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Interaction of polyamines and mTOR signaling in the synthesis of antizyme (AZ)

Ramesh M. Ray, **Mitul Bavaria**, and **Leonard R. Johnson**

Department of Physiology, University of Tennessee Health Science Center, Memphis, TN 38163, USA

Abstract

Tissue polyamine levels are largely determined by the activity of ornithine decarboxylase (ODC, EC 4.1.17), which catalyzes the conversion of ornithine to the diamine putrescine. The activity of the enzyme is primarily regulated by a negative feedback mechanism involving ODC antizyme (AZ). Our previous studies demonstrated that AZ synthesis is stimulated by the absence of amino acids, the levels of which are sensed by the mTOR complex containing TORC1, which is stimulated by amino acids and inhibited by their absence, and TORC2 the function of which is not well defined. Polyamines, which cause a +1 ribosomal frameshift during the translation of AZ mRNA are required to increase AZ synthesis in both the presence and absence of amino acids. Amino acid starvation increases TORC2 activity. We have demonstrated that mTORC2 activity is necessary for AZ synthesis in the absence of amino acids. Tuberous sclerosis protein (TSC), a negative regulator of mTOR function regulates the activities of both the TORC1 and TORC2. TSC2 knockdown increased mTORC1 activity with concomitant inhibition of mTORC2 activity eliminating AZ induction in the absence of amino acids as well as that induced by spermidine. Thus, these results clearly demonstrate that in addition to polyamines, mTORC2 activity is necessary for AZ synthesis. Moreover, our results support a role for mTORC2 in the synthesis of a specific protein, AZ, which regulates growth of intestinal epithelial cells.

Corresponding Author: Ramesh M. Ray, Department of Physiology, 894 Union Avenue, Memphis, TN38163, rray3@uthsc.edu, Phone: 901 448 7168, Fax: 901 448 7126.

Author contribution

R. M. Ray designed the study, planned and performed experiments, analyzed and interpreted data, and wrote manuscript; M. Bavaria performed experiments, analyzed data and drafted the materials and methods section and figure legends; L.R. Johnson designed the study, interpreted experiments, and wrote the manuscript together with R. M. Ray.

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Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

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Keywords

Spermidine; ornithine decarboxylase; amino acids; TSC2; AKT; p70S6 kinase; Mouse embryonic fibroblasts; mTORC1/2

1. Introduction

Cell growth and proliferation are regulated by the integration of multiple signals, such as nutrients, hormones, and growth factors. The mechanistic target of rapamycin (mTOR) plays a central role in regulating these signal transduction pathways (1). mTOR forms two distinct complexes named mTOR complex 1 (mTORC1) and complex 2 (mTORC2). mTORC1 consists of mTOR, raptor, and mLST8 and is sensitive to inhibition by rapamycin. While mTORC2 components include mTOR, RICTOR (Rapamycin Insensitive Component of TOR), Sin1, and mLST8 (2–6). The central role of mTOR in protein synthesis is attributed to mTORC1, but increasing evidence suggests a role for mTORC2 in this basic cellular function (7). Nutrients, such as glucose and amino acids, regulate mTORC1 activity, but their role in mTORC2 function is undefined.

The polyamines putrescine, spermidine, and spermine are essential for cell growth and are elevated in many types of cancer (8–10). Polyamines have been shown to regulate cell proliferation, apoptosis and cell migration, and thereby, tissue homeostasis (8, 11–13). Tissue polyamine levels are determined in large part by the activity of ornithine decarboxylase (ODC, EC 4.1.17), which catalyzes the conversion of ornithine to the diamine putrescine, the first rate-limiting step in polyamine synthesis. ODC activity itself is highly controlled, increasing dramatically from basal levels in response to tissue damage, growth factors, trophic hormones and other proliferative stimuli (14,15). The activity of the enzyme is primarily regulated by a negative feedback mechanism involving ODC antizyme (AZ) (16). AZ levels increase via a frameshifting mechanism that is triggered by increasing tissue polyamine levels (17–19). Optimal intracellular polyamine levels are maintained by tightly regulated synthesis, degradation, uptake, and release (20, 21). We have shown that amino acid starvation, produced by incubating cells in a glucose salt solution (EBSS), or the addition of putrescine to EBSS induced AZ1 within 2h, and that ASN prevented the induction of AZ synthesis in both cases (22). Furthermore, amino acids like GLN and ASN inhibited, while LYS, VAL, and ORN increased AZ in EBSS. Recently we have established that among the three polyamines, spermidine is a direct and physiological regulator of AZ synthesis (23).

Various growth promoting proteins, cyclin D1 (24), aurora-A (25), and smad1 (26) are targeted for destruction by AZ. In addition, the antiapoptotic delta Np73 (DNp73) is degraded through the AZ-mediated pathway (27). These reports strongly indicated a role for AZ in regulating the growth of mammalian cells. Since AZ regulates intracellular polyamine levels by inhibiting ODC activity and the cellular uptake of polyamines, the question arises as to whether the effects of AZ on growth are direct or mediated through polyamines. We recently showed that AZ inhibits proliferation independently of the levels of polyamines (28) and that AZ is not only regulated by polyamine mediated ribosomal frameshifting but

also by amino acids through mTORC2 activity (22). However, the relationship between polyamines and mTOR activity is unclear.

Our previous studies demonstrated that AZ synthesis occurred in the absence of amino acids, and thereby, in the absence of mTORC1 activity but required mTORC2 activity in the presence of basal levels of intracellular polyamines (22). Furthermore, polyamines were absolutely required for the induction of AZ both in the presence and absence of amino acids (23). In the current study, we determined the role of mTORC1, mTORC2 and polyamines in AZ synthesis. Interestingly, both spermidine and spermine increased mTORC1 activity in the absence of amino acids. We previously have shown that mTORC2 activity is necessary for AZ synthesis in the absence of amino acids. In addition, TSC2 (Tuberous sclerosis complex 2) inhibition increased mTORC1 activity with concomitant inhibition of mTORC2 activity and eliminated AZ induction in the absence of amino acids as well as that induced by spermidine. Thus, these results demonstrate that in addition to polyamines, mTORC2 activity is necessary for AZ synthesis. Moreover, our results support roles for mTORC2 and PI3 kinase in the synthesis of a specific protein, AZ, which regulates the growth of intestinal epithelial cells.

2. Materials and Methods

2.1. Materials

Cell culture medium and fetal bovine serum (FBS) were obtained from Mediatech (Herndon, VA). Dialyzed FBS (dFBS), Putrescine, Spermidine, Spermine, L-Asparagine, and mouse monoclonal anti-actin antibody were purchased from Sigma (St. Louis, MO), and trypsin-EDTA, antibiotics, and insulin were from GIBCO-BRL (Grand Island, NY). Rabbit polyclonal anti-AZ1 antibody was a gift from Dr. Senya Matsufuji. Anti-rabbit antibodies for phospho-AKT (Ser473), -AKT, -phospho (Thr371 or Thr389) p70 S6 Kinase, -phospho-S6 Ribosomal protein (Ser235/236), -Phospho-4E-BP1 (Ser65) and -Tuberin were purchased from Cell Signaling Technology (Beverly, MA). Mouse anti-actin antibody was purchased from Millipore (Billerica, MA). Protease inhibitors, phosphatase inhibitors, phosphate buffer saline (PBS), Dulbecco's PBS (DPBS), Bicinchoninic acid (BCA), and Mammalian Protein Extraction Reagent (MPER) were purchased from Thermo Fisher Scientific (Rockford, IL). The enhanced chemiluminescence substrate Western Lightning TM (ECL) was purchased from PerkinElmer Life and Analytical Sciences (Shelton, CT). DFMO was a gift from ILEX Oncology (San Antonio, TX). Disposable culture ware was purchased from Corning Glass.

2.2. Cell culture

The IEC-6 cell line was obtained from the American Type Culture Collection (Manassas, VA) at passage 13. The stock was maintained in T-150 flasks in a humidified, 37°C incubator in an atmosphere of 90% air-10% CO_2. The medium consisted of Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL) with 5% heat-inactivated FBS, 10μg/ml insulin, and 50-μg/ml gentamicin. The stock was passaged weekly and fed three times per week. Passages 15–20 were used in the experiments (23, 29, 30).

TSC1- and 2-null mouse embryonic fibroblasts (MEFs) from Dr. David Kwiatkowski at Brigham and Women's Hospital have been described earlier (31). WT ($TSC2^{+/+}$) and knock-out ($TSC2^{-/-}$) MEFs were maintained in T-150 flasks in DMEM supplemented with 5% dFBS, Penstrep with glutamine at 37°C and 5% CO_2 (31). Stock cells were passaged once a week, and medium was changed three times a week. Before an experiment, cells were trypsinized, counted using a Beckman Coulter counter, and grown for 3 days followed by serum starvation for 24 h. Serum starved cells were exposed to serum free DMEM with or without amino acids in the presence or absence of spermidine for 4h.

2.3. Experimental protocol

For most experiments, the cells were taken up with 0.05% trypsin plus 0.53 mM EDTA in Hanks' balanced salt solution without Ca^{2+} and Mg^{2+}. They were counted and plated (day 0) at 6.25×10^4 cells/cm^2 in DMEM plus 5% dialyzed FBS, 10 μg insulin, and 50 μg gentamicin sulfate/ml with or without DFMO (5mM). Cells were fed on day 2 with respective growth media. On day 3, the medium was removed, the cells were washed once with Hanks' balanced salt solution, and the medium was replaced with serum-free DMEM containing 10 μg/ml insulin and 50 μg/ml gentamicin. On day 4, the medium was removed, and the cells were washed once with Hanks' balanced salt solution followed by the addition of serum free medium with (AA+) or without amino acids (AA−) and the appropriate treatment. Putrescine (10 μM), spermidine (5 μM), spermine (5 μM) was used in those concentrations unless stated otherwise. In experiments involving Rapamycin, PP242 and LY294002 cells were preincubated (30–60 min) in serum free DMEM, washed once with HBSS and then exposed to AA+ or AA− DMEM containing inhibitors and/or the indicated amounts of putrescine or spermidine for 4h.

2.4. Western blot analysis

At the end of an experiment, cells were washed twice with DPBS. The DPBS was removed, 500 μl of MPER containing protease and phosphatase inhibitors was added, and the plates were frozen overnight and scraped. The cell lysate was centrifuged at 10,000 rpm for 10 min and the supernatant was used to determine the protein concentration by the BCA method. Total cell protein (30 μg) was separated on 12% or 15% SDS-PAGE and transferred to a PVDF membrane for Western blotting with a specific primary antibody (dilution 1:1000 in PBS/5% BSA) and appropriate secondary antibody (dilution 1: 10,000 in PBS/5% BSA) labeled with horseradish peroxidase (HRP). Immune complexes were detected by chemiluminescence system (23, 29,30).

2.5. Statistical analysis

All data are expressed as mean +/− SE. Experiments were repeated three times. Representative Western blots from three experiments are shown. ANOVA and appropriate post-hoc testing determined the significance of the differences. Values of $P<0.05$ were regarded as significant.

3. Results

3.1. Polyamines are necessary for AZ synthesis in the absence of amino acids

We have shown that AZ synthesis is induced in response to amino acid starvation (22) and that spermidine induced AZ in the presence of amino acids (23). However, it is unclear whether polyamines are necessary for AZ induction during amino acid starvation. To study this, IEC-6 cells grown in the presence of DFMO for 4 days (depleted of intracellular polyamines) were exposed to DMEM with (AA+) or without (AA−) amino acids for 4h. Cell extracts were analyzed by western blot to determine mTORC1 and mTORC2 activities. Activation of mTORC1 was predicted based on the levels of p70S6 kinase phosphorylation on threonine 389 (p-p70S6K-Thr389) and the subsequent phosphorylation of its downstream target, ribosomal protein S6 (p-S6Rbp- Ser235/Ser236). Phosphorylation of AKT-Ser473 was considered as an index of mTORC2 activity. Results in Fig. 1A show a robust p70S6K activity leading to phosphorylation of S6Rbp in control cells, which was completely eliminated by amino acid starvation. Polyamine depleted cells (DFMO) had a similar response to amino acid starvation. Amino acid starvation increased mTORC2 activity as indicated by increased AKT phosphorylation. Importantly, polyamine depleted cells had higher mTORC2 activity indicated by increased p-AKT (Ser473) compared to control cells in response to amino acid starvation. Robust AZ synthesis was detected in control cells in response to amino acid starvation. Although, polyamine depleted cells had higher mTORC2 activity, amino acid starvation failed to induce AZ in these cells. These results indicate that polyamines are necessary for AZ induction. To establish the requirement of polyamines for AZ synthesis, cells depleted of polyamines were exposed to putrescine (5 μM) for 4h in the presence (AA+) or absence (AA−) of amino acids. Putrescine induced AZ in polyamine depleted (DFMO) cells, but the effect was much greater in the absence of amino acids (Fig. 1B). mTORC2 activity was approximately the same in the presence and absence of putrescine. Putrescine slightly attenuated the response to amino acid starvation as indicated by residual activity of mTORC1 as detected by pS6Rbp levels (Fig. 1B, lanes 2 and 4). These results confirmed the requirement for polyamines for AZ induction in response to amino acid starvation and suggested that polyamines may modulate mTOR function.

3.2. Polyamines and mTOR activity

The mechanistic target of rapamycin (mTOR) senses the presence of nutrients and energy status to control protein synthesis (1, 32, 33). Since AZ synthesis during amino acid starvation required polyamines, it is likely that polyamines might act on the mTOR complex to influence AZ synthesis. Serum starved IEC-6 cells were exposed to putrescine, spermidine or spermine for 4h in the absence of amino acids. Results in Fig. 2A show that all three polyamines increased mTORC2 activity as judged by AKT-Ser473 phosphorylation. As expected, amino acid starvation decreased p70S6K activity compared to that seen in the presence of amino acids (Fig. 2A, p-p70S6K-Thr389 and p-S6Rbp). Addition of putrescine (5 μM) during amino acid starvation (AA−) had no effect on mTORC1 activity (Fig. 2A, lane 3). However, spermidine and spermine prevented the decrease in p70S6 kinase activity as seen by the levels of p-S6Rbp in AA− (Fig. 2A). Spermine appears to augment p70S6K activity compared to that seen in AA+ and AA−/SPD groups. Although, spermidine maintained S6Rbp phosphorylation at the level of AA+,

p70S6K activity decreased slightly upon amino acid starvation compared to that seen in the presence of amino acids (Fig. 2A, lane 1). Spermine completely prevented the decrease in p70S6K activity produced by the absence of amino acids. Thus, polyamines appear to control mTORC1 activity in the absence of amino acids. Therefore, we determined whether polyamines stimulated mTORC1 activity or prevented its inactivation. We have observed that while amino acid deprivation has no effect on mTORC2 activity it decreases mTORC1 activity within 1h and that it remains inhibited for up to 4h (data not shown). Serum starved cells were deprived of amino acids for 2h to decrease p70S6K activity and then exposed to polyamines, amino acids or insulin for an additional 2h. Fig. 2B shows that putrescine, spermidine, and spermine added 2h after amino acid starvation caused different responses in mTORC1 activity. Spermine increased activity to a greater extent than spermidine, while putrescine had almost no effect on mTORC1 activity. These results are identical to those shown in Fig. 2A, reaffirming that spermidine and spermine stimulated mTORC1 activity rather than preventing its deactivation in response to amino acid starvation. mTORC2 activity followed the same pattern albeit to a lower extent compared to that seen for mTORC1. Exposure of amino acid deprived cells to medium containing amino acids (2h) stimulated mTORC1 activity while decreasing mTORC2 activity (Fig. 2B, lane 5). Furthermore, insulin, a known modulator of mTOR function, stimulated mTORC2 activity but failed to induce mTORC1 activity (Fig. 2B, lane 6), indicating the requirement of amino acids for the stimulation of mTORC1 activity. Increased mTORC2 activity by insulin and the absence of mTORC1 activity restored AZ synthesis to the level seen in the absence of amino acids (data not shown). Thus, AZ is synthesized in the absence of mTORC1 activity (AA−) and in the presence of basal levels of intracellular polyamines and mTORC2 activity. Consistent with our previous results, both putrescine and spermidine increased AZ synthesis (23). Interestingly, spermine failed to increase AZ, which correlated with increased p70S6K activity in the absence of amino acids. Thus, it appears that increased p70S6K and, thereby, mTORC1 activity may counteract the effects of amino acid starvation, and decrease AZ synthesis. Furthermore, in polyamine depleted cells, putrescine failed to induce AZ (Fig. 3A, lane 2) in the presence of amino acids (AA+), while it robustly induced AZ in the absence of amino acids (AA−) (Fig. 3B, lane 2). Interestingly, spermine prevented mTORC2 activity in cells depleted of polyamines and completely blocked AZ induction in the absence of amino acids (Fig. 3B, lane 4). These results suggest that spermidine efficiently induces AZ with basal TORC2 activity independently of the status of mTORC1, suggesting that mTORC2 plays a crucial role in AZ synthesis.

3.3. Role of mTORC1 and mTORC2 in AZ synthesis

We have shown that rapamycin treatment for 4h failed to inhibit AZ synthesis in AA− medium, however, it mimicked amino acid starvation when added to cells incubated in AA+ medium and stimulated AZ synthesis (22). p70S6K and AKT regulate the activities of RICTOR and TSC1/TSC2, and thereby, mTORC1 and mTORC2 complex formation. Prolonged inhibition of mTORC1 by rapamycin is known to influence mTORC2 activity. Since putrescine failed to induce AZ in polyamine depleted cells containing higher mTORC1 activity (Fig. 3A, lane 2), we speculated that inhibition of mTORC1 by overnight treatment with rapamycin may allow putrescine to increase AZ synthesis in polyamine depleted cells. To test this, rapamycin was added in DMEM (AA+) containing DFMO

medium during a 24h period of serum starvation. Cells were washed with EBSS and further incubated in DMEM (AA−)/DFMO in the presence or absence of putrescine and/or rapamycin for an additional 4h. Since rapamycin was prepared in DMSO (vehicle), cells treated with DMSO were considered the control group (V). Cell extracts were analyzed to determine mTORC1 and mTORC2 activities and AZ levels. Results in Fig. 4A show that prolonged rapamycin treatment (24h) completely inhibited mTORC1 activity as seen by the absence of pS6Rbp (Fig. 4A, lane 3) in polyamine-depleted cells exposed to putrescine (4h). Putrescine increased mTORC2 activity (pAKT-Ser473) in rapamycin treated (mTORC1 inhibited) cells compared to that seen with cells exposed to putrescine without rapamycin (Fig. 4A, lane 2). As predicted, prolonged rapamycin treatment mimicked the effects of amino acid starvation enabling the stimulation of AZ synthesis by putrescine in polyamine depleted cells (Fig. 4A, lanes 2 and 3). These results suggested that mTORC2 might be sufficient for AZ induction. To confirm the involvement of mTORC1/2 in the induction of AZ by putrescine, serum starved, polyamine depleted cells were incubated with DMEM (AA−)/DFMO in the presence or absence of either rapamycin (Rapa) or PP242 for 4h. As expected, amino acid starvation abolished p70S6K activity, and putrescine slightly increased p70S6K, which was prevented by both rapamycin and PP242. Interestingly, levels of phosphorylated S6Rbp remained high in cells treated with putrescine and those treated with inhibitors along with putrescine (Fig. 4B, lanes 2–4) indicating a role for phosphatase/s in controlling the levels of phosphorylated Rbp. Notably, short-term (4h) treatment with rapamycin had no effect on putrescine induced AZ levels. Unlike rapamycin, cells treated with PP242 completely inhibited mTORC2 activity as judged by AKT-Ser473 phosphorylation without having any effect on the levels of p-S6-Rbp. The inhibition of mTORC2 by PP242 decreased putrescine-induced AZ synthesis (Fig. 4B, lane 4). These results clearly indicate that mTORC2 activity is required for AZ synthesis and confirms the role of mTORC2 in the regulation of protein translation.

We have shown that spermidine is a direct inducer of AZ and that putrescine is rapidly converted to spermidine in polyamine-depleted cells, allowing it to also induce AZ (23). Therefore, we used spermidine to examine the role of mTORC2 activity on AZ synthesis. Serum starved cells grown in control and DFMO containing medium (polyamine depletion) were pretreated with PP242 in serum free DMEM (AA+) for 1h prior to washing and exposure to DMEM (AA−) with or without spermidine and/or PP242 for 4h. Results in Fig. 5 show that PP242 inhibited basal as well as spermidine induced AZ synthesis in both control and polyamine depleted cells. PP242 completely inhibited mTORC2 mediated AKT-ser473 phosphorylation. Interestingly, mTORC1 activity as judged by phosphorylation of p70S6K and 4EBP1 was also completely inhibited by PP242. However, the phosphorylation of ribosomal protein S6 (S6Rbp), a down stream target of p70S6K was not altered in spermidine treated groups. These results clearly show that decreased AKT activity correlated with inhibition of AZ synthesis. Since mTOR is a member of a family phosphatidylinositol-3-kinase-related PIKKs, we used PI3K inhibitor LY294002 (LY) to determine whether PI3K activity is also necessary for AZ synthesis during amino acid starvation. Amino acid starvation decreased mTORC1 activity, and LY inhibited PI3K mediated AKT activity. LY decreased AZ synthesis in the absence of amino acids, however, to a lesser extent compared to that seen with PP242 (Fig. 6).

Hermatin and Tuberin, products of TSC1 and TSC2 tumor suppressor genes respectively, are important regulators of cell proliferation and tumor development (34). The TSC1-TSC2 complex known to inhibit mTORC1 activity is required for proper activation of mTORC2 (35). TSC2 has been predicted to directly regulate mTORC2 activity (36, 37). Therefore, we used TSC2 knockout (KO, $TSC2^{-/-}$) mouse embryonic fibroblasts (MEFs) to clarify the role of mTORC2 in AZ synthesis. Results in Fig. 7A confirmed the phenotype of these cells. TSC2 knockout cells had higher basal levels of p70S6 kinase activity as judged by the phosphorylation on Ser371 and Thr389 residues and subsequent phosphorylation of its downstream substrate ribosomal protein S6 (S6Rbp). Furthermore, TSC2 knockdown completely prevented mTORC2 activity as seen by complete loss of AKT phosphorylation (p-AKT-Ser473). TSC2 knockdown completely eliminated AZ synthesis in response to amino acid starvation compared to that seen in WT ($TSC^{+/+}$) MEFs. In addition, spermidine induced AZ synthesis in wild type MEFs while it failed to do so in $TSC2^{-/-}$ MEFs (Fig. 7B and C). Together, these results clearly demonstrate that both spermidine and mTORC2 cooperatively regulate AZ synthesis.

4. Discussion

The importance of polyamines (PAs) has been established for both normal and abnormal functions of cells. Spermidine, spermine and their diamine precursor, putrescine, are essential for eukaryotic cell proliferation and the growth of normal tissues (38). Furthermore, cellular functions including trans-membrane ion flux (39), apoptosis (12) and migration (13, 40) either require polyamines or are influenced by them. Numerous studies have affirmed the importance of polyamines to the growth of cancer cells and the effects of polyamine analogs on inhibiting abnormal cell growth (8, 9).

Most studies including our own were carried out using the concept of “polyamine depletion”, where cells are grown in the presence of DFMO, a suicide inhibitor of ODC, which completely depletes intracellular putrescine within 6h and spermidine by 24h, and decreases spermine to 40% by 96h in IEC-6 cells (40). Times for the depletion of polyamines vary among cell types. That the effects of DFMO are due to the depletion of polyamines is shown by the prevention of all effects by the addition of polyamines along with the DFMO. We have shown that addition of either spermidine or spermine along with DFMO significantly increased AZ within few a hours compared to levels seen in cells grown in the absence of added polyamines (23). Increased AZ levels in response to polyamines led to inhibition of growth, which was restored by inhibiting AZ synthesis by asparagine (ASN) in the presence of spermidine or spermine (23). These findings indicate that AZ acts directly to inhibit growth independently of its effects on polyamine levels.

AZ induction by polyamines both in the presence and absence of amino acids and its inhibition by specific amino acids (ASN and GLN) suggest the involvement of both polyamines and mTOR signaling in the regulation of growth by AZ. Furthermore, figure 1 demonstrates that the induction of AZ in cells incubated in the absence of amino acids is accompanied by decreased activity of mTORC1 with a concomitant increase in mTORC2 activity. Although, polyamine depleted cells (Fig. 1A, DFMO) had higher mTORC2 activity compared to that seen in cells grown under control conditions, amino acid starvation failed

to induce AZ in these cells. Addition of putrescine to polyamine depleted cells in the absence of amino acids resulted in a significantly greater induction of AZ compared to that seen in the presence of amino acids (Fig. 1B). These results indicate that both mTORC2 activity and polyamines are necessary for the synthesis of AZ. We have shown that addition of either putrescine or spermidine induced AZ in control cells incubated in AA+ medium, but putrescine failed to do so in polyamine depleted cells (Figs. 2 and 3). Spermidine induced AZ irrespective of the presence or absence of amino acids in polyamine depleted cells (Fig. 3). Surprisingly, spermine prevented mTORC2 activity and AZ synthesis in polyamine-depleted cells undergoing amino acid starvation (Fig. 3B, p-Akt-ser473). These findings indicate that both the polyamines and mTORC2 control AZ synthesis. Although, it is well established that polyamines, specifically, spermidine, induce AZ synthesis through a frameshifting mechanism (41), it is unclear whether polyamines directly alter mTOR activity, and thereby, affect AZ mRNA translation following ribosomal frameshifting.

The mammalian target of rapamycin (mTOR) controls growth and is also known to be deregulated in cancer and diabetes (33, 42, 43). mTORC1 is studied extensively and is regulated by growth factors, hormones, nutrients (amino acids and glucose), and stress stimuli (32, 42, 44). However, we are beginning to understand how mTORC2 can also be triggered by some of these signals. Since mTORC2 is a critical regulator of AKT, which is deregulated in cancer, it is gaining attention as a potential target for cancer therapy (5, 35, 45). Furthermore, the addition of leucine to starved cells increases migration via mTORC2 suggesting that amino acids could activate mTORC2. (46)

While the central role of mTOR in protein synthesis is attributed to mTORC1 (7, 42), increasing evidence suggests a role for mTORC2 in protein translation. mTORC2 mediates AKT phosphorylation by associating with translating ribosomes (45, 47). A recent review by Oh and Jacinto (35) mentions that both mammalian and yeast TORC2 localize with membranes of the endoplasmic reticulum (ER) and the Golgi apparatus. Furthermore, mTORC2 localizes with polysomes and associates with ribosomal proteins, and rictor forms complexes with L23a and L26 ribosomal proteins at the exit tunnel suggesting that mTORC2 could play a role in co-translational processes (48). This review points out that identification of other nascent proteins that require mTORC2 for maturation would provide clues to the function of mTORC2 in ribosomes and in translation complex formation. In this paper, we have defined AZ as a novel target whose synthesis is regulated by mTORC2. Furthermore, the requirement of polyamine-induced frameshifting (18), and mTORC2 activity (22) suggest that mTOR signaling is an integral part of AZ regulation in the absence of amino acids as well as polyamine induced frameshift-mediated AZ synthesis.

mTORC1 signaling is essential for growth and autophagy in response to nutrients and growth factors (1, 44, 49, 50). Signaling through mTORC1 promotes protein synthesis by inhibiting the activity of the translation repressor 4E-BP1 and stimulating the activity of p70S6 kinase (S6K1) (51). We have demonstrated that inhibition of mTORC1 by rapamycin to mimic amino acid starvation eliminated p70S6 kinase (p70S6K) and induced AZ in medium containing amino acids (AA+) similar to that detected in response to amino acid starvation (22). Furthermore, results in Figs. 2 and 3 show that stimulation of AZ synthesis by polyamines in the absence of amino acids is accompanied by increased mTORC1 and

mTORC2 activities. mTORC1 stimulation by spermidine and spermine in the absence of amino acids indicates that polyamines may regulate or control mTOR activity. Our results in Fig. 2B show that among three polyamines, spermine stimulated both mTORC1 and mTORC2 activities, but the degree of stimulation were greater for mTORC1 compared to mTORC2.

We clarified the role of polyamine-induced mTORC1 activity using rapamycin, a specific inhibitor of the mTORC1 complex. Data in Fig. 4 show that rapamycin (4h) had no effect on putrescine induced AZ synthesis in cells depleted of polyamines (Fig. 4B). Furthermore, S6Rbp phosphorylation was not altered in polyamine depleted cells incubated with AA+ medium containing putrescine (Fig. 4A, lanes 1 and 2). However, it decreased during amino acid starvation (Fig. 4B, lane 1) and was restored by putrescine (Fig. 4B, lane 2) to the levels seen in the presence of amino acids (Fig. 4A, lanes 1 and 2). Rapamycin (4h) failed to prevent putrescine induced AZ synthesis as well as phosphorylation of S6Rbp (Fig. 4B, lane 3). Interestingly, the activity of p70S6 kinase was completely inhibited by rapamycin while there was no effect on mTORC2 activity. Prolonged exposure of polyamine depleted cells to rapamycin (24h) completely prevented phosphorylation of S6Rbp, which accompanied the increased mTORC2 activity and AZ synthesis stimulated by putrescine (Fig. 4A, lane 3). The role of mTORC2 is evident from the results in Fig. 4B (lane 4) showing that inhibition of mTORC2 by PP242 decreased putrescine induced AZ synthesis. Unlike putrescine, spermidine restored mTORC1 activity in the absence of amino acids (Fig. 2) and in polyamine depleted (Fig. 5) cells. PP242 prevented increases in both mTORC1 and 2 activities in response to spermidine with concomitant inhibition of AZ synthesis in both control and polyamine depleted cells (Fig. 5). The two-mTOR complexes (mTORC1/2) share components, and increased activity of downstream targets like S6 kinase and AKT controls the activities of both complexes by feedback mechanisms (52, 53). The inhibition of PI3 kinase by LY294002 inhibited AKT activity and AZ synthesis in amino acid starved cells (Fig. 6), further supporting the role of mTORC2 in AZ synthesis. Interestingly, increased phosphorylated ribosomal protein S6 in the absence of S6 kinase activity in cells incubated with putrescine or spermidine suggests that polyamines might directly regulate phosphorylation by preventing the dephosphorylation of pS6Rbp by controlling the activity of PP2A. We have observed p-S6Rbp binding to the catalytic subunit of PP2A (PP2Ac) using a microcystin-sepharose pull down assay (data not shown). Thus, these results suggest that decreased mTORC1 activity led to increased mTORC2 and allowed the synthesis of AZ in polyamine depleted cells. These results clearly show that mTORC2 activity is crucial for the synthesis of AZ in IEC-6 cells.

Recent emerging evidence indicates that disruption of mTORC2, or its inhibition, severely represses translation compared to that seen with rapamycin (48, 54, 55). Embryonic fibroblasts from embryos in which mTORC2 has been knocked out and tissue-specific knockouts of mTORC2 components have provided strong evidence regarding cellular functions and the role of mTORC2 in organ development and tissue homeostasis (56, 57). The TSC1/2 complex, a negative regulator of mTORC1 activity, is required for the activation of mTORC2 (34, 36, 37, 58). The TSC1/2 complex associates with mTORC2 via rictor, and TSC2-deficient cells had increased levels of mTORC1 (59). We confirmed the role of mTORC2 in AZ synthesis using wild type, (WT, $TSC2^{+/+}$) and knockout (KO,

$TSC2^{-/-}$) MEFs. TSC2 KO MEFs had higher S6 kinase activity (p-p70S6K (Ser371/Thr389), p-S6Rbp) but lacked mTORC2 activity (p-AKT-Ser473) (Fig. 7A). It has also been reported that p70S6 kinase mediated phosphorylation of rictor on threonine 1135 inhibits mTORC2 activity (52, 60, 61). Amino acid starvation induced AZ in WT ($TSC^{+/+}$) MEFs while it failed to induce AZ in KO ($TSC2^{-/-}$) MEFS (Fig. 7B). Furthermore, TSC2 knockdown prevented spermidine induced AZ synthesis suggesting that mTORC2 activity might be necessary for the resumption of translation of AZ following ribosomal frameshifting.

5. Conclusions

Our results provide strong evidence supporting the role of mTORC2 in AZ synthesis. We demonstrated that AZ synthesis requires 1) spermidine for frameshifting and 2) mTORC2 activity for translation of AZ mRNA or co-translational stabilization of the AZ protein. Furthermore, our results provide evidence that polyamines like spermidine or spermine at a very low concentration (5 μM) can stimulate mTOR activity in the absence of amino acids. This is important because of the emergence in the importance of mTOR function in growth, diabetes and cancer progression, and that modulators of mTOR function have gained renewed attention for the design of novel treatment strategies.

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Abbreviations

ODC	ornithine decarboxylase
TSC1/2	tuberous sclerosis complex 1/2
mTOR	mechanistic target of rapamycin
DMEM	Dulbecco’s modified eagles medium
DPBS	Dulbecco’s phosphate buffer saline
EBSS	Earle’s balanced salt solution

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Highlights

- Amino acid starvation increases mTORC2 activity and antizyme synthesis.
- Spermidine induced +1 ribosomal frameshifting of AZ mRNA translation requires mTORC2 activity.
- TSC2 knockdown inhibits mTORC2 activity and prevents AZ induction by spermidine.

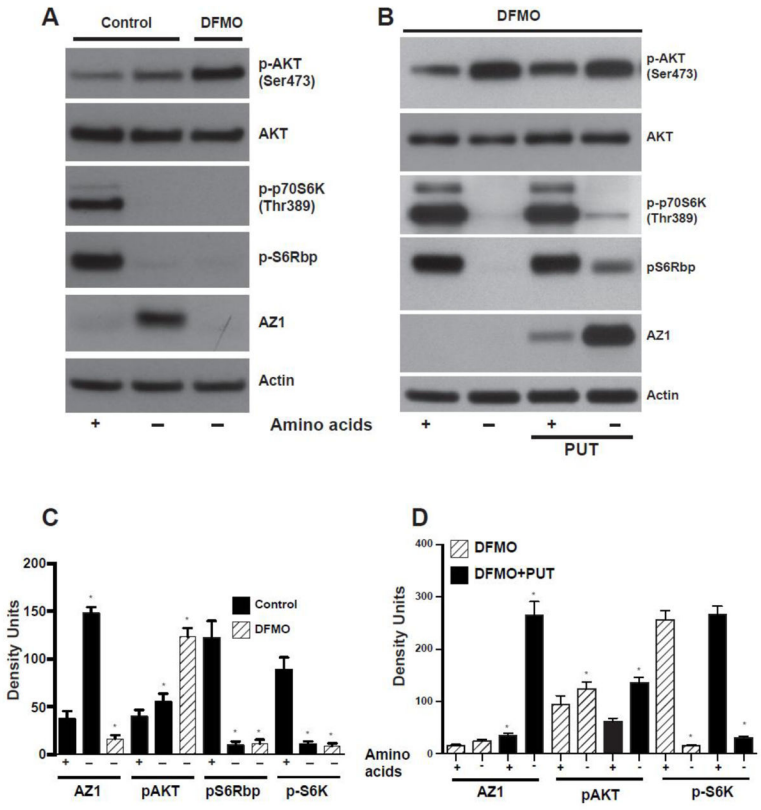

Fig. 1.

(A) Serum starved IEC-6 cells grown in control and DFMO containing medium for 4 days were exposed to DMEM with (AA+) or without (AA−) amino acids with or without DFMO for 4h. (B) Serum starved cells grown in the medium containing DFMO for 4 days were exposed to DMEM containing DFMO with (AA+) or without (AA−) amino acids with or without putrescine for 4h. Cell extracts were analyzed to determine mTORC1 activity (p70S6K and pS6Rbp levels) and mTORC2 (p-AKT-Ser473 levels) activity, AZ and actin levels. Western blots shown are representative of three experiments. (C and D) Quantification of western blots shown in fig. 1A and 1B respectively. Density units represent light intensity values measured by Image J software and normalized to β-actin and/or total protein. Values expressed are mean ± SE, n=3. (C) *, significantly different compared to respective control AA+ groups ($p<0.05$). (D) *, significantly different compared to respective DFMO AA+ groups ($p<0.05$).

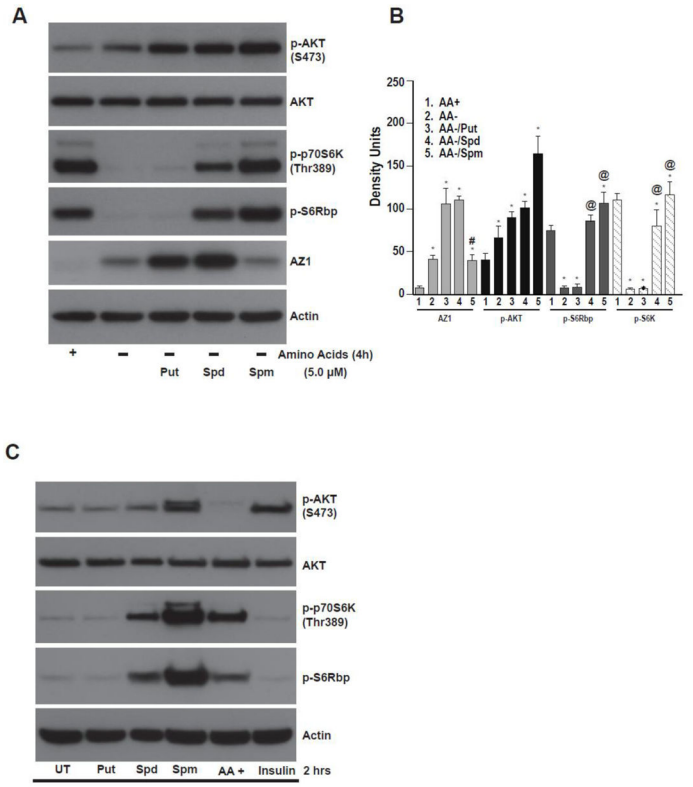

Fig. 2.

(A) Serum starved IEC-6 cells grown in control medium for 4 days were exposed to DMEM with amino acids (AA+) or DMEM without amino acids (AA−) for 4h. During 4h amino acid starvation, cells were also exposed to putrescine (Put), spermidine (Spd) or spermine (Spm). (B) Quantification of western blots shown in fig. 2A. Density units represent light intensity values measured by Image J software and normalized to β-actin and/or total protein. Values expressed are mean ± SE, n=3. *, significantly different compared to respective AA+ groups ($p<0.05$); #, significantly different compared to AA−/Put and AA −/Spd groups ($p<0.05$); @, significantly different compared to respective AA− groups, ($p<0.05$). (C) Serum starved IEC-6 cells grown in control medium for 4 days exposed to DMEM (AA−) for 2h were left untreated (UT) or exposed to amino acids (AA+), Put, Spd, Spm or insulin for an additional 2h. Cell extracts were analyzed to determine mTORC1 (p70S6K and pS6Rbp levels) and mTORC2 (p-AKT-Ser473 levels) activities and actin levels. Western blots shown are representative of three experiments.

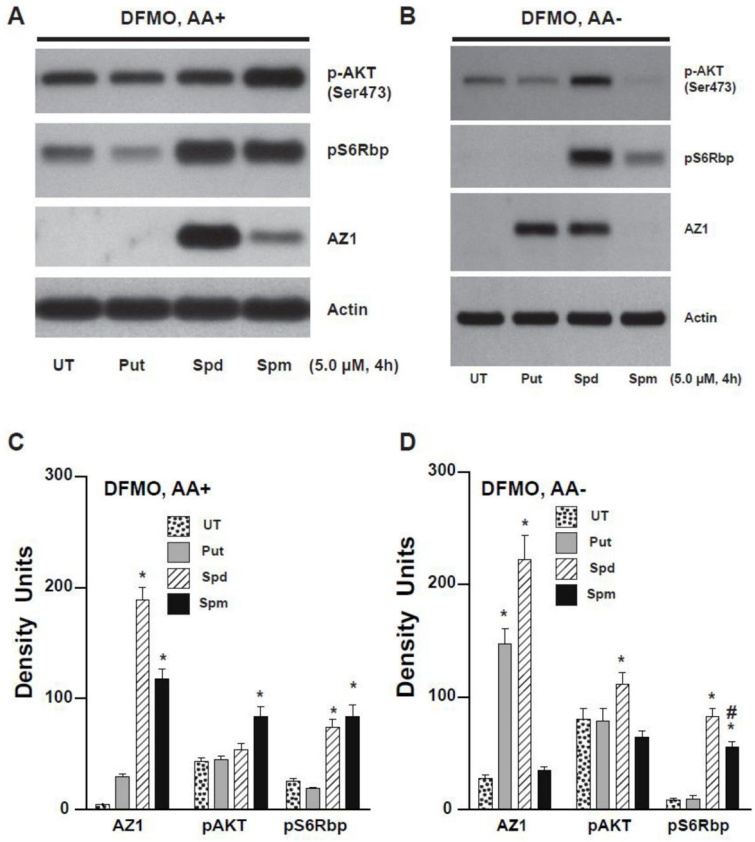

Fig. 3.

Serum starved IEC-6 cells grown in DFMO containing medium for 4 days to deplete polyamines were exposed to (A) DMEM+DFMO with amino acids (AA+) or (B) DMEM +DFMO without amino acids (AA−) for 4h in the presence or absence of Put, Spd, Spm for 4h. Cell extracts were analyzed to determine mTORC1 activity (p70S6K and pS6Rbp levels) and mTORC2 (p-AKT-Ser473 levels) activity, AZ and actin levels. Western blots shown are representative of three experiments. (C and D) Quantification of western blots shown in fig. 3A and B respectively. Density units represent light intensity values measured by Image J software and normalized to β-actin. Values expressed are mean ± SE, n=3. (C) *, significantly different compared to respective untreated (UT) groups ($p<0.05$). (D) *, significantly different compared to respective untreated (UT) groups ($p<0.05$); #, significantly different compared to Spd group ($p<0.05$).

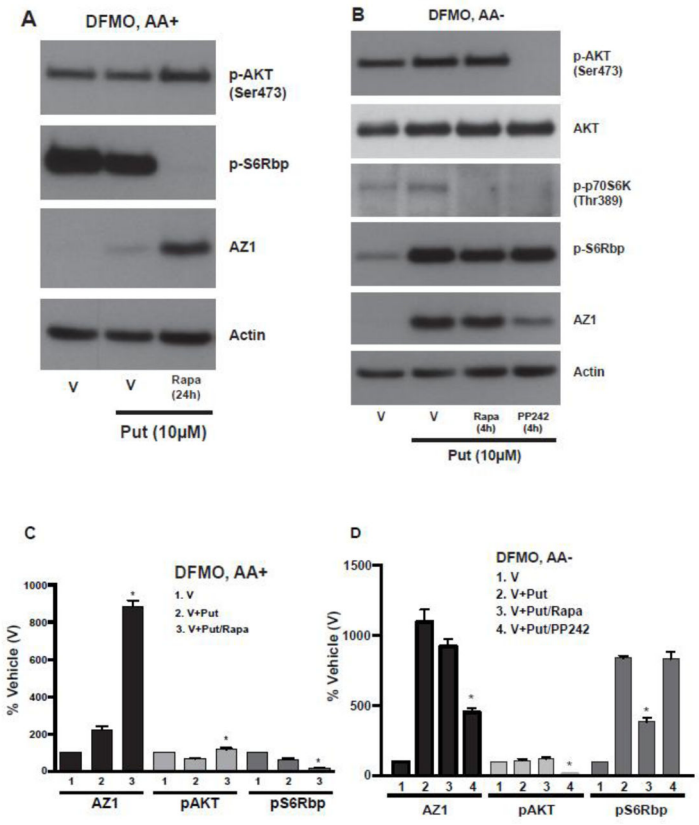

Fig. 4.

Serum starved IEC-6 cells grown in DFMO containing medium for 4 days to deplete polyamines were exposed to DMEM+DFMO with amino acids (AA+) or DMEM+DFMO without amino acids (AA−) for 4h in the presence or absence of putrescine (Put), rapamycin (Rapa), or PP242. Cell extracts were analyzed to determine mTORC1 activity (p70S6K and pS6Rbp levels) and mTORC2 (p-AKT-Ser473 levels) activity, AZ and actin levels. Western blots shown are representative of three experiments. (C and D) Quantification of western blots shown in fig. 4A and B respectively. Density units represent light intensity values measured by Image J software and normalized to β-actin. Values expressed are mean ± SE, n=3. *, significantly different compared to respective vehicle (V) groups ($p<0.05$).

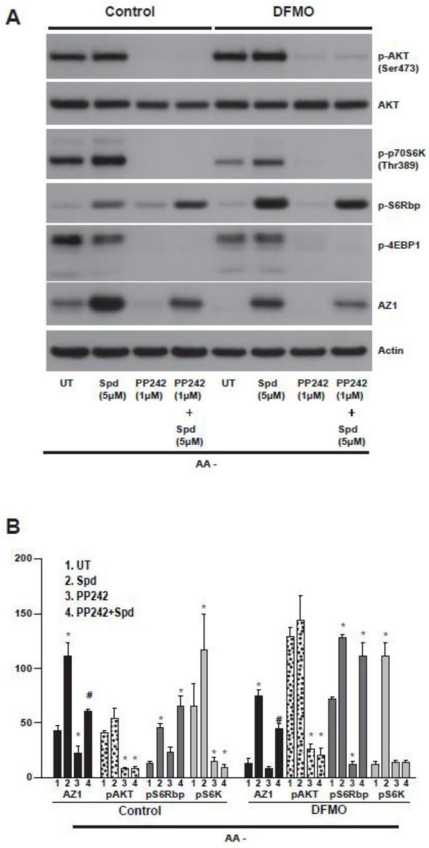

Fig. 5.

Serum starved IEC-6 cells grown in control and DFMO containing medium for 4 days were exposed to DMEM without (AA−) amino acids with or without DFMO for 4h. During 4h amino acid starvation cells were also exposed to spermidine (Spd), PP242 or Spd+PP242. Cell extracts were analyzed to determine mTORC1 activity (p70S6K and pS6Rbp levels) and mTORC2 (p-AKT-Ser473 levels) activity, AZ and actin levels. Western blots shown are representative of three experiments. (B) Quantification of western blots shown in fig. 5A. Density units represent light intensity values measured by Image J software and normalized to β-actin and or total protein. Values expressed are mean ± SE, n=3. *, significantly different compared to respective untreated (UT) groups ($p<0.05$); #, significantly different compared to respective Spd groups ($p<0.05$).

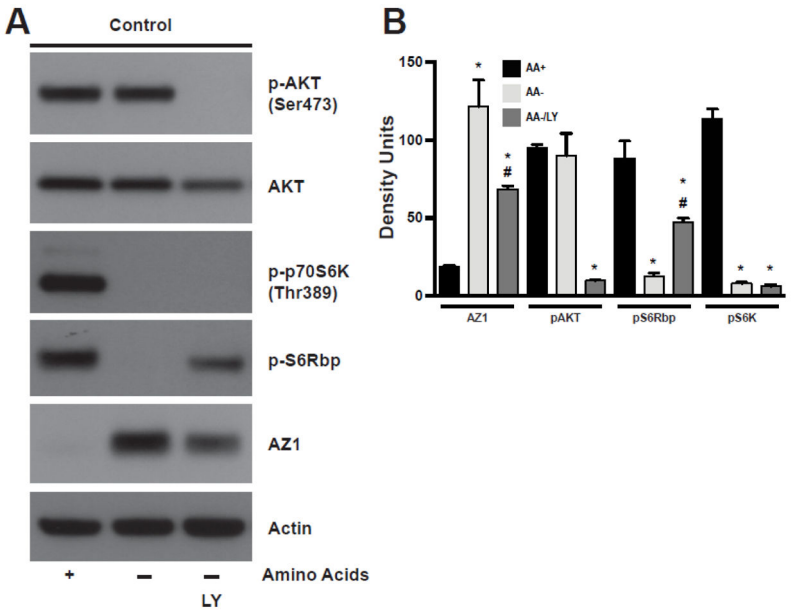

Fig. 6.

(A) Serum starved IEC-6 cells grown in control medium for 4 days were exposed to DMEM with amino acids (AA+) or DMEM without amino acids (AA−) or AA− containing LY294002 (10 μM) for 4h. Cell extracts were analyzed to determine mTORC1 activity (p70S6K and pS6Rbp levels) and mTORC2 (p-AKT-Ser473 levels) activity, AZ and actin levels. Western blots shown are representative of three experiments. (B) Quantification of western blots shown in fig. 6A. Density units represent light intensity values measured by Image J software and normalized to β-actin and or total protein. Values expressed are mean ± SE, n=3. *, significantly different compared to respective AA+ groups ($p<0.05$); #, significantly different compared to respective AA− groups ($p<0.05$).

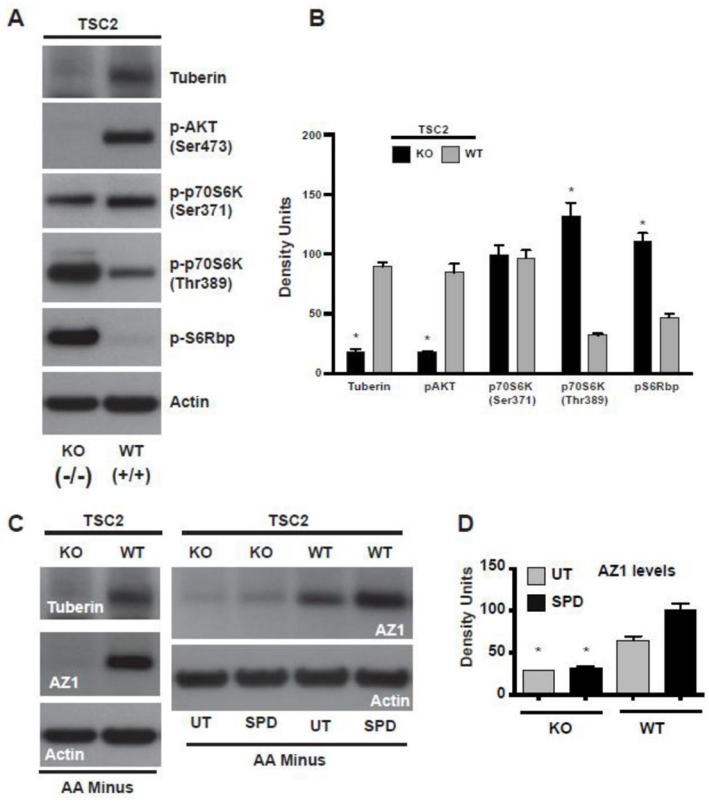

Fig. 7.

(A) Wild type (WT, $TSC2^{+/+}$) and knockout (KO, $TSC2^{-/-}$) mouse embryonic fibroblasts (MEFs) grown as described in methods were serum starved overnight. (C) Cells were incubated in medium lacking amino acids (AA−) or AA− medium containing 5μM spermidine (AA−/SPD) for 4h. Cell extracts were analyzed to determine mTORC1 activity (p70S6K and pS6Rbp levels) and mTORC2 (p-AKT-Ser473 levels) activity, AZ and actin levels. Western blots shown are representative of three experiments. (B and D) Quantification of western blots shown in fig. 7A and 7C respectively. Density units represent light intensity values measured by Image J software and normalized to β-actin and or total protein. Values expressed are mean ± SE, n=3. *, significantly different compared to respective WT groups ($p<0.05$).