Biochemical and Biophysical Research Communications 437 (2013) 146–150

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

Impaired lipid accumulation in the liver of Tsc2-heterozygous mice during liver regeneration

Yoko Obayashi [a,*,1], Jean S. Campbell [a,1], Nelson Fausto [a,1], Raymond S. Yeung [b,2]

[a] Department of Pathology, University of Washington School of Medicine, Seattle, WA, USA
[b] Department of Surgery, University of Washington School of Medicine, Seattle, WA, USA

ARTICLE INFO

Article history:
Received 12 June 2013
Available online 26 June 2013

Keywords:
Lipid
Liver regeneration
Tuberin
TSC2
mTOR
P70S6K

ABSTRACT

Tuberin is a negative regulator of mTOR pathway. To investigate the function of tuberin during liver regeneration, we performed 70% hepatectomy on wild-type and *Tsc2*+/− mice. We found the tuberin phosphorylation correlated with mTOR activation during early liver regeneration in wild-type mice. However, liver regeneration in the *Tsc2*+/− mice was not enhanced. Instead, the *Tsc2*+/− livers failed to accumulate lipid bodies, and this was accompanied by increased mortality.

These findings suggest that tuberin plays a critical role in liver energy balance by regulating hepatocellular lipid accumulation during early liver regeneration. These effects may influence the role of mTORC1 on cell growth and proliferation.

1. Introduction

Tuberin and hamartin, which are the products of genes TSC1 and TSC2, form a complex that negatively regulates mammalian target of rapamycin (mTOR). This complex works as a critical nutrient sensor, which regulates cell growth and proliferation via mTOR pathway. Mammalian target of rapamycin is activated by Ras-related small GTPase Rheb, which is a target of tuberin-hamartin complex. Tuberin is a GTPase-activating protein and stimulates the intrinsic GTPase activity of Rheb, thereby converting Rheb from its GTP-bound active state to GDP-bound inactive state. The insulin signaling pathway inactivates tuberin via the protein kinase, Akt. Inoki et al. reported that tuberin is directly phosphorylated by Akt at Thr1462, followed by an inactivation of tuberin, a disruption of its interaction with hamartin, and the activation of mTOR [1].

Under energy starvation (e.g., increased AMP:ATP ratio), AMPK becomes activated and phosphorylates tuberin to enhance its activity to inhibit mTOR signaling and cell growth/proliferation. Furthermore, phosphorylation of tuberin by AMPK protects cells from energy deprivation-induced apoptosis [2]. Taken together, these results indicate that tuberin inhibits cell growth/proliferation through the inactivation of mTOR under energy deprivation, and stimulates growth through the activation of mTOR when it receives growth-stimulating signals.

Jiang et al. reported that p70S6K, the downstream targets of mTORC1, was highly activated by phosphorylation at Thr389 in response to partial hepatectomy, and this activation was inhibited by rapamycin administration, leading to the attenuation of liver regeneration [3]. Accordingly, one would predict that enhanced mTORC1 activity could accelerate liver regeneration following partial hepatectomy by promoting cell growth and proliferation.

In the present study, we investigated the role of tuberin in liver regeneration by determining the time-dependent change of p70S6K phosphorylation at Thr389 and tuberin phosphorylation following 70% hepatectomy in C57BL/6 mice. We further performed 70% hepatectomy on *Tsc2*+/− mice, whose livers expressed half of the normal level of tuberin, and examined if liver regeneration is accelerated with hyperactivation of mTOR.

The results of these analyses indicate that tuberin phosphorylation strongly correlated with p70S6K activation during liver regeneration, but the process was not accelerated in the *Tsc2*+/− mice. Instead, we found impaired accumulation of lipid vesicles in the liver of these animals during the initial phase of liver regeneration, which serves as a primary energy source during early regeneration. These findings provide insights into the multiple functions of tuberin during liver regeneration.

Abbreviations: TSC, tuberous sclerosis; mTOR, mammalian target of rapamycin; GAP, GTPase-activating protein; MAPK, mitogen-activated protein kinase; p90 RSK, p90 ribosomal S6 kinase; ERK, extracellular signal-regulated kinase; P70S6K, p70 ribosomal S6 kinase; AMPK, 5′AMP-activated protein kinase; PH, partial hepatectomy; SH, sham operation; C57BL/6, c57 black 6; Rheb, ras homolog enriched in brain; AEBSF, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride.

* Corresponding author. Present address: Ajinomoto Co., Inc., 1-15-1, Kyobashi, Chuo-ku, Tokyo 104-8315, Japan. Fax: +81 3 5250 8403.
E-mail address: youko_oobayashi@ajinomoto.com (Y. Obayashi).
[1] Address: UW Medicine Pathology, Box 357470, Seattle, WA 98195-7470, USA.
[2] Address: UW Department of Surgery, Box 356410, Seattle, WA 98195-6410, USA.

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.06.056

2. Materials and methods

2.1. Antibodies

Anti-tuberin (C-20) antibody (cat# sc-893) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-β-actin antibody (cat# A5441) was obtained from Sigma (St. Louis, MO). Anti-p70S6 kinase (cat# 9202), anti-p-p70S6 kinase (Thr389, 1A5)(cat# 9206), anti-phospho-Akt-substrate (RXRXXS*/T*, 110B7) (cat# 9614) were obtained from Cell Signaling (Beverly, MA). Anti-adipophilin antibody (cat# GP40) was obtained from Progen Biotechnik (Germany).

2.2. Partial hepatectomy

Eight to nine-week old C57 black 6 (C57BL/6) or *Tsc2*+/− (gift of D. Kwaitkowski, Harvard) male mice were fasted overnight. The next morning between 8:00 and 10:00, the mice were anesthetized with isoflurane (Abbott, cat# 05260-05) and the median and left lateral lobes of the liver were ligated at their stem and excised. Control mice were subjected to sham operation, which consisted of laparotomy and a brief manipulation of the liver with a cotton swab prior to wound closure. Food was re-introduced 6–8 h after surgery. The animals were sacrificed by cervical dislocation at the indicated time points following surgery. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Washington, which is certified by the Association for Assessment and Accreditation of Laboratory Animal Care International.

2.3. Adipophilin immunostaining

Liver sections were fixed in 10% formalin, paraffin embedded, and stained with anti-adipophilin antibody using Vectastain Elite ABC Kit (Vector Laboratories, cat# PK-6101). Sections were reacted with 3,3′-diaminobenzizine (DAB) hydrochloride (Sigma, cat# D4293), followed by counterstaining with Harris hematoxylin.

2.4. Oil red O staining

Liver sections were fixed in 10% formalin for 2–3 h, equilibrated in 30% sucrose, and embedded in OCT. Frozen liver sections were stained with oil red O and counterstained with Harris hematoxylin.

2.5. Detection of phospho-tuberin recognized with anti-phospho-akt substrate (RXRXXS*/T*) antibody

Liver tissue was homogenized in NP-40 lysis buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 2.5 mM ethylenediaminetetraacetate·$2H_2O$, 1% Nonidet P-40, 50 mM sodium fluoride, 0.5 mM AEBSF, 1 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 1 mM sodium Orthovanadate, 1 mM dithiothreitol, 10 μg/ml leupeptin, 50 μg/ml SBTI, 200 nM Microcystin). Four micrograms anti-tuberin antibody was added to 5 mg protein and was rocked at 4 °C overnight for immunoprecipitation. Tuberin-antibody complex was absorbed with 40 μl Protein A Sepharose (1 g/4 ml, Sigma, cat# P3391, MO, USA), followed with washing by 1 ml NP-40 lysis buffer three times. The Protein A Sepharose was boiled with 25 μl of protein loading buffer at 1.5 time higher concentration for 5 min., and was subject to western blot analysis with anti-Phospho-Akt Substrate (RXRXXS*/T*) antibody as a primary antibody.

2.6. Western blot analysis

Liver tissue was homogenized in lysis buffer (50 mM Tris–HCl (pH7.4), 1% Triton X100, 150 mM NaCl, 50 mM β-glycerohosphate, 10% glycerol, 2 mM 2.5 mM ethylenediaminetetraacetate·$2H_2O$, 0.48 mM AEBSF, 1 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 10 μg/ml leupeptin, 50 μg/ml SBIT, 0.2 μM microcystin) and then centrifuged for 15 min at 4 °C. Protein concentration of the supernatant was quantified with BCA Protein Assay Kit (Pierce, cat#2161297A). An aliquot of protein was boiled with protein loading buffer for 5 min, and was loaded on SDS polyacrylamide gel. (7% for tuberin, 10% for p70S6K) After electrophoresis at constant voltage of 100 V, proteins were transferred onto PVDF membranes and blotted against primary antibodies. Membranes were washed with Tris-buffered saline with 0.1% Tween-20 and incubated with a 1:5000 dilution of horseradish peroxidase-conjugated secondary antibodies for 1 h. Protein bands were visualized by chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate (Pierce, cat#34080).

2.7. Expression of data and statistical analysis

The results are expressed as the mean ± SEM. The Student's *t* test was used for the comparison of data from two groups. The difference between groups was considered significant when *P* was less than 0.05.

3. Results and discussion

3.1. Hepatectomy induces P70S6K activation, downstream of mTOR signaling, during early liver regeneration

In order to investigate the time-dependent change of themTOR activation after 70% PH in C57BL/6 mice, we quantified the level of p-P70S6K (Thr389), using western blot analysis after PH.

We found that P70S6K was specifically phosphorylated in PH mice in contrast to sham-operated mice (Fig. 1A). The PH-specific phosphorylation already began at 0.5 h, peaked around 4–8 h, and gradually decreased by 24 h (Fig. 1A and B). These results indicate that mTOR signaling is activated at very early phase of liver regeneration.

3.2. Hepatectomy induces tuberin phosphorylation, which strongly correlates with P70S6K activation

Given that tuberin regulates mTORC1 and hence, p70S6K activity, we next investigated tuberin activity based on its phosphorylation state. Following partial hepatectomy, Hong et al. reported rapid activation of Akt, which in turn, can phosphorylates tuberin at multiple sites to suppress its function [4]. Based on the consensus sequence, RXRXX(S/T), Akt phosphorylation can be detected using a well-characterized anti-phospho PKB/Akt-substrate antibody. To determine such sites in tuberin following partial hepatectomy, we performed western blotting with this antibody following immunoprecipitation of tuberin from liver lysates. The western blot analysis at 2 h after surgery showed specific phosphorylation of tuberin in the PH group in contrast to minimally detectable phosphorylation in the sham group (Fig. 2A). In addition, there was strong correlation between the levels of phosphorylation of tuberin and that of P70S6K (Thr389) in the liver from multiple mice at 2 h. The temporal pattern of tuberin phosphorylation as detected by the Akt-substrate antibody paralled that of p70S6K phosphorylation beginning at 0.5 h, peaked around 2–6 h, and continued up to 12 h (Fig. 2C).