was used to provide gene annotation and gene ontology term enrichment analysis.

**Immunoblot Analysis—**

Immunoblotting was used to verify increased expression of branched-chain amino acid transferase (Bcat2), medium-chain specific acyl-CoA dehydrogenase (Acadm), and short-chain specific acyl-CoA dehydrogenase (Acads) in WT S49 cells incubated with CPT-cAMP. Whole cell lysates prepared from WT and kin^− cells incubated with CPT-cAMP for 0–24 h were separated by 10% NuPAGE Bis-Tris gels (Invitrogen) in MOPS running buffer and transferred using an iBlot according to the manufacturer’s instructions. Antibodies for Acadm were from Santa Cruz Biotechnology, for Bcat2 and anti-rabbit secondary antibodies were from Cell Signaling Technologies, and for GAPDH antibody were from Abcam. Protein expression was quantitated by densitometry using ImageJ 1.41o software (imagej.nih.gov).

**Real-time PCR of Metabolic Genes—**

Cell pellets were collected and snap-frozen from untreated WT and kin^− S49 cells, cells were incubated with CPT-cAMP for 16 h, or WT S49 cells were incubated for 40 min with the PKA inhibitor H89 (20 μM) and then with CPT-cAMP for 0 or 16 h. Pellets were stored at −80 °C until used. RNA was isolated from frozen pellets using Direct-zol RNA MiniPrep Kit (Zymo) according to the manufacturer’s instructions and converted to cDNA using SuperScript III Reverse Transcriptase (Invitrogen) using the manufacturer’s recommended protocol for random hexamer priming. Real-time PCR reactions contained 1 × SYBR Green Master Mix (Eurogentec), 30–60 ng of cDNA, and primers at a final concentration of 0.2 μM. Primer sequences were as follows: Acads, forward 5′-GAC TGG CGA CGG TTA CAC A-3′; reverse 5′-GGC AAA GTC ACG GCA TGT C-3′; Acadm forward 5′-AAC ACA ACA CTC GAA AGC GG-3′; reverse 5′-TTC TGC TGT TCC GTC AAC TCA-3′; Bcat2 forward 5′-ACA GAC CAC ATG CTG ATG GTG-3′; reverse 5′-CTG GGT GTA GCG TGA GGT TC-3′.

**Culture of S49 Cells in Media Lacking Glutamine or Glucose—**

WT and kin^− S49 cells were grown in suspension culture in a humidified atmosphere containing 10% CO₂ at 37 °C in media for each tested condition. Culture media formulations were as follows: regular (high glucose) media (DMEM with 4.5 g/liter glucose supplemented with 10% heat-inactivated horse serum, 1 mM sodium pyruvate, and 10 mM HEPES (pH 7.4)); minimal glucose media (DMEM without glucose supplemented with 10% heat-inactivated horse serum, 1 mM sodium pyruvate, and 10 mM HEPES (pH 7.4)); glucose-deficient media (DMEM with 4.5 g/liter glucose and no l-glutamine supplemented with 10% heat-inactivated horse serum, 1 mM sodium pyruvate, and 10 mM HEPES (pH 7.4)). Cultures were initiated at a density of 5 × 10⁵ cells/ml and incubated with CPT-cAMP as described above, with 10 μM forskolin or with...
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H89 (1–20 μM) for 40 min before incubation with CPT-cAMP. Viability was determined using a Coulter Z2 Particle analyzer (Beckman Coulter); further analysis of apoptotic versus necrotic death was by flow cytometry. Freshly isolated cells were pelleted and washed twice in PBS, stained with annexin V-FITC (BD Biosciences) for 30 min at room temperature, diluted to a final volume of 500 μl in PBS and propidium iodide (BD Biosciences) was added just before reading on a FACSARia (BD Biosciences). Data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR). Events were gated to exclude debris; the data shown are the percentages of gated events.

Oxygen Consumption and Proton Production Measurements—WT, kin− and CPT-cAMP-treated cells (1.25 x 10⁶ cells/well) were placed in XF96 microplates pretreated with Cell-Tak (BD Biosciences) and assayed in a Seahorse XF96 Analyzer (24). Plates were spun at 500 x g for 5 min, and growth medium was replaced with unbuffered DMEM (Sigma #D5030) supplemented with 8 mM glucose, 3 mM glutamine, 1 mM pyruvate, and 0.5 mM carnitine. ATP-linked respiration was measured as the respiration rate sensitive to 2 μM oligomycin. Maximal respiration was reported as the difference between the rate of protonophore-stimulated respiration (calculated by using sequential additions of FCCP; final concentrations, 400 – 800 nM) and the rate of respiration in the presence of 1 μM rotenone and 2 μM antimycin A (non-mitochondrial respiration). The response to inhibitors of specific oxidative pathways was assessed using UK5099 (Tocris) (25), BPTES (36), or etomoxir (20) (27) added to cells 1 h before measurements.

Adherent cells were permeabilized, and ADP-stimulated respiration (State 3 respiration) was measured as previously described (28). Briefly, cells were permeabilized with recombinant perfringolysin O (commercially XF PMP; Seahorse Bioscience) and provided with 4 mM ADP and the following concentrations of respiratory substrates: 10 mM pyruvate with 1 mM malate, 5 mM glutamate with 5 mM malate, 10 mM succinate with 2 mM rotenone, 40 μM palmitoyl carnitine or octanoyl carnitine with 1 mM malate, and 4 mM L-α-ketoisocaproate (the keto acid of leucine) or 4 mM L-α-keto-β-methyl valerate (the keto acid of isoleucine) with 1 mM malate. All bioenergetic experiments were conducted with a minimum of three biological replicates and at least five technical replicates per experimental condition.

Electron Microscopy (EM)—S49 cells were pelleted and prepared for EM according to the method of Niikura et al. (29) before imaging using an FEI spirit transmission electron microscope operated at 120 kV.

Data Analysis—Statistical analyses were performed using GraphPad Prism 6 (La Jolla, CA). We performed one-way analysis of variance followed by Dunnett’s multiple comparison test to determine statistical differences for untreated WT S49 cells or cells incubated with CPT-cAMP or forskolin and for WT compared with kin− S49 cells. Where only t tests were applied, we used the Holm-Sidak method, with α = 5.0%, analyzing each comparison without the assumption of a consistent standard deviation.

Results

Quantitative Proteomic Analysis of Mitochondrial Proteins—Fig. 1A illustrates our workflow for quantitative proteomic analysis of mitochondria-enriched fractions of WT and kin− S49 cells. We conducted this analysis in three biological replicates using 4-plex iTRAQ labeling (20) and identified and quantified the proteins with Spectrum Mill. Applying a 1% false discovery rate cutoff for peptide identification (to minimize detection of false positives) and requiring detection of at least 5 unique peptides for each protein, we identified 2013 proteins (supplemental Table 1). Known or predicted mitochondrial localization was determined using the COMPARTMENTS database (30); 1871 of the proteins we detected were listed in COMPARTMENTS, 1305 of which had known or predicted mitochondrial localization based on text mining or protein sequence.

Differences in Proteins between WT and kin− S49 Cells under Basal Conditions—Under basal conditions, 75 proteins demonstrated differences (36 decreases, 39 increases) in abundance in kin− compared with WT S49 cells (supplemental Table 2). Most of these proteins were not previously identified as regulated by or interacting with PKA. Analysis by the DAVID bioinformatics tool indicated that these proteins include ones involved in formation of intracellular structures, trafficking, oxidation/reduction, and metabolism. Table 1 lists a subset of differentially expressed proteins in the oxidation/reduction and metabolism categories.

kin− cells have lower basal expression of a set of proteins involved in mitochondrial oxidative metabolism including MPC2 (a component of the mitochondrial pyruvate carrier), trimethyllysine dioxygenase (Tmlhe), the first enzyme in the carnitine biosynthesis pathway, glycerol-3-phosphate acyltransferase (Gpam), the first enzyme in the synthesis of glycerolipids, and two components of Complex I of the electron transport chain, Nduv3 and Ndufa12.

Changes in Protein Expression in Response to Incubation with CPT-cAMP—Incubation of WT S49 cells with CPT-cAMP for 6 h altered the expression of 83 (52 increases, 31 decreases) proteins in the mitochondria-enriched fraction, but none of these changes occurred in CPT-cAMP-incubated kin− cells. The 16-h incubation with CPT-cAMP altered the expression of 110 proteins (67 increases, 43 decreases) in WT cells but produced no changes in protein expression in kin− S49 cells (supplemental Table 3). Thus, under basal conditions and in response to cAMP, PKA regulates the expression of numerous proteins in a mitochondria-enriched fraction of WT S49 cells.

The DAVID Bioinformatics tool identified seven gene ontology classifications (oxidation/reduction, cell cycle/cell cycle process/cell proliferation, BCAA degradation, FA metabolism, and leukocyte (lymphocyte) activation) of proteins whose expression increased in WT S49 cells incubated for 16 h with CPT-cAMP (Table 2). Proteins with decreased abundance after 16 h of CPT-cAMP treatment include ones involved in regulation of the cell cycle and oxidation/reduction.

Proteins classified as “oxidation/reduction” have diverse roles. Proteins with decreased abundance in CPT-cAMP-treated WT cells include C-1-tetrahydrofolate synthase
(Mthfd1), which is associated with methionine, thymine, and purine synthesis (31) and squalene epoxidase (Sqle), the first oxygenation step in cholesterol biosynthesis (32). Foxred1, an assembly factor specific to Complex I, may have a role in metabolism, as bacterial FOXRED genes appear in operons that regulate degradation of creatine and creatinine, resulting in glycine, a component of the antioxidant glutathione (33).

Oxidation/reduction proteins that are increased by incubation with CPT-cAMP include electron transfer flavoprotein subunit α (Etfa), which catalyzes the initial step of FA β-oxidation, and aldehyde dehydrogenase family 3 member B1 (Aldh3b1), which oxidizes long-chain FAs (34) and may protect cells from oxida-

![Figure 1A](https://example.com/figure1a.png)

**Figure 1A.** Diagram of the workflow of mitochondrial proteomic studies. Proteins from mitochondria-enriched fractions of WT and kin S49 cell were digested in three biological replicates of cells (WT control, WT treated for 6 or 16 h with CPT-cAMP (100 μM), kin control, and kin cells treated for 6 or 16 h with 100 μM CPT-cAMP). After digestion, peptides were desalted and labeled with iTRAQ reagents, and the labeled peptides from each sample were pooled and then separated by online two-dimensional LC with (PQD/CAD) pulsed Q dissociation-collision/collision activated dissociation acquisition on an LTQ-Orbitrap. Raw data for protein identification and quantitation were obtained using Spectrum Mill. Functional categories of proteins whose level of expression was increased or decreased were assigned according to the results of the DAVID bioinformatics tool.

![Figure 1B](https://example.com/figure1b.png)

**Figure 1B.** Location of proteins with PKA-dependent increases within BCAA degradation pathway. Proteins related to degradation of leucine, isoleucine, and valine show PKA-dependent increases in WT S49 cells incubated for 16 h with CPT-cAMP. 1, Bcat2 (BCAA transferase, mitochondrial precursor) mediates the first step in the degradation of each BCAA. 2, Acadm (medium-chain specific acyl-CoA dehydrogenase, mitochondrial precursor) catalyzes a reversible step in the degradation of all three BCAA. Acads (short-chain specific acyl-CoA dehydrogenase, mitochondrial precursor) (3) and Acadsb (short/branched-chain specific acyl-CoA dehydrogenase, mitochondrial precursor) (4) catalyze steps in valine and isoleucine degradation. 5, Hibch (3-hydroxyisobutyryl-CoA hydrolase, mitochondrial precursor) is unique to valine degradation. 6, Acat1 (acetyl-CoA acetyltransferase, mitochondrial precursor) reversibly catalyzes the conversion of acetyl-CoA to acetoacetyl-CoA. 7, Mccc1 (methylcrotonoyl-CoA carboxylase subunit α, mitochondrial precursor is unique to leucine degradation. 8, Hmgcs2 (hydroxymethylglutaryl-CoA synthase, mitochondrial precursor) converts acetoacetyl-CoA to (S)-3-hydroxy-3-methylglutaryl-CoA, which can be converted further to acetoacetate and acetyl-CoA and is, therefore, involved in ketone body oxidation. This pathway map is based on the (human) KEGG (Kyoto Encyclopedia of Genes and Genomes) valine, leucine, and isoleucine degradation pathway.
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Subset of proteins with greater or lower abundance in untreated kin− S49 relative to untreated WT (kin−:WT ratio >1.5 or <0.67)

| Gene symbol | Accession no. | Average ratio | Protein Description | Unique peptide no. | Spectrum no. | % Protein coverage |
|-------------|---------------|---------------|---------------------|--------------------|--------------|------------------|
| LOC100047372 | IPI00222546 | 1.91 | Rp22, LOC100047372 60S ribosomal protein L22 | 8 | 117 | 47 |
| Hk2 | IPI00114342 | 1.59 | Hk2 hexokinase-2 | 55 | 1122 | 44 |
| Gln1 | IPI00328028 | 1.51 | Gln1 elongation factor G 1, mitochondrial | 20 | 119 | 27 |
| Mpc2 | IPI00131896 | 0.64 | Mitochondrial pyruvate carrier 2 | 7 | 23 | 29 |
| Tmnh2 | IPI00129163 | 0.60 | Tmnh2 trimethyllysine dioxygenase, mitochondrial | 5 | 13 | 8 |
| Taok3 | IPI00670075 | 0.44 | Taok3 similar to Serine/threonine-protein kinase | 5 | 9 | 6 |
| Ssb | IPI00134300 | 0.36 | Ssb lupus La protein homolog | 9 | 18 | 19 |
| Lipid metabolic process | | | | | | |
| Oshb | IPI00755161 | 1.61 | Oshb oxysterol binding protein | 6 | 9 | 8 |
| Cyb5r3 | IPI00759904 | 1.53 | Cyb5r3 Isomor 2 of NADH-cytochrome b5 reductase 3 | 12 | 56 | 34 |
| Gpam | IPI00387288 | 0.57 | Gpam glycerol-3-phosphate acyltransferase, mitochondrial | 8 | 46 | 12 |
| Fech | IPI00608064 | 0.25 | Fech Fech protein (fragment) | 10 | 36 | 9 |
| Oxidation/reduction | | | | | | |
| Mthfd2 | IPI00109824 | 3.36 | Mthfd2 bifunctional methylenetetrahydrofolate dehydrogenase/cyclohydrolase, mitochondrial precursor | 20 | 223 | 69 |
| LOC100046934 | IPI00854030 | 2.98 | LOC100046934 similar to amine oxidase (flavin-containing) domain 2 | 8 | 20 | 11 |
| Nduf3 | IPI00128285 | 0.67 | NADH dehydrogenase (ubiquinone) flavoprotein 3 | 9 | 19 | 22 |
| Nduf12 | IPI00344004 | 0.54 | Nduf12 NADH dehydrogenase (ubiquinone) 1a subcomplex, 12 | 7 | 27 | 54 |

Comparison of microarray and protein expression data demonstrated that mRNA expression of genes associated with cell cycle or regulation of cell proliferation, oxidation/reduction, and FA and BCAA metabolism correlated with protein abundance at 16 h.

Acads, Acadsb, Hmgcs2, and Bcat2, each involved in BCAA, FA, and ketone body catabolism, increased at 6 h; expression of Hibch and Acadm increased by 24 h. Altered abundance of the corresponding proteins (at 16 h) was as predicted by their gene expression changes. Thus, there is clearly a shift as a result of CPT/cAMP incubation toward expression of proteins involved in oxidative pathways for FA and BCAA. We next sought to validate these proteomic changes with alternative methods.

Increased Expression of Protein and mRNA of BCAA Metabolizing Genes in WT, but Not kin− S49 Cells—Immunoblot analysis confirmed that incubation of WT S49 cells with CPT/cAMP increased the protein expression of Bcat2, Acads, and Acadm; the expression of Bcat2 in kin− S49 cells did not significantly change with CPT/cAMP treatment, and expression of Acads and Acadm was too low to quantify (Fig. 2, A–F).

We used real-time qPCR analysis to assess the mRNA expression of Acads and Acadm, two genes in the FA oxidation pathway, and Bcat2, a mitochondrial BCAA aminotransferase.
Table 2

Proteins showing significant changes in CPT-cAMP-treated WT S49 cells, 16h

| Gene Symbol | Average Ratio | Unique Peptide | Spectrum Num | Protein Description | DAVID Classification | 6h mRNA Change | 24h mRNA Change |
|-------------|---------------|----------------|--------------|---------------------|----------------------|----------------|----------------|
| Fabh1       | 3.6           | 6              | 10           | delta-5 desaturase   | Oxidation reduction  |                |                |
| Bcat2       | 2.8           | 10             | 11           | Branched-chain amino acid transferase, mitochondrial precursor | Cell cycle           |                |                |
| H2afx       | 2.7           | 13             | 631          | Histone H2A.s       | Cell cycle process   |                |                |
| Atpi1       | 2.5           | 5              | 11           | ATPase inhibitor, mitochondrial precursor | Regulation of cell proliferation |                |                |
| Prdx3       | 2.6           | 9              | 75           | Thioredoxin-dependent peroxide reductase, mitochondrial precursor | Oxidation reduction |                |                |
| Mbd2        | 2.5           | 5              | 9            | Isoform 1 of Methyl-CpG-binding domain protein 2 | Regulation of cell proliferation |                |                |
| Hic1h       | 2.3           | 11             | 54           | 3-hydroxysterol-CoA hydrolase, mitochondrial precursor | Branch chain amino acid degradation |                |                |
| Acadm       | 2.2           | 9              | 55           | Medium-chain specific acyl-CoA dehydrogenase, mitochondrial precursor | Fatty acid metabolism, Branch chain amino acid degradation |                |                |
| Acadh       | 2.2           | 19             | 170          | Short-chain specific acyl-CoA dehydrogenase, mitochondrial precursor | Fatty acid metabolism, Branch chain amino acid degradation |                |                |
| Acadh2      | 2.1           | 5              | 21           | Short-branched chain specific acyl-CoA dehydrogenase, mitochondrial precursor | Fatty acid metabolism, Oxidation reduction, Branch chain amino acid degradation |                |                |
| Pdha1       | 2.1           | 23             | 174          | Pyruvate dehydrogenase E1 component alpha subunit, somatic form, mitochondrial precursor | Oxidation reduction |                |                |
| Hmgcr2      | 2.1           | 26             | 381          | Hydroxymethylglutaryl-CoA synthase, mitochondrial precursor | Branch chain amino acid degradation |                |                |
| Mec1        | 1.8           | 12             | 34           | Methylcrotonyl-CoA carboxylase subunit alpha, mitochondrial precursor | Branch chain amino acid degradation |                |                |
| Muc1        | 1.8           | 29             | 43           | Microtubule-actin crosslinking factor 1b | Cell cycle |                |                |
| Gld1        | 1.7           | 30             | 270          | Glutamate dehydrogenase 1, mitochondrial precursor | Oxidation reduction |                |                |
| Thef3       | 1.7           | 7              | 14           | Thyroid hormone receptor beta | Leukocyte (lymphocyte) activation, Leukocyte (lymphocyte) differentiation |                |                |
| Acat1       | 1.7           | 24             | 781          | Acetyl-CoA acetyltransferase, mitochondrial precursor | Fatty acid metabolism, Branch chain amino acid degradation |                |                |
| Cpt2        | 1.6           | 6              | 49           | Carnitine O-palmitoyltransferase 2, mitochondrial precursor | Fatty acid metabolism |                |                |
| Gfi1        | 1.6           | 19             | 874          | Electron transfer flavoprotein subunit alpha, mitochondrial precursor | Oxidation reduction |                |                |
| Me2         | 1.6           | 26             | 264          | NAD-dependent malic acid dehydrogenase subunit alpha, mitochondrial precursor | Oxidation reduction |                |                |
| Ptprec      | 1.5           | 29             | 260          | Isoform 3 of Leukocyte common antigen precursor | Oxidation reduction, Leukocyte (lymphocyte) activation, Leukocyte (lymphocyte) differentiation, Positive regulation of cell proliferation, Regulation of cell proliferation |                |                |
| Mthfd1      | 0.6           | 28             | 108          | C-1-tetrahydrofolate synthase, cytoplasmic | Oxidation reduction |                |                |
| Atb3b1      | 0.5           | 5              | 13           | Aldehyde dehydrogenase 3B1 | Oxidation reduction |                |                |
| Sple        | 0.5           | 6              | 26           | Squalene monooxygenase | Oxidation reduction |                |                |
| Atn         | 0.5           | 21             | 61           | Actin-binding protein anillin | Cell cycle, Cell cycle process |                |                |
| Exp1        | 0.4           | 13             | 28           | Separase | Cell cycle, Cell cycle process |                |                |
| Incenp      | 0.3           | 8              | 33           | Isoform 1 of inner centrosome protein | Cell cycle |                |                |
| Foxred1     | 0.3           | 6              | 11           | Isoform 2 of FDRS-dependent oxidoreductase domain-containing protein 1 | Oxidation reduction |                |                |
The DAVID bioinformatics tool classifies Bcat2 as a cell cycle related protein, but it catalyzes the first step in BCAA catabolism. CPT-cAMP increased the expression \((/H_{1022}^{2}\text{fold}\) at 16 h compared with basal) of mRNA for Acads, Acadm, and Bcat2 (Fig. 2G). None of these significantly changed in kin\(^{-}\) S49 cells. Consistent with this finding, treatment of WT cells with the PKA inhibitor H89 \((20 \mu M)\) blunted the increase by CPT-cAMP in mRNA expression of Acads, Acadm, and Bcat2 (Fig. 2G).

**cAMP/PKA Protects WT, but Not kin\(^{-}\) S49 Cells from Death in Media That Lacks Glutamine**—The lower abundance of proteins related to pyruvate and FA metabolism in kin\(^{-}\) than WT S49 cells and the higher abundance of HK, Gls, and Pck2 (Table 1) suggested that kin\(^{-}\) cells may have a higher dependence upon glucose or amino acids such as glutamine to meet energetic requirements. CPT-cAMP-stimulated elevations in genes and proteins involved in BCAA degradation in WT but not kin\(^{-}\) cells indicate a role for PKA in regulation of proteins involved in BCAA degradation. To metabolically stress the cells, we cultured WT and kin\(^{-}\) S49 cells in media lacking added glucose or glutamine. WT cells grown in media with DMEM with no added glucose for 72 h had a \(40\%\) decrease in viability but the addition of CPT-cAMP mitigated this loss in viability (Fig. 3A). kin\(^{-}\) cells were more resistant to glucose withdrawal, and CPT-cAMP had no effect on viable cell number (Fig. 3A), thus implying that the increased survival of WT cells is mediated by PKA. Both WT and kin\(^{-}\) S49 cells grown for 36 h in (high glucose) media that lacks glutamine had a \(50\%\) loss in viability; the addition of CPT-cAMP to WT, but not kin\(^{-}\) cells protected them from this loss in viability (Fig. 3B). Moreover, the two cell types differed in their rates of loss in viability in media that lacked glutamine; WT cells had a gradual decrease in viability,
which was slowed (up to 48 h) by the addition of CPT-cAMP (Fig. 3C). By contrast, kin− S49 cells had lower viability, which was detectable within 18 h (Fig. 3D) and appears to reflect an increase in apoptosis. The inability of CPT-cAMP to sustain the increase in the viability of WT S49 cells likely results from its pro-apoptotic action that occurs in these cells by 48 h (7, 11) and by findings with cAMP-deathless (D-) S49 cells, which do not undergo CPT-cAMP-promoted apoptosis (Fig. 3E) (3).

CPT-cAMP and forskolin (which increases endogenous cAMP via activation of adenylyl cyclase) protected WT S49 cells from death in media lacking glutamine; the PKA inhibitor H89 eliminated this protection (Fig. 3, F and G). Flow cytometry analysis revealed a higher percentage of early apoptotic events compared with necrototic events in kin− cells (glutamine-free media). Inhibition of necroptosis with necrostatin appears to accelerate apoptotic cell death in kin− cells in glutamine-free media. Data shown are the mean ± S.D., n = 2, *** = p < 0.001 versus kin− untreated, ** = p < 0.01 versus kin− untreated.
creased endogenous (basal) PPR (Fig. 4). Uncoupler-stimulated rates of cellular ATP turnover by the addition of oligomycin and the activity of the electron transport chain was disengaged from ATP production to glycolysis, or an overall ATP-linked respiration (Fig. 4), implying mitochondrial dysfunction and sensitivity to nutrient deprivation manifested in changes in cellular energetics, respiration and proton production rates were measured. Cellular ATP needs are predominantly supplied by oxidative phosphorylation and glycolysis. Mitochondrial ATP production can be estimated by the respiration rate sensitive to the ATP synthase inhibitor oligomycin. Glycolysis, the catabolism of uncharged glucose into anionic lactate, can be indirectly measured by extracellular acidification and expressed quantitatively as the proton production rate (PPR; Refs. 36 and 37). The contribution of respiratory CO₂ to medium acidification was broadly similar across groups and unlikely to confound interpretation, as basal respiration (Fig. 4A) and cellular substrate preference (see below) were similar in WT and kin⁻ cells. This renders PPR a reliable indicator of glycolytic turnover for this specific comparison (36, 37).

CPT-cAMP treatment of WT cells decreased the rate of ATP-linked respiration (Fig. 4A), implying mitochondrial dysfunction, a shift of ATP production to glycolysis, or an overall decrease in the rate of ATP utilization by the cells (37). To determine if CPT-cAMP induces mitochondrial dysfunction, activity of the electron transport chain was disengaged from cellular ATP turnover by the addition of oligomycin and the protonophore FCCP (Fig. 4B). Uncoupler-stimulated rates of respiration were similar between control and CPT-cAMP-treated WT cells, suggesting that PKA activation does not induce a defect in electron transport chain function.

With regard to glycolytic flux, CPT-cAMP treatment decreased endogenous (basal) PPR (Fig. 4C). These data, combined with the findings discussed above (Fig. 4, A and B), strongly suggest that CPT-cAMP diminishes cellular ATP demand in the WT cells. Oligomycin significantly stimulates PPR (Fig. 4D), blocking mitochondrial ATP production and forcing glycolysis to meet the cellular ATP demand. Under these conditions, CPT-cAMP negatively regulated glycolytic turnover (Fig. 4D). Taken together, the results in Fig. 4 imply that CPT-cAMP acts via PKA to regulate the ATP utilization rate as well as the poise between oxidative phosphorylation and glycolysis to meet the energy demand of the cell.

WT and kin⁻ cells had similar rates of ATP-linked respiration (Fig. 4A), suggesting that the absence of PKA does not change the mitochondrial contribution to overall cellular ATP production. In contrast, maximal rates of FCCP-stimulated respiration were decreased in kin⁻ compared with WT cells (Fig. 4B), indicating that the lack of PKA-mediated signaling lowers the cells’ respiratory capacity. As expected, respiratory rates in the kin⁻ cells were insensitive to CPT-cAMP (Fig. 4, A and B).

With regard to PPR in kin⁻ cells, rates of glycolytic flux were not significantly different in the basal state (Fig. 4C) or in the presence of oligomycin (Fig. 4D) from WT cells. As expected, there was no change in the glycolytic rate upon CPT-cAMP treatment in kin⁻ cells under either condition (Fig. 4, C and D).

To examine whether PKA can mediate not only global changes in cellular bioenergetics but also the flux through specific respiratory pathways, rates of State 3 (ADP-stimulated, phosphorylating) respiration were measured in permeabilized cells. Unlike with intact cells, this approach allows experimental control over the specific substrates offered to mitochondria, allowing for the direct interrogation of specific metabolic pathways (28).

The proteomic and Western analysis described above suggested that PKA-mediated regulates of expression of proteins involved in BCAA and FA catabolism. To test whether this regulatory control can translate into an altered capacity for carbon flux through these pathways, we measured the oxygen consumption on several substrates, including branched-chain keto acids and fatty acyl carnitines. First, we found that maximal rates of State 3 respiration on a variety of substrates were lower in kin⁻ than WT S49 cells (Fig. 5, A–C), suggesting a generalized decrease in oxidative capacity in kin⁻ cells consistent with the decrease in uncoupler-stimulated respiration seen in intact cells. CPT-cAMP acting via PKA increased the capacity to oxidize branched-chain keto acids of leucine and isoleucine (Fig. 5A) as well as the medium- and long-chain FA conjugates octanoyl carnitine and palmitoyl carnitine (Fig. 5B). Such changes were not seen in kin⁻ cells, indicating that PKA upregulates the capacities for BCAA and FA oxidation. CPT-cAMP did not change the maximal, ADP-stimulated rates of permeabilized cells for pyruvate, glutamate, or succinate (Fig. 5C), thus ruling out a global effect of CPT-cAMP on mitochondrial function, branched-chain keto acids, and FA conjugates.
To determine whether the relative contribution of different substrates was altered in whole cells, rates of maximal, uncoupler-stimulated respiration in intact cells were measured in response to UK5099 (which inhibits mitochondrial pyruvate uptake), BPTES (a glutaminase inhibitor), and etomoxir (an inhibitor of the carnitine palmitoyl transferase-1). CPT treatment did not alter the sensitivity to blocking pyruvate uptake or glutamine oxidation, but blocking of mitochondrial long-chain FA uptake was observed in WT but not \( \text{kin}^- \) cells, suggesting an increased capacity to oxidize endogenous long-chain FAs (Fig. 5D). These results support the idea that CPT treatment induces a shift toward use of FAs as an energy substrate in WT cells.

**FIGURE 5.** PKA activity affects BCAA and FA substrate usage. In WT, but not \( \text{kin}^- \) S49 cells, CPT-cAMP increased the capacity to oxidize the branched chain keto acids for leucine (\( \text{l-}\alpha\text{-ketooisocaproate} \) (KIC)) and isoleucine (\( \text{l-}\alpha\text{-keto-}\beta\text{-methyl valerate} \) (KMV)) (A) as well as the FA conjugates palmitoyl carnitine and octanoyl carnitine (B). C, other substrates showed no effect, and the relative usage of glucose and glutamine for oxidative metabolism was not changed. D, inhibitors were used to determine the extent to which cells oxidize pyruvate (UK5099), glutamine (BPTES), and long-chain FAs (etomoxir) and to assess for differences in substrate oxidation in the mitochondria of WT and \( \text{kin}^- \) S49 cells. Only etomoxir produced a significant change in substrate oxidation between WT S49 control and WT S49 + CPT-cAMP (* \( p < 0.05 \) versus WT control, ** \( p < 0.01 \) versus WT control). All data are the mean \( \pm \) S.E. (n \( = 3 \)).

**FIGURE 6.** CPT-cAMP treatment changes mitochondrial structure and increases autophagy. Electron microscopy showed medium to large mitochondria typically with long cristae (arrowhead) in WT S49 cells (a), similar mitochondrial structure (arrowhead) in \( \text{kin}^- \) S49 cells (b), and CPT-cAMP-treated WT S49 cells (c) have smaller mitochondria with fewer cristae (arrowhead) than do untreated WT cells. Scale bar, 500 nm for panels a, b, and c. d and e, lysosomal vesicles (arrowheads) are more prevalent in CPT-cAMP-treated WT cells (e) than in untreated WT cells (d), consistent with measurements (Table 3) that show decreased mitochondrial volume density and size, but no change in number, suggesting degradation of a portion of individual mitochondria, likely by fission of the dysfunctional part and its subsequent autophagosomal digestion. Scale bar, 1000 nm and applies to panels d and e.
cAMP/PKA Regulation of Mitochondria in S49 Cells

These findings are consistent with data showing that treatment of WT cells with CPT-cAMP induces loss of mitochondrial membrane potential, mitochondrial release of cytochrome c, and second mitochondria-derived activator of caspases (SMAC) and increases caspase-3 activity (3, 6).

The decrease in volume density and size of mitochondria but not their number implies that CPT-cAMP may increase autophagy in WT cells. CPT-cAMP treatment increased lysosomal vesicle number (Fig. 6, d and e), which suggests that some mitochondria have been degraded, perhaps by fission of the dysfunctional part and its subsequent digestion by an autophagosome.

Discussion

We studied S49 lymphoma cells and utilized multiple complementary approaches to determine if cAMP/PKA regulates the mitochondrial proteome and influences the structure and function of mitochondria. Prior studies of the mitochondrial proteome (38–40) have not assessed a role for cAMP or PKA nor the contribution of mitochondria to cellular actions of cAMP/PKA. We reasoned that a comparison of WT and kin− cells would be useful for such an assessment.

Under basal conditions, 36 and 39 proteins had decreased and increased expression, respectively, in kin− cells compared with WT S49 cells, which implies that under steady-state conditions PKA both stimulates and inhibits the expression of proteins in the mitochondrial proteome. Consistent with these bidirectional effects of PKA, incubation of WT, but not kin− S49 cells, with CPT-cAMP both decreased and increased protein expression. In CPT-treated WT S49 cells, expression of 31 proteins decreased after 6 h and of 43 proteins after 16 h, whereas 52 and 67 proteins increased after 6 and 16 h, respectively. The DAVID bioinformatics tool predicted that numerous cell cycle-related proteins were among the cAMP/PKA-regulated proteins with increased expression after 16 h, consistent with the G1 phase growth arrest of WT cells in response to increases in intracellular cAMP concentration (6, 8, 10).

Incubation of WT cells with CPT-cAMP prominently increased expression of proteins involved in FA oxidation, ketogenesis, and BCAA degradation, changes not seen in kin− S49 cells treated with CPT-cAMP. Immunoblot analysis confirmed the increases in expression of the BCAA and FA degradation pathway proteins, and real-time PCR showed that these increases result from their increased gene expression. Functional studies revealed a cAMP/PKA-dependent survival response and increased BCAA oxidation in WT S49 cell under glutamine-deprived conditions. Furthermore, analysis of respiratory rates revealed that CPT-cAMP treatment of WT cells results in an increased capacity to oxidize branched-chain α-ketoacids and fatty acyl carnitines, whereas no effect was observed on other oxidizable substrates including succinate, pyruvate, and glutamate. Together, these data imply that the regulation by cAMP/PKA of BCAA and FA degradation is transcriptional; the increase in BCAA and FA oxidation may be part of the pro-apoptotic events promoted by cAMP in WT S49 cells (3, 6, 7, 14).

In agreement with this effect of cAMP/PKA in S49 cells, yeast CAMP-dependent protein kinase type 1 (TPK1), a PKA, regulates BCAA metabolism through the de-repression of genes for BCAA aminotransferase, a homolog of murine Bcat1/2, and ketol-acid reductoisomerase (ILV5), involved in BCAA synthesis (41). Murine Acadm is transcriptionally regulated by PKA (42), but we believe ours is the first demonstration that Bcat2 or Acads is transcriptionally regulated by PKA.

The ability of forskolin to promote survival of WTS49 cells in media lacking glutamine supports the idea that cAMP/PKA mediates the increased transcription of metabolic genes. Actions of cAMP can be spatially limited by phosphodiesterase activity and complexes between PKA and its targets (43, 44).
Mitochondria-located membrane adenylyl cyclases have not been identified (5), but a soluble adenylyl cyclase is present in mitochondria and regulated by changes in bicarbonate, Ca$^{2+}$, and ATP levels (45, 46).

A phosphoproteomic analysis of mouse liver mitochondria revealed changes in phosphorylation of Hmgcs2, but not other proteins, related to BCAA degradation or FA oxidation (4). Assessment of the Jurkat T cell PKA-regulated phosphoproteome (47) did not identify the proteins that we found. An unexpected aspect of PKA deficiency in the kin$^{-}$ S49 cells is their sensitivity to a lack of glutamine, which includes apoptotic death (Fig. 3H). kin$^{-}$ S49 cells rapidly proliferate in complete media and remain viable in low glucose media. K-ras-transformed NIH-3T3 cells display aerobic glycolysis (the Warburg effect) and are sensitive to glucose withdrawal, undergoing apoptosis that can be prevented by PKA stimulation (48) and have decreased proliferation in the absence of glutamine (49). Because S49 cells do not possess a Ras mutation (50), the sensitivity of WT S49 cells to reduced glucose and its alleviation by stimulation of PKA occurs without constitutive Ras activity. Perhaps PKA-mediated up-regulation of alternative mitochondrial pathways for energy metabolism, including BCAA and FA oxidation, enhances survival in the face of glucose or glutamine deprivation. We identified two proteins encoded by mitochondria DNA mitochondrial cytochrome c oxidase subunit 2 (mt- COX$_{2}$ or COX2) and NADH-ubiquinone oxidoreductase chain 1 (mt-Nd1), but their basal expression is similar in WT and kin$^{-}$ S49 cells, and CPT-cAMP did not increase their expression.

The current mitoproteomic analysis identified 2013 proteins, whereas our previous proteomic study of lysates from WT and kin$^{-}$ S49 cells identified 1056 proteins (15); 469 proteins were in common between these studies. The greater number of proteins identified in this study may result from the use of iTRAQ 4-plex in place of 8-plex (51). One protein, LOC100046934 (similar to amine oxidase (flavin-containing) domain 2) was increased in both studies. Other proteins had significantly high or low kin$^{-}$/WT ratios but not in at least two experimental sets or lacked statistical significance in the other study. DAVID analysis of proteins in common showed enrichment for nucleotide binding proteins. Other highly enriched categories include protein folding/chaperone function, vesicle, mitochondria, and oxidation/reduction. The mitochondrial proteomic analysis thus revealed more proteins but shared similarities in terms of basal differences between WT and kin$^{-}$ cells, the effect of cAMP/PKA on protein abundance and enriched categories. The relatively small number of “overlapping” proteins in the two datasets may, at least in part, reflect our rather conservative approach for protein identification.

Future phosphoproteomic analyses of WT and kin$^{-}$ S49 cells should enhance understanding of PKA-specific substrates and mechanisms involved in cAMP/PKA-mediated actions (6). Such analyses may not only reveal the PKA-mediated phosphorylation profile but also if other kinases compensate for the lack of PKA in kin$^{-}$ cells. In yeast, deletion of cAMP-dependent protein kinase type 2 or 3 (TPK2 or TPK3) had a prominent impact on the phosphoproteome, whereas deletion of TPK1 had a smaller effect but changed the morphology of the yeast (23).

The current study provides new insights regarding the regulation by cAMP/PKA of mitochondrial function, including stimulated expression of enzymes involved in BCAA and FA oxidation, especially under states of nutritional stress. Our findings raise the possibility that the regulation of mitochondrial metabolism contributes to, and perhaps helps mediate, cellular responses that are altered by cAMP acting via PKA.

**Author Contributions**—S. S. T. and P. A. I. conceived and coordinated the study. Y. G. and L. Z. performed and analyzed the data in the proteomics experiments. A. W. performed and analyzed the experiments shown in Figs. 2 and 3. A. S. D. and A. N. M. designed, performed, and analyzed the experiments shown in Figs. 4 and 5. G. P. performed and analyzed the electron microscopy shown in Fig. 6. A. W. and P. A. I. wrote the manuscript, but all authors contributed to the writing and approved the final version.

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