Biochimica et Biophysica Acta 1863 (2016) 2658–2667

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

TSC2 N-terminal lysine acetylation status affects to its stability modulating mTORC1 signaling and autophagy

Ana García-Aguilar [a], Carlos Guillén [a,b,*], Mark Nellist [c], Alberto Bartolomé [d], Manuel Benito [a,b]

[a] Faculty of Pharmacy, Department of Biochemistry and Molecular Biology II, Complutense University of Madrid, Madrid 28040, Spain
[b] Spanish Biomedical Research Center in Diabetes and associated metabolic disorders (CIBERDEM), Instituto de Salud Carlos III, Spain
[c] Department of Clinical Genetics, Erasmus Medical Center, Rotterdam, The Netherlands
[d] Naomi Berrie Diabetes Center, Department of Medicine, Columbia University, New York, NY 10032, USA

ARTICLE INFO

Article history:
Received 21 March 2016
Received in revised form 7 August 2016
Accepted 13 August 2016
Available online 16 August 2016

Keywords:
Acetylation
TSC2
mTORC1
Stability
Ubiquitination
Proliferation

ABSTRACT

There is a growing evidence of the role of protein acetylation in different processes controlling metabolism. Sirtuins (histone deacetylases nicotinamide adenine dinucleotide-dependent) activate autophagy playing a protective role in cell homeostasis. This study analyzes tuberous sclerosis complex (TSC2) lysine acetylation, in the regulation of mTORC1 signaling activation, autophagy and cell proliferation. Nicotinamide 5 mM (a concentration commonly used to inhibit SIRT1), increased TSC2 acetylation in its N-terminal domain, and concomitantly with an augment in its ubiquitination protein status, leading to mTORC1 activation and cell proliferation. In contrast, resveratrol (RESV), an activator of sirtuins deacetylation activity, avoided TSC2 acetylation, inhibiting mTORC1 signaling and promoting autophagy. Moreover, TSC2 in its deacetylated state was prevented from ubiquitination. Using MEF *Sirt1* +/+ and *Sirt1* −/− cells or a SIRT1 inhibitor (EX527) in MIN6 cells, TSC2 was hyperacetylated and neither NAM nor RESV were capable to modulate mTORC1 signaling. Then, silencing *Tsc2* in MIN6 or in MEF *Tsc2* −/− cells, the effects of SIRT1 modulation by NAM or RESV on mTORC1 signaling were abolished. We also observed that two TSC2 lysine mutants in its N-terminal domain, derived from TSC patients, differentially modulate mTORC1 signaling. TSC2 K599M variant presented a lower mTORC1 activity. However, with K106Q mutant, there was an activation of mTORC1 signaling at the basal state as well as in response to NAM. This study provides, for the first time, a relationship between TSC2 lysine acetylation status and its stability, representing a novel mechanism for regulating mTORC1 pathway.

1. Introduction

Type 2 diabetes mellitus (T2DM) is characterized by insulin resistance in different tissues [1]. Two major phases can be distinguished in progression to T2DM. During the first one, an increase in pancreatic β cell mass by hyperplasia and hypertrophy is important to compensate for systemic insulin resistance. In the second one, β cells start to fail and die by apoptosis, leading to overt diabetes [2]. Under insulin resistance, mammalian/mechanistic target of rapamycin complex 1 (mTORC1) is hyperactive and essential for the compensatory mechanisms that lead to augmented beta cell mass, but might act as a double-edged sword in a later phase and contribute to beta cell apoptosis, involved in the pathogenesis of T2DM [3]. Autophagy is a physiologic process that eliminates damaged organelle and aggregated proteins. Our group and others have determined that autophagy, is a protective mechanism under different stress situations in several cell lines and tissues, promoting cell survival [4–7].

Insulin signaling modulates longevity in a great variety of organisms such as worms, flies, and mice [8], and rapamycin or knockdown of *MTOR* or *S6K1* can extend life span in different species [9–13]. In addition, tuberous sclerosis complex 1 (TSC1) and 2 (TSC2) activation, which negatively controls mTORC1 signaling through its GAP (GTPase activating protein) towards *Rheb* (Ras homolog enriched in brain), prolongs longevity in *Drosophila* [14]. Aging or a hypercaloric diet is associated with mTORC1 hyperactivity, leading to a defective autophagy and an increase in ER stress, contributing to insulin resistance [15]. In this regard, calorie restriction modulates changes in both nicotinamide adenine dinucleotide (NAD^+) and sirtuins levels, activating autophagy, and has been related with mammalian longevity [16]. Mammalian

Abbreviations: ARD1, arrest defective type 1; Atg, autophagy related; CHX, cycloheximide; GAP, GTPase activating protein; LC3B, microtubule-associated protein 1 light chain 3 beta; mTORC1, mammalian/mechanistic target of rapamycin complex 1; NAD, nicotinamide adenine dinucleotide; NAM, nicotinamide; NAT, N-terminal acetyl transferase; OAADPr, *O*-acetyl ADP-ribose; P70S6K, ribosomal protein S6 kinase polypeptide; RAPA, rapamycin; RESV, resveratrol; Rheb, Ras homologue enriched in brain; Sir2, silent information regulator 2 protein; SIRT1, sirtuin 1; shRNA, short-hairpin RNA; TSC1, tuberous sclerosis complex 1; TSC2, tuberous sclerosis complex 2; T2DM, type 2 diabetes mellitus.

* Corresponding author at: Faculty of Pharmacy, Department of Biochemistry and Molecular Biology II, Complutense University of Madrid, Madrid 28040, Spain.
E-mail address: cguillen@ucm.es (C. Guillén).

http://dx.doi.org/10.1016/j.bbamcr.2016.08.006
0167-4889/

sirtuins are composed of seven members (SIRT1–7) stimulated by energy deprivation, calorie restriction and resveratrol [17,18]. Sirtuins are histone deacetylases NAD^+-dependent homologous to the yeast silent information regulator 2 (Sir2) protein. SIRT1–3 catalyzed deacetylation reaction yielding nicotinamide and *O*-acetyl ADP-ribose (OAADPr) along with the deacetylated lysine [19]. Then, the nicotinamide product is a noncompetitive inhibitor of sirtuins [20].

The different localizations of SIRT proteins in the cell have an important implication in physiologic or pathologic metabolism processes. SIRT1 and SIRT2 shuttle between the nucleus and cytoplasm [21–23]. Protein acetylation modulates autophagy and can increase life span in yeasts as well as in nematodes [24]. SIRT1 interacts with TSC2 in HeLa cells, without affecting TSC2 acetylation [25]. The N-terminal acetyl-transferase (NAT), arrest-defective protein 1 (ARD1) interacts with TSC2, acetylates the first residue methionine, stabilizing it and inhibiting mTORC1 activity [26]. Furthermore, it has recently been published the interaction between SIRT1 and p70S6K. This interaction diminishes p70S6K acetylation degree, favoring the phosphorylation by mTORC1 [27].

Many reports indicate a differential regulation of TSC2 by both activating and inactivating phosphorylation sites [28]. However, the consequences of TSC2 lysine acetylation remain to be explored. Here, in this report we describe TSC2 lysine acetylation as a novel mechanism in the control of mTORC1 signaling and its consequences affecting essential processes, such as autophagy and proliferation.

2. Material and methods

2.1. Antibodies and reagents

The following antibodies were obtained from Cell Signaling Technology (Beverly, MA): anti pan-acetylated Lysine, #9441, anti-LC3B #4108, anti-p70S6K #9202, anti-phospho-p70S6K (Thr389), #9205, anti-TSC2, #9442, anti-phospho-AMPK (T172), #2531. From Sigma-Aldrich: anti-HA H6908, anti-Flag M2F1804, anti-β-actin A5316. From Santa Cruz Biotechnology: anti-GFP sc-9996, anti-SIRT1 sc-15404, anti-TSC2 sc-893. Other antibodies were used as follows: anti-phospho-S6 Ribosomal Protein (Ser235/236) from Thermo Scientific, MA5-15140, and anti-mono- and polyubiquitinylated proteins FK2 conjugated with peroxidase (HRP) or FK2H from Enzo Life Sciences, BML-PW0150. Chloroquine C6628, cycloheximide C7698, 6-chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxamide (EX-527) E7034, nicotinamide N3376 and propidium iodide P4170, were from Sigma-Aldrich; rapamycin 553210 and resveratrol R5010 were from Merck; and geneticin was from Santa Cruz (G418).

2.2. Cell culture

Mouse insulinoma 6 (MIN6) cell line originally described in [29] were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose supplemented with 15% FBS (fetal bovine serum), 100 units/ml penicillin, 100 μg/ml streptomycin and 50 μM β-mercaptoethanol. MIN6 Scr and *Tsc2* shRNA cell lines were generated as described in [7]. Cells were maintained at 37 °C in an atmosphere of 5% CO_2. Mouse embryonic fibroblasts (MEFs) and human embryonic kidney 293 (HEK 293T) cells were grown in DMEM containing 25 mM glucose supplemented with 10% FBS. MEF *Tsc1* −/− *and Tsc2* −/− were generous gifts of Dr. Kwiatkowski (Harvard Medical School, Boston). Primary cultures of MEF *Sirt1* +/+ and *Sirt1* −/− MEFs were generous gifts of the Maria Monsalve Lab (IIB, CSIC), and were immortalized by retrovirus-mediated transfection of attenuated SV40 T-antigen. After 5 h, the medium was refreshed and 72 h later, cells were selected with puromycin (1 μg/ml) for three weeks. Alternatively we used immortalized *Sirt1* +/+ and *Sirt1* −/− MEFs generously provided by Leonard Guarente (MIT, Boston).

2.3. Flow cytometry

For cell cycle analysis, trypsinised adherent and non-adherent cells were collected by centrifugation and fixed with cold ethanol (70% v/v). The cells were then washed, resuspended in PBS, and incubated with RNase for 30 min at 37 °C. After addition of 0.05% propidium iodide (w/v), cellular DNA content was quantified by flow cytometry.

2.4. Immunoprecipitation and Western blot

After treatment, cells were washed twice with PBS and lysed for protein extraction according to standard procedures. Protein determination was performed by the Bradford dye method, using the Bio-Rad (Hercules, CA) reagent and BSA as the standard. For immunoprecipitation, equal amounts of protein (200–600 μg) were immunoprecipitated at 4 °C o/n with the corresponding antibodies. The immune complexes were collected on protein A-agarose beads (Roche Applied Sciences) and protein samples were submitted to Western blot analysis. After SDS–PAGE, gels were transferred to Immobilon P PVDF membranes (Merck–Millipore). Then, membranes were blocked with 5% non-fat dried milk and incubated overnight with antibodies at 4 °C. Immunoreactive bands were visualized using the ECL Western blotting protocol (GE Healthcare, Little Chalfont, UK).

2.5. Lentivirus production and cell infection

To generate MIN6 *Tsc2 shRNA* cells, HEK 293T cells were co-transfected using Lipofectamine2000 with lentiviral packaging plasmid pMD2.G (Addgene, 12259) and psPAX2 (Addgene, 12260), along with lentiviral vector pLKO.1 neo or hygro for shRNA production (Addgene, 13425 and 24150 respectively). Different sequences were cloned between EcoRI (Roche, 10-200-310-001) and AgeI (New England Biolabs, R-0552S) sites of pLKO.1 lentiviral vector, following the recommendations from Addgene. Sequences of oligonucleotides used for knocking-down Tsc2 were as follows:

Tsc2-sense: 5′-CCGGcccgatatgtgttctccaaCTCGAGttggagaacacatatcgggTTTTTG-3′,

Tsc2-antisense: 5′ AATTCAAAAAcccgatatgtgttctccaaCTCGAGttggagaacacatatcggg-3′.

Pairs were annealed and cloned into pLKO.1 using restriction enzymes from Roche and New England Biolabs and a T4 ligation kit (Roche Applied Sciences, 11635379001).

Supernatants containing lentiviral particles from 24 and 48 h after HEK293T transfection were collected and passed through 0.45 μm filters. MIN6 cells were infected with lentiviral particles in polybrene (8 μg/ml) supplemented-media. After 24 h cells were used for experiments.

2.6. Measurement of TSC2 protein stability

HEK293T cells were seeded in 60 mm plates with fresh medium (DMEM 10% FBS). The following day, cells were stimulated with cycloheximide (100 μg/ml) for 30 min, and then, cells were treated with NAM (5 mM) or RESV (50 μM) for 4 h. After treatment, cells were washed twice with PBS and lysed for protein extraction according to standard procedures.

2.7. Retroviral production and cell infection

To generate stably transfected *Tsc2* +/+ MEF cells with EGFP-LC3B, the Phoenix packaging cells were transfected with retroviral vector p-EGFP-LC3B (Addgene, 11546) [30] using Lipofectamine2000 (Invitrogen, 11668-019). Supernatants containing retroviral particles were collected 48 h after transfection and passed through 0.45 μm filters. Cells were