The Mammalian Target of Rapamycin (mTOR) Pathway Regulates Mitochondrial Oxygen Consumption and Oxidative Capacity*

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Metabolic rate and the subsequent production of reactive oxygen species are thought to contribute to the rate of aging in a wide range of species. The target of rapamycin (TOR) is a well conserved serine/threonine kinase that regulates cell growth in response to nutrient status. Here we demonstrate that in mammalian cells the mammalian TOR (mTOR) pathway plays a significant role in determining both resting oxygen consumption and oxidative capacity. In particular, we demonstrate that the level of complex formation between mTOR and one of its known protein partners, raptor, correlated with overall mitochondrial activity. Disruption of this complex following treatment with the mTOR pharmacological inhibitor rapamycin lowered mitochondrial membrane potential, oxygen consumption, and ATP synthetic capacity. Subcellular fractionation revealed that mTOR as well as mTOR-raptor complexes can be purified in the mitochondrial fraction. Using two-dimensional difference gel electrophoresis, we further demonstrated that inhibiting mTOR with rapamycin resulted in a dramatic alteration in the mitochondrial phosphoproteome. RNA interference-mediated knockdown of TSC2, p70 S6 kinase (S6K1), raptor, or rictor demonstrates that mTOR regulates mitochondrial activity independently of its previously identified cellular targets. Finally we demonstrate that mTOR activity may play an important role in determining the relative balance between mitochondrial and non-mitochondrial sources of ATP generation. These results may provide insight into recent observations linking the TOR pathway to life span regulation of lower organisms.

For nearly a century it has been appreciated that an organism’s intrinsic metabolic rate is an important determinant of life span. This theory, initially known as the “rate of living” hypothesis, has merged with another proposed mechanism for aging first enunciated by Denham Harman (1) and often called the “free radical theory of aging.” The basis for combining these two hypotheses came from observations demonstrating that mitochondria determine both cellular and organismal metabolic rate and that these organelles also produce a continuous stream of reactive oxygen species. Although both the rate of living and the “free radical” theories of aging remain viable and attractive explanations for determining the rate of aging in a wide range of species, neither hypothesis has been conclusively proven (2–4).

Surprisingly relatively little is known regarding what regulates the intrinsic metabolic rate of an organism. Similarly on a cellular level the molecular regulation of mitochondrial activity is incompletely understood. Resting oxygen consumption presumably is set at a point to meet overall energetic demands. Nonetheless for most cells this basal respiration is considerably below the maximum oxidative capacity of the mitochondria. The maximal oxidative or ATP synthetic capacity can be easily assessed by treating the cell with various chemical uncouplers and thereby producing the maximal degree of respiration. It is important to note that although mitochondria are the most efficient generators of ATP, cells can also produce ATP through the cytosolic metabolism of glucose. This cytosolic process is particularly important during hypoxic conditions. Nonetheless even under normal aerobic laboratory conditions, most cultured cells rely on both aerobic mitochondrial metabolism and aerobic glycolysis to generate their basal ATP needs. Indeed for many cells in culture these two sources of ATP generation each appear to contribute to roughly half of the overall ATP supply (5). It is presently unclear what determines the relative balance between cytosolic glycolysis and mitochondrial metabolism and why cells with significant unused mitochondrial capacity rely so heavily on less efficient non-mitochondrial energy sources.

The use of lower organisms including yeast, flies, and worms has provided significant insight into the molecular mechanisms underlying aging. Both genetic and environmental interventions have been studied as potential modifiers of life span. Interestingly in simple organisms, structural mutations in mitochondrial subunits or knockdown of mitochondrial components has been shown to significantly alter life span albeit in both positive and negative directions (4, 6–9). Caloric restriction is another non-genetic intervention that appears to be effective in extending life span in a wide range of organisms (10). The molecular basis for the antiaging effects of caloric restriction remains incompletely understood. In the budding yeast Saccharomyces cerevisiae, reducing the glucose concentration in the culture medium can increase the replicative life span of the organism by 20–40% (11, 12). Using a collection of yeast strains harboring...
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single gene deletions, it was shown recently that the life span extension occurring with very low extracellular glucose requires the target of rapamycin (TOR) pathway (13). A subsequent study suggested that this pathway is also involved in the chronological life span of yeast (14). These results are also supported by earlier observations in Caenorhabditis elegans and Drosophila where it was demonstrated that RNAi-mediated knockdown of the TOR pathway can lead to life span extension in these organisms (15, 16).

The TOR pathway is a well-conserved pathway from yeast to mammalian cells (17, 18). Evidence suggests that this pathway is sensitive to both the energetic supply and demand of the cell. TOR itself is a large serine/threonine protein kinase of ~280 kDa and forms a multisubunit complex with numerous protein partners. In mammalian cells two distinct complexes have been identified: mTORC1 in which mTOR is bound to the protein partner raptor and mTORC2 in which mTOR is bound to another protein partner called rictor (17, 18). These protein complexes appear to have distinct biological functions. Given the role of metabolism in aging and the role of the TOR pathway in life span determination, in this study we sought to understand the role of mTOR in regulating mitochondrial function and activity.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Jurkat T cell leukemia clone E6-1 (ATCC, Manassas, VA) was maintained in RPMI 1640 medium containing 10% fetal calf serum; HEK-293T (ATCC) was maintained in Dulbecco’s modified Eagle’s medium containing 10% calf serum. In general, cell sorting was performed in Hanks’ balanced salt solution. Nucleofection of small interfering RNA (non-targeting control 2, TSC2, S6K, raptor, and rictor small interfering RNA from Dharmacon, Lafayette, CO) was performed according to the manufacturer’s protocol (Nucleofector kit V, Amaza, Gaithersburg, MD), and cells were routinely analyzed 72 h after transfection. Using a control expression plasmid encoding for green fluorescent protein we could demonstrate that ~80% of Jurkat and HEK-293T cells could be transfected using this electroporation strategy. Where indicated, cells were incubated with either carbonyl cyanide-3-chlorophenylhydrazone (FCCP; 10 μM) or oligomycin (0.2 μg/ml), or rapamycin (10 μM), all from Sigma.

Immunoblotting, Immunoprecipitation, and Isolation of Mitochondria—Antibodies for immunoblotting were as follows: mTOR, raptor, phospho-p70 S6K1 (Thr-421/Ser-424), p70 S6K1, phospho-S6 (phosphorylated ribosomal protein S6) (Ser-235/236), S6, phospho-4E-BP1 (Thr-37/46), 4E-BP1, and p70 S6K1, phospho-S6 (phosphorylated ribosomal protein S6). Immunoprecipitations were performed with the FACSCalibur cytometer (BD Biosciences Immunocytometry Systems). Excitation light source was 15 milliwatts from an argon laser tuned to 488 nm.

For sorting of cells with different mitochondrial membrane potential, Jurkat cells were sorted into three pools corresponding to the cells with the lowest (5%), median (10%), and highest (5%) TMRM fluorescence. For fluorescence determination of mitochondrial mass, cells were stained with MitoTracker Deep Red 633 (20 nM for 15 min). Cell sorting was performed on a MoFlo flow cytometer (Dako Cytomation, Glostrup, Denmark). Mitochondria purification kit for cultured cells (Pierce).

Membrane Potential and Cell Sorting—Membrane potential was assessed using the potentiometric dye tetramethylrhodamine methyl ester (TMRM) at a final concentration of 25 nM for 15 min. At this concentration, significant autoquenching is thought to be minimized (20). Flow cytometric analysis was performed with the FACSCalibur cytometer (BD Biosciences Immunocytometry Systems). Excitation light source was 15 milliwatts from an argon laser tuned to 488 nm.

Oxygon Consumption, Lactate, and ATP Measurements—Oxygen consumption was determined using the BD Oxygen Biosensor System (BD Biosciences) as described previously (21). Cells were resuspended in culture medium and subsequently transferred to a 96-well plate where the same numbers of cells (106) were placed in each well. Levels of oxygen consumption were measured under base-line conditions and in the presence of FCCP (1 μM) or oligomycin (0.2 μg/ml). Fluorescence was recorded using a GENios multimode reader (Tecan, San Jose, CA) at 2-min intervals for 2 h at an excitation of 485 nm and emission of 630 nm. For semiquantitative data analysis the maximum slope of fluorescence units/s was used and converted into arbitrary units. The respiration rates shown are triplicate determinations of a single experiment that are representative of at least three similar experiments. Alternatively, levels of oxygen consumption were determined using a fiber optic oxygen monitor (Institute Laboratories, Plymouth Meeting, PA) as we have described previously (22). Lactate was measured using the lactate reagent (Trinity Biotech, Bray, Ireland). Simultaneous measurement of oxygen consumption and lactate production allowed for the calculation of the percentage of ATP derived from mitochondrial respiration rates and aerobic glycolysis as described previously (5). ATP was measured using the ATP determination kit (Invitrogen) according to the manufacturer’s recommendations.

Proteomics—Mitochondrial proteins isolated from ~1 × 106 cells were solubilized using 100 μl of 1% SDS (w/v) in Tris buffer (100 mM Tris-HCl, pH 7.0) at 95 °C for 5 min. A chloroform/methylene chloride mixture was added to form a 2:1 ratio, and the sample was vortexed and incubated for 5 min at 4 °C. Proteins were precipitated with acetone, washed, and resuspended in 192 μl of 1% SDS (w/v) in Tris buffer (100 mM Tris-HCl, pH 8.0) containing 50 mM NaCl. These proteins were then dialyzed against the same buffer, diluted 1:1 with 2× SDS sample buffer, and boiled for 5 min prior to loading onto a 4-12% Bis-Tris gel. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes as previously described (19). Membrane proteins were visualized by Ponceau-S staining, quantified by laser densitometry images, and autoradiography performed with [35S]methionine-labeled proteins. The proteins were visualized by Western blot analysis using mouse monoclonal antibodies specific for each protein of interest. Western blot membranes were stained with Ponceau-S and visualized by UV transillumination. The bands were quantified by densitometry and the percentage of total protein derived from each band was determined. The data were analyzed by ANOVA followed by Tukey’s post-hoc test as previously described (19) using the GraphPad software package (San Diego, CA). The appearance of distinct bands was consistent with our previous findings (19).

Oxidative metabolism was measured by determining the percentage of ATP derived from mitochondrial respiration rates and aerobic glycolysis as described previously (5). ATP was measured using the ATP determination kit (Invitrogen) according to the manufacturer's recommendations.

The abbreviations used are: TOR, target of rapamycin; mTOR, mammalian target of rapamycin; raptor, regulatory associated protein of mTOR; rictor, rapamycin-insensitive companion of mTOR; TMRM, tetramethylrhodamine methyl ester; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; 4E-BP1, eukaryotic translation initiation factor eIF4E-binding protein 1; HEK-293T, human embryonic kidney-293T; Cy2, Cy3, or Cy5, cyanine dye 2, 3, or 5; RN1, RNA interference; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; MS, mass spectrometry.
methanol precipitation step was performed to remove salts and lipids (23). The resulting pellet was dried and resuspended in 100 μL of a lysis buffer containing 15 mM Tris-HCl, pH 8.5, 7 M urea, 2 M thiourea, and 4% CHAPS. Fifty micrograms each of the control and rapamycin-treated samples were labeled on lysine residues with Cy5 and Cy3, respectively. As an internal standard, 50 μg of a mixture consisting of 25 μg of the control and 25 μg of rapamycin-treated samples were labeled with Cy2 as described previously (24). The first dimension isoelectric focusing was carried out using the IPG (immobilized pH gradient) strips (pH 3–10 non-linear) for a total of 63 kV-h (Ettan, IPGphor, GE Healthcare). The strips were then loaded onto an Ettan DALT-12 electrophoresis unit (GE Healthcare), and the proteins were separated on a 10–15% SDS-polyacrylamide gel (Jule Inc., Milford, CT) at room temperature for 16 h under a constant voltage (105 V). Image analysis was performed using cross-stain analysis function with Progenesis Discovery software (NonLinear Dynamics, Durham, NC). An automated Ettan Spot Handling Workstation (GE Healthcare) was used for gel spot picking, in-gel protein digestion with trypsin, and spotting on matrix-assisted laser desorption ionization (MALDI) plates as described previously (24). Peptides were analyzed using the Proteomics Analyzer (ABI 4700 MALDI-TOF/TOF). Full scan mass spectrometry (MS) spectra were obtained first followed by MS/MS spectra. Protein identification was carried out using the search engine MASCOT (Matrix Science, Boston, MA). Pro-Q Diamond staining for direct phosphoprotein determination was performed as described recently (25).

RESULTS

We were interested in the role of mTOR in mitochondrial metabolism and therefore sought a system to explore the endogenous variation in metabolic rate for cells in culture. To begin to address this issue, we used fluorescence-activated cell sorting to purify populations of cells based on the measurement of mitochondrial membrane potential using the mitochondrial potential-sensitive dye TMRM. Furthermore because the number of cells that can be obtained by flow
cytometry is limited, we adapted a recently described method that allows for the measurement of oxygen consumption of intact cells plated in microtiter wells (21). This method uses microtiter wells coated with an oxygen-sensitive fluorophore and thus provides a means for real-time measurement of metabolic rates. Such methodology reduced the number of cells required for reliable oxygen consumption by nearly 2 orders of magnitude.

We chose to primarily study a Jurkat T cell line because this cell is routinely grown in suspension and therefore easy to sort and analyze by flow cytometry. Jurkat cells were initially loaded with the mitochondrial potential-sensitive dye TMRM and sorted based on low, medium, or high TMRM fluorescence (Fig. 1A). We next used a miniaturized assay to measure oxygen consumption. Approximately $1 \times 10^6$ M. Fl. cytometer-sorted M. Fl. cells were transferred to individual microtiter wells, and the oxygen consumption of intact cells was measured under normal growth conditions or in the same media supplemented with oligomycin (to assess respiratory leak) or FCCP (to measure maximal oxidative capacity). As seen in Fig. 1B, there was a dramatic difference in the basal metabolic rate as well as the oxidative capacity of these differentially sorted cells. This microtiter-based technique does not allow for absolute quantification, and therefore oxygen consumption is expressed in terms of relative fluorescence units/s. Nonetheless when we compared the relative metabolic rate of unsorted Jurkat cells using the microtiter plate format with conventional assays using an oxygen electrode we saw a good concordance between these methods (Fig. 1, C and D). The basal level of ATP was also slightly higher in the $\Delta \psi_m$ low cells (relative increase to $\Delta \psi_m$: $19.2 \pm 1.01\%$, n = 3, p < 0.01).

Based on these results we next asked whether there was any evidence that levels of mTOR activity might be correlated to these observed differences in endogenous oxygen consumption and oxidative

**FIGURE 2.** mTOR-raptor complex formation and mTOR activity correlate with mitochondrial metabolism. A, analysis of total mTOR and raptor levels in cells sorted for low (L), medium (M), or high (H) TMRM fluorescence. B, corresponding levels of mTOR-raptor complex formation in these three metabolic conditions. Extracts were first immunoprecipitated (IP) for mTOR and then analyzed by Western blot for either mTOR or raptor. C, assessment of mTOR activity as determined by the degree of phosphorylation of S6K1, S6, and 4E-BP1. Phosphorylation-specific antibodies (p) and total protein levels are shown for low, medium, and high TMRM fluorescent cells.

**FIGURE 3.** Rapamycin regulates mitochondrial activity. A, treatment with rapamycin inhibits mTOR-raptor complex formation in Jurkat cells. B, rapamycin treatment reduces mitochondrial membrane potential. C, levels of oxygen consumption and oxidative capacity in cells grown in normal media (–) or in the presence of rapamycin for 12 h. FU, fluorescence units; IP, immunoprecipitated; RAP, rapamycin.
capacity. As seen in Fig. 2A, there were no differences in the absolute level of mTOR or the mTOR-interacting protein raptor in our sorted Jurkat cell populations. In contrast, we did observe that the degree of mTOR-raptor complex formation tightly correlated with the observed metabolic parameters (Fig. 2B). Similarly the level of phosphorylation of known mTOR targets such as p70 S6 kinase (S6K1) and 4E-BP1 appeared to also correlate with the observed metabolic phenotype (Fig. 2C).

To further test whether mTOR-raptor association was an important regulator of mitochondrial metabolism we made use of rapamycin, a specific pharmacological inhibitor of mTOR-raptor complex formation (Fig. 3A). The dissociation of mTOR-raptor complexes with rapamycin is consistent with past observations (26, 27). Consistent with a role for the mTOR-raptor complex in regulating mitochondrial function, treatment of Jurkat cells with rapamycin resulted in a significant decrease in mitochondrial membrane potential (Fig. 3B). Similarly measurement of oxygen consumption and oxidative capacity of intact Jurkat cells with and without rapamycin treatment revealed that inhibiting mTOR-raptor complex formation dramatically altered metabolic rate and reduced overall oxidative capacity (Fig. 3C).

Oxidative capacity is thought to represent an intrinsic property of the mitochondria suggesting that mTOR could potentially directly interact with the mitochondria. Interestingly the subcellular localization of mTOR is not well characterized, although a previous report has demonstrated that a fraction of mTOR directly associates with the mitochondria (28). Consist-

FIGURE 4. Association of mTOR-raptor with the mitochondria. A, cell fractionation into cytosolic (C) or mitochondrial (M) fractions demonstrates that mTOR and raptor are contained within the mitochondria subcellular fraction. Purity of the fractions was assessed by Western blotting for the cytosolic tubulin or the mitochondrial VDAC1/porin gene product. B, detection of intact mTOR-raptor complexes within the mitochondrial fraction. C, two-dimensional difference gel electrophoresis analysis of mitochondrial proteins isolated from control (labeled red, Cy5) and rapamycin (labeled green, Cy3)-treated cells. D, nine proteins demonstrating noticeable isoelectric shifts following rapamycin treatment were selected and identified by mass spectrometry. E, two proteins identified by mass spectrometry exhibiting decreased Pro-Q Diamond staining after rapamycin treatment. In this assay, the degree of phosphorylation is proportional to the spot intensity, which is reduced in the rapamycin-treated samples. Top panel is HSP 90, and bottom panel is VDAC1. IP, immunoprecipitated; RAP, rapamycin.
ent with that report, in our cells, fractionation experiments revealed that mTOR and raptor could both be identified in the mitochondrial fraction (Fig. 4A). Furthermore we could also immunoprecipitate intact mTOR-raptor complexes from both the cytosolic and mitochondrial fractions (Fig. 4B).

Given that the mTOR-raptor complex appears to physically associate with the mitochondria and the stability of the mTOR-raptor complex correlates with mitochondrial activity, we next sought to assess whether we could observe any direct biochemical changes to the mitochondria following disruption of this complex. We therefore performed comparative proteomic analysis using two-dimensional difference gel electrophoresis of mitochondria extracts from untreated and rapamycin-treated cells. Over 1300 presumptive mitochondrial proteins could be resolved by this strategy. Comparison of control and rapamycin samples revealed no large difference in the level of proteins associated with oxidative phosphorylation or substrate oxidation. This similarity in enzyme content between control and rapamycin-treated samples suggested that the decrease in ATP synthetic capacity observed following rapamycin treatment (Fig. 3C) was most likely due to post-translational modifications. In support of this notion, a number of proteins appeared to undergo shifts in their isoelectric focusing point consistent with a change in phosphorylation status (Fig. 4, C and D). Many of the proteins exhibiting differences in their isoelectric focusing point following rapamycin treatment could be identified by subsequent mass spectroscopic analysis. The proteins identified appeared to be enriched for enzymes involved in either intermediary metabolism or electron transport (Fig. 4D). Rapamycin treatment appeared to uniformly involve isoelectric shifts consistent with the dephosphorylation of these metabolic proteins, a result also consistent with the known role of rapamycin as an mTOR kinase inhibitor. Nonetheless it is currently unclear whether the proteins identified represent direct or indirect targets of mTOR or in fact whether these shifts represent changes in phosphorylation or oxidation. However, the supposition that these isoelectric shifts represented dephosphorylation of numerous mitochondrial proteins was further supported by Pro-Q Diamond staining of additional two-dimensional gels obtained from mitochondria extracts derived from untreated and rapamycin-treated cells. The intensity of the Pro-Q Diamond stain has been shown previously to correlate with the degree of protein phosphorylation (25, 29, 30). Mass spectrometry allowed us to again identify a number of individual Pro-Q Diamond-positive proteins, and as before, treatment with rapamycin appeared to reduce the level of phosphorylation (Fig. 4E).

Although rapamycin is viewed as a specific inhibitor of the mTOR-raptor complex, we sought additional and complementary methods to alter the level of complex formation to assess the regulatory role of mTOR activity on mitochondrial function. We first made use of previous observations demonstrating that mTOR activity is negatively regulated by the TSC2 gene product (17, 18, 31). Transient expression of RNAi specific for TSC2 resulted in a significant decrease in TSC2 protein levels without affecting the levels of either mTOR or raptor (Fig. 5A). Consistent with previous results, TSC2 knockdown also augmented S6K activity (17, 18, 30). In our cell type, however, TSC2 knockdown did

**FIGURE 5.** Knockdown of TSC2 regulates mitochondrial activity. A, efficacy of TSC2 knockdown was assessed by Western blot analysis demonstrating reduced TSC2 expression and activation of S6K1 but not 4E-BP1. B, TSC2 knockdown results in increased mTOR-raptor complex formation. Shown is one representative Western blot from three similar experiments. C, determination of basal oxygen consumption (shaded bars) and oxidative capacity (open bars) in control transfected (non-targeting) and TSC2-targeted cells. D, levels of mTOR and the targeted TSC2 protein in transiently transfected HEK-293T cells. E, oxygen consumption under basal conditions (black bar) or in the presence of FCCP (open bar) in either control transfected (non-targeting sequence) or TSC2 RNAi-transfected HEK-293T cells. RFU, relative fluorescent units; IP, immunoprecipitated.
FIGURE 6. Metabolic effect of RNAi knockdown of mTOR components. A, Western blot analysis of non-targeted and S6K-targeted Jurkat cells. B, corresponding basal oxygen consumption (shaded bars) or oxidative capacity (open bars). C, Western blot analysis of non-targeting or raptor-targeted cells. D, corresponding basal oxygen consumption (shaded bars) and oxidative capacity (open bars) demonstrating that knockdown of raptor reduces mitochondrial activity. E, Western blot from non-targeted transfected or rictor RNAi-transfected cells. F, corresponding levels of basal respiration (black bars) and oxidative capacity (open bars) demonstrating that rictor knockdown increases mitochondrial respiration. G, mitochondrial mass as assessed by MitoTracker (MTR) Deep Red fluorescence (relative scale) in cells electroporated with a non-targeting RNAi or an RNAi targeted against TSC2, S6K1, raptor, or rictor. H, corresponding cell cycle distribution of these five different conditions. The cell cycle phases include G1 (Gap 1), S phase (DNA synthesis), and G2/M (Gap 2 and mitosis). FU, fluorescence units; siRNA, small interfering RNA; n.s., not significant; VDAC, VDAC1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
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not appear to affect 4E-BP1 phosphorylation (Fig. 5A). Interestingly TSC2 knockdown also consistently resulted in an ~2-fold increase in mTOR-raptor complex formation (Fig. 5B). Consistent with a role for mTOR in regulating mitochondrial activity, TSC2 knockdown increased basal oxygen consumption as well as oxidative capacity (Fig. 5C). Similar increases in oxygen consumption were also seen following TSC2 knockdown in other cell types (Fig. 5, D and E).

One possible explanation of these results is that mTOR stimulates an increase in energetic demand by augmenting protein translation via an increase in S6K1 activity. In an effort to test this we directly targeted S6K1 by RNAi (Fig. 6). Translation via an increase in S6K1 activity. In an effort to test this intervention had no measurable effect on mTOR activity. Intracellular source of ATP production in non-targeted control cells and TSC2 knockdown and raptor knockdown cells sorted for spontaneous differences in resting mitochondrial metabolism. The second possibility is that mTOR stimulates an increase in energetic demand by augmenting protein translation via an increase in S6K1 activity. In an effort to test this we directly targeted S6K1 by RNAi (Fig. 6). As demonstrated in Fig. 6B, this intervention had no measurable effect on mitochondrial metabolism. In contrast, knockdown of raptor resulted in a decrease in oxygen consumption (Fig. 6, C and D), whereas knockdown of rictor increased oxygen consumption and oxidative capacity (Fig. 6, E and F). Interestingly levels of mTOR-raptor complex were increased over 1.5-fold in rictor knockdown cells suggesting that, as reported previously in other cell types, raptor and rictor might compete for binding to mTOR (19). The alteration in mitochondrial metabolism seen with either TSC2, S6K1, raptor, or rictor knockdown was not associated with significant change in mitochondrial mass as assessed by either VDAC1 levels (see Fig. 6, A, C, and F) or by staining with MitoTracker Deep Red, a membrane potential-independent fluorescent marker of mitochondrial mass (Fig. 6G). Similarly although inhibiting mTOR can cause G1 (Gap 1) delay we observed no alteration in the cell cycle in our various RNAi conditions (Fig. 6H).

We next asked whether the observed perturbations in mitochondrial respiration significantly altered the relative balance of ATP generation. There are two main pathways for ATP generation in mammalian cells: the first is aerobic respiration in the mitochondria, whereas the second involves the metabolism of glucose in the cytosol. In control Jurkat cells, direct and simultaneous measurement of coupled respiration and lactate production revealed that these cells derive a little less than half of their ATP from mitochondrial metabolism (Table 1), a result not atypical for cells maintained in culture (5). Knockdown of TSC2 and raptor appears to alter this balance. In particular, TSC2 knockdown cells derived ~60% of their basal ATP needs from respiration, whereas raptor knockdown cells derived only 30% of their ATP from this source (Fig. 7A).

**DISCUSSION**

In summary, we have provided evidence that mTOR activity and more precisely mTOR-raptor complex formation is tightly correlated with mitochondrial metabolism. In particular, cells sorted for spontaneous differences in resting mitochondrial respiration showed corresponding differences in their mTOR-raptor complexes and mTOR activity. Similarly disrupting mTOR-raptor complexes by either pharmacological means with rapamycin or genetically with RNAi resulted in a corresponding change in metabolism. There are at least two possible explanations for this relationship. The first is that increasing mTOR activity places a significant energetic load on the cell presumably through an increase in protein synthesis, ribosomal biogenesis, etc. that results in a corresponding reactive increase in mitochondrial activity. The second possibility is that mTOR can directly modulate mitochondrial metabolism in what might be described as a feed forward mechanism. Clearly these two possibilities need not be mutually exclusive. Although we cannot conclusively conclude which mechanism predominates we believe that our observations do provide evidence for a feed forward pathway. This primary regulation is supported by three observations. First, mTOR-raptor directly associated with the mitochondria, and disruption of mTOR-raptor resulted in a significant alteration in the mitochondrial phosphoproteome. Second, altering mTOR-raptor did not just affect the load-dependent property of basal oxygen consumption but also the total oxidative capacity of the cell, a measurement that is believed to reflect an intrinsic mitochondrial property. Finally if the alteration in

**TABLE 1**

| O2 consumption | Lactate production | Total ATP | Percentage of ATP production from Respiration | Glycolysis |
|---------------|-------------------|-----------|---------------------------------------------|-----------|
| nmol/min/10^8 | nmol/min/10^8     | nmol/min/10^8 | %                                          | %         |
| 5.47 ± 0.71   | 26.1 ± 0.73       | 70.0 ± 2.48 | 44                                          | 56        |

**FIGURE 7.** mTOR and mitochondrial metabolism. A, the balance of ATP production is regulated by mTOR activity. Intracellular source of ATP production in non-targeted control cells and TSC2 knockdown and raptor knockdown cells is shown. Percentage of ATP derived from aerobic respiration is shown with hatched bars, and that from cytosolic glycolysis is shown with stippled bars. B, model of mTOR-raptor interaction with mitochondria. An increase in mTOR-raptor association augments mitochondrial function through an S6K1 and 4E-BP1-independent pathway. Mitochondrial dysfunction via a retrograde pathway has also been demonstrated previously to stabilize the mTOR-raptor complex suggesting an apparent homeostatic loop.
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Mitochondrial metabolism were merely secondary to changes in energetic demand we would not expect the relative balance of aerobic to glycolytic ATP generation to be altered. Rather we would expect that if cellular demand went up or down mitochondrial metabolism and cytosolic glycolysis would go correspondingly up or down but do so while maintaining their initial balance. Although our oxygen consumption measurements have limitations with regard to quantification, our data (Fig. 7A) do suggest that knockdown of raptor and TSC2 appears to alter the relative contribution of cytosolic glycolysis to mitochondrial metabolism. These observations, in our view, further support a primary regulatory role of mTOR on mitochondrial activity.

We performed our respiration experiments using intact cells, whereas the majority of previous studies have used isolated mitochondria. One somewhat surprising aspect of our data is that in our sorted cells the level of aerobic ATP production (i.e. oligomycin-sensitive oxygen consumption) and the mitochondrial membrane potential positively correlate (Fig. 1B). This is somewhat unusual because most events that increase the synthesis of ATP in isolated mitochondria that act solely through the F1,F0-ATPase result in a lowering of membrane potential. A higher mitochondrial membrane potential in the face of higher respiration supports the notion that the overall capacity of the mitochondria to produce ATP has been enhanced. This supposition is also supported by our observation that oxidative capacity (maximal FCCP-stimulated respiration) increased as a function of mitochondrial membrane potential. We believe these observations highlight the potential utility of studying intact cells with intrinsic differences in endogenous mitochondrial activity and capacity rather than isolating and pooling mitochondria from metabolically heterogeneous tissues.

Although both S6K1 and 4E-BP1 are important downstream effectors of the mTOR pathway, our data demonstrate that the ability of mTOR to regulate oxygen consumption and oxidative capacity appears to be independent of these known effectors. For instance, although TSC2 knockdown increased oxygen consumption it did not appear in Jurkat cells to significantly affect 4E-BP1. Similarly knockdown of S6K1 had no effect on overall oxygen consumption. Interestingly a recent report has demonstrated that S6K1−/− mice have a 5-fold increase in their rate of lipolysis. This large increase in substrate supply, as well as an increase in uncoupling, led to an observed 27% increase in total body oxygen consumption in these mice (32). As such, it is important to emphasize that observed in vivo metabolic rate reflects both intrinsic cellular parameters as measured here including mitochondrial number, activity, and coupling as well as a variety of extrinsic factors (nutrient supply, temperature, and overall demand).

Previous results have suggested that perturbing mitochondrial function can influence mTOR activity by a property known as retrograde signaling (17, 18, 25, 26). We confirm that this pathway is also operational in Jurkat cells as agents that lower mitochondrial membrane potential rapidly inactivated mTOR activity (data not shown). Our rapamycin and RNAi results suggest, however, that the reciprocal regulation is also operative. As such, these results suggest that mitochondria can signal to mTOR and that mTOR may in turn regulate mitochondrial activity (Fig. 7B). It is possible that such a homeostatic loop may be essential for coordinating energy supply and demand during complicated and important mTOR-regulated processes such as cell cycle progression.

Finally there is growing evidence that mitochondrial metabolism may play an important role in aging (2–4). Previous reports have indicated that in yeast, C. elegans, and Drosophila, inhibition of the TOR pathway results in life extension (13–16). An analysis of a large number of life-extending mutations in C. elegans has led to a hypothesis that these diverse genetic alterations may all function by inducing energetic shifts away from the mitochondria and toward alternative ATP-generating pathways (33). Our observation that mTOR activity tightly correlates with oxygen consumption and serves to regulate the balance between glycolysis and aerobic metabolism appears consistent with that theoretical framework.

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