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RESEARCH ARTICLES

Localization of polypyrimidine-tract-binding protein is involved in the regulation of albumin synthesis by branched-chain amino acids in HepG2 cells

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Abstract

Long-term supplementation of branched-chain amino acids (BCAA) improves hypoalbuminemia in patients with cirrhosis. Our previous findings have suggested that the binding of polypyrimidine-tract-binding protein (PTB) to rat albumin mRNA attenuates its translation. The aim of the present study was to investigate the role of PTB in the regulation of albumin synthesis by BCAA in human hepatoma cells. HepG2 cells were cultured in a medium containing no amino acids (AA-free medium), a medium containing only 1 amino acid (a BCAA: valine, leucine or isoleucine) or a medium containing all 20 amino acids (AA-complete medium). HepG2 cells cultured in AA-complete medium secreted much more albumin than cells cultured in AA-free medium, with no difference in albumin mRNA levels. In cells cultured in AA-free medium, nuclear export of PTB was observed, and the level of the albumin mRNA–PTB complex was greater than in cells cultured in AA-complete medium. Addition of amino acids stimulated nuclear import of PTB. However, addition of amino acids with rapamycin inhibited the nuclear import of PTB. The addition of leucine, but not of valine or isoleucine, to AA-free medium increased albumin secretion and stimulated the nuclear import of PTB. These data indicate that the mammalian target of rapamycin is involved in the regulation of PTB localization and that leucine promotes albumin synthesis by inhibiting the formation of the albumin mRNA–PTB complex.

Keywords: Albumin; Polypyrimidine-tract-binding protein; Mammalian target of rapamycin; Branched-chain amino acids; HepG2

1. Introduction

Albumin is the most abundant protein in plasma, and the colloid pressure of plasma is maintained principally by the level of circulating albumin [1]. Albumin also performs important metabolic functions by transporting free fatty acids, bilirubin and many drugs [2]. There is a high prevalence of hypoalbuminemia among patients with chronic hepatic failure, including patients with decompensated liver cirrhosis or hepatocellular carcinoma [3–6]. It has been reported that prognosis significantly differs between patients with a serum albumin concentration of >3.5 g/dl and patients with a serum albumin concentration of ≤3.5 g/dl [6,7].

Serum albumin concentration is a result of numerous processes, including albumin synthesis, catabolism and body distribution [8]. Previous studies have suggested that albumin synthesis is decreased in patients with cirrhosis [7,9]. In Japan, pharmacological supplementation of branched-chain amino acids (BCAA) is used widely to improve decreased albumin synthesis in patients with decompensated liver cirrhosis [10–12]. It is thought that supplementation with BCAA increases albumin synthesis by increasing the translation efficiency of albumin mRNA via posttranscriptional events [13–15]. Some studies suggest

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that mammalian target of rapamycin (mTOR) signaling plays an important role in the promotion of albumin synthesis [15,16]. In a recent study, we found that polypyrimidine-tract-binding protein (PTB) in extracts prepared from rat liver interacts with the coding region of rat albumin mRNA and that immunodepletion of PTB from rabbit reticulocyte lysate causes an increase in albumin mRNA translation in the lysate [17]. Also in that study, we found that extracts prepared from the liver of rats with acute liver failure contained higher levels of the albumin mRNA–PTB complex than liver extracts from normal rats and that the level of this complex decreased with the infusion of BCAA-enriched total parenteral nutrition solution [17].

PTB is an abundant eukaryotic RNA-binding protein that has been implicated in several aspects of mRNA metabolism, including splicing regulation [18], RNA nuclear export [19], internal ribosome entry-site-mediated translation initiation [20], mRNA stability [21] and cytoplasmic RNA localization [22]. Although PTB is mainly found in the nucleus at steady state [19,21], it shuttles rapidly between the nucleus and the cytoplasm [19,23,24]. In addition, recent studies indicate that PTB translocates to the cytoplasm under certain conditions [21,25]. The cytoplasmic localization of PTB is an important factor in its function.

To improve the nutritional status of chronic liver disease patients, current European Society for Clinical Nutrition and Metabolism (ESPEN) guidelines recommend supplementing their diet with BCAA [26]. In the present study, we investigated molecular mechanisms whereby BCAA affect albumin synthesis.

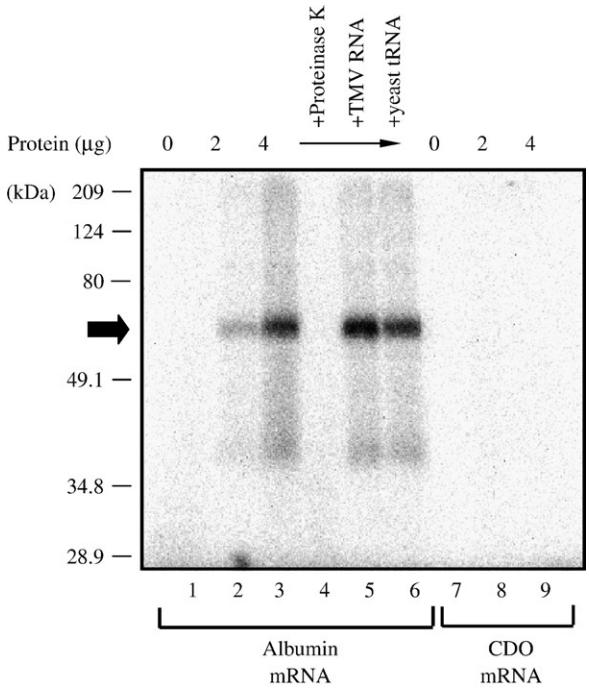

Fig. 1. Formation of a specific RNA–protein complex between albumin mRNA and protein contained in cell extracts prepared from HepG2 cells. UV cross-linking analysis was performed using albumin (Lanes 1–6) and CDO (Lanes 7–9) cRNA probes. ^{32}P-labeled RNA probes were incubated alone (Lanes 1 and 7) or with 2 μg (Lanes 2 and 8) or 4 μg (Lanes 3–6 and 9) of cell extracts prepared from HepG2 cells, followed by UV cross-linking analysis. In some cases, before the addition of the RNA probe, cytosolic fractions were preincubated with 5 μg of proteinase K for 30 min at 37°C (Lane 4), or with 100 ng of TMV RNA (Lane 5) or yeast tRNA (Lane 6) for 15 min. Solid arrow indicates the RNA–protein complex.

2. Materials and methods

2.1. Materials

A human albumin cDNA clone (FCC-101) was purchased from TOYOBO Co. Ltd. (Osaka, Japan). [α-^{32}P]UTP (3000 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Anti-PTB and anti-PGC-1 antibodies were purchased from Zymed Lab (San Francisco, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Anti-tubulin antibodies were purchased from Sigma (St. Louis, MO). Anti-S6 and anti-phospho-S6 (Ser235/236) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-mouse Alexa488-conjugated secondary antibody and Alexa568-conjugated phalloidin were purchased from Molecular Probes (Eugene, OR).

Table 1
Sequences of primers used for PCR

		Sequence	Position
Plasmid construction			
CDO	Sense	ATTCCTAGTGACTCCAAGCG	1–20
	Antisense	CCATTAAAATCGTCATGTTT	1582–1563
Preparation of templates for in vitro transcription			
pSP70	Sense	GGCCGATTCATTAATGCAGG	113–94
	Antisense	TGTCGTTAGAACGCGGCTAC	2346–2365
Albumin	Antisense	GAATAAGCCGAGCTAAAGAG	92–73
	Antisense	GAAAAAGAAGGGAAATAAAG	73–54
	Antisense	GTTACCCACTTCATTGTGCC	53–34
Real-time PCR			
Albumin	Sense	ACAGAATCCTTGGTGAACAGGCGA	1543–1566
	Antisense	TCAGCCTTGCAGCACTTCTCTACA	1796–1773
GAPDH	Sense	CTGCACCACCAACTGCTTAGC	513–533
	Antisense	CTTCTGGGTGGCAGTGATGGC	618–598

Primer sequences are written from 5′ end to 3′ end.

2.2. Cell culture

The human cell line HepG2 was grown in α-minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 50 IU/ml penicillin and 50 μg/ml streptomycin. The cell culture methods used for the amino acid supplementation experiment are described in detail elsewhere [15]. Briefly, the cells were precultured for 12 h in a medium that contained all 20 amino acids (AA-complete medium) and was supplemented with 1% FBS. The medium was then changed, and the cells were incubated in a medium containing no amino acids (AA-free medium) for 2 h. Then, the cells were incubated either in AA-free medium, in a medium containing only one amino acid (a BCAA: valine, leucine or isoleucine) or in AA-

complete medium. The concentration of each amino acid in AA-complete medium was as follows (in mM): glycine, 0.40; alanine, 0.40; serine, 0.40; threonine, 0.80; cystine, 0.20; methionine, 0.20; glutamine, 4.00; asparagine, 0.40; glutamic acid, 0.40; aspartic acid, 0.40; valine, 0.80; leucine, 0.80; isoleucine, 0.80; phenylalanine, 0.40; tyrosine, 0.40; tryptophan, 0.08; lysine, 0.80; arginine, 0.40; histidine, 0.20; proline, 0.40. Some cells cultured in AA-complete medium were treated with 25 ng/ml rapamycin (Wako Pure Chemical Industries, Osaka, Japan).

2.3. Preparation of cell extracts

For UV cross-linking analysis, cells were homogenized in a 0.35-M sucrose buffer containing 0.2 M Tris–Cl (pH 8.5), 50 mM KCl and 10 mM Mg-acetate. Cell homogenates were centrifuged at 10,000×*g* for 30 min at 4°C. The supernatant fraction was recentrifuged at 80,000×*g* for 1 h at 4°C. This supernatant fraction was dialyzed against a buffer containing 10 mM Tris–Cl (pH 7.6), 1 mM K-acetate, 1.5 mM Mg-acetate, 2 mM dithiothreitol (DTT) and 10% glycerol.

Nuclear and cytoplasmic fractions were prepared using the method described by Andrews and Faller [27]. Briefly, cells were suspended in a buffer containing 10 mM HEPES–KOH (pH 7.9), 1.5 mM KCl, 0.5 mM DTT and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). The cells were allowed to swell on ice for 10 min and were then vortexed for 10 s. Samples were centrifuged for 10 s, and the resulting supernatant was recentrifuged at 8000×*g* for 30 min at 4°C. This supernatant fraction was used as a cytoplasmic fraction. The pellet containing nuclei was resuspended in a buffer containing 20 mM HEPES–KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT and 0.2 mM PMSF, and was incubated on ice for 20 min. Cellular debris was removed by centrifugation for 2 min at 4°C, and the resulting supernatant fraction was used as a nuclear fraction. These two fractions, cytoplasmic and nuclear, were used for Western blot analysis using anti-PTB antibody.

For Western blot analysis using anti-S6 and anti-phospho-S6 antibodies, cells were homogenized in a buffer containing 20 mM HEPES (pH 7.4), 100 mM KCl, 0.2 mM EDTA, 2 mM EGTA, 1 mM DTT, 50 mM NaF, 50 mM β-glycerophosphate, 0.1 mM PMSF, 1 mM benzamidine and 0.5 mM sodium vanadate. Homogenates were centrifuged at

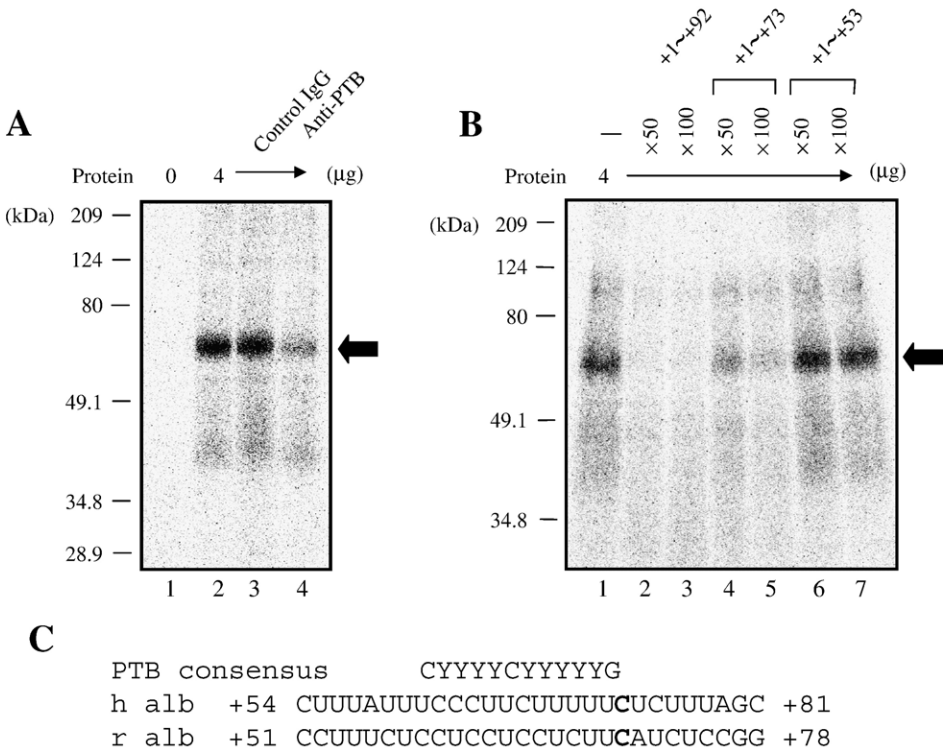

Fig. 2. PTB is contained in the albumin mRNA–protein complex. (A) Cell extracts prepared from HepG2 cells were incubated with 0.4 μg of control IgG (Lane 3) or anti-PTB antibody (Lane 4) for 30 min. After incubation with the antibody, UV cross-linking analysis was performed. Lane 2 shows a control reaction with no antibody. Lane 1 contains only a probe. Solid arrow indicates the RNA–protein complex. (B) UV cross-linking analysis using albumin cRNA probe was performed with competitor transcripts corresponding to nucleotides 1–92 (Lanes 2 and 3), 1–73 (Lanes 4 and 5) and 1–53 (Lanes 6 and 7) of albumin mRNA, respectively. Competitor transcripts were preincubated with 4 μg of cell extracts before the addition of ^{32}P-labeled RNA. After incubation for 15 min, these reaction mixtures were subjected to UV cross-linking analysis. Competitor transcripts were added at 50-fold (Lanes 2, 4 and 6) and 100-fold (Lanes 3, 5 and 7) molar excess. Lane 1 shows a control reaction with no competitor. Solid arrow indicates the RNA–protein complex. (C) Comparisons of the PTB consensus-binding sequence with albumin mRNA. The PTB consensus-binding sequence [17] is aligned with mRNA sequences of human albumin (GenBank accession no. V00495) and rat albumin (GenBank accession no. V01222). "Y" denotes pyrimidines. Letters in bold indicate positions of transition that differ from the PTB consensus-binding sequence.

8000×*g* for 30 min at 4°C. The resulting supernatant fraction was subjected to Western blot analysis.

Protein concentration was determined using the BCA protein assay reagent (Pierce, Rockford, IL).

2.4. Plasmid construction

Full-length albumin cDNA was excised from a cloning vector (TOYOBO) using the restriction enzyme *Xho*I and was ligated to the pSP70 expression vector (Promega, Madison, WI) [17]. Full-length cysteine dioxygenase (CDO) cDNA was amplified from first-strand cDNA synthesized from the total RNA prepared from HepG2 cells. Specific primers are shown in Table 1. Polymerase chain reaction (PCR) product was sequenced and ligated to the pSP70 expression vector.

2.5. In vitro transcription and UV cross-linking analysis

In vitro transcription and UV cross-linking analysis were performed as described elsewhere [17]. Templates for in vitro transcription were prepared by PCR using pSP70-specific primers (Table 1). A sense primer binds 5′ upstream of the T7 promoter of pSP70, and an antisense primer binds 3′ downstream of inserted full-length cDNA fragments. Templates for competitor RNA were prepared by PCR using pSP70-specific sense primers and albumin-specific antisense primers (Table 1). These templates were incubated with T7 RNA polymerase (TOYOBO) in the presence of 10 μCi of [α-^{32}P]UTP and nucleotide triphosphates [17]. Competitor RNA was synthesized using 0.5 mM of unlabeled nucleotide triphosphates.

2.6. Enzyme-linked immunosorbent assay

After preculture for 2 h in AA-free medium, cells were cultured for 6 h in one of the three types of experimental media described in Section 2.2. The medium was collected, and the amount of albumin that had been secreted into the medium was measured using ALBWELLII (EXOCELL, Inc., Philadelphia, PA).

2.7. Preparation of total RNA and quantitative real-time PCR

Total RNA was extracted using the ISOGEN system (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. First-strand cDNA synthesis and real-time PCR were performed as described elsewhere [28]. The human-albumin-specific primers and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific primers used in this experiment are shown in Table 1. Albumin mRNA levels were normalized to the GAPDH mRNA level.

2.8. Western blot analysis

Protein samples were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis under standard reducing conditions. Separated proteins were transferred by electrophoresis into Hybound-P polyvinylidene difluoride transfer membranes (GE Healthcare Bio-Science Corp., Piscataway, NJ). The membranes were incubated with antibodies to PTB, tubulin, S6 or phospho-S6, respectively. Horseradish-peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody was utilized as a secondary antibody (Jackson Immunoresearch Laboratories, Inc., West Grove, PA), and signals were detected using the ECL Plus system (GE Healthcare Bio-Science Corp.).

2.9. Immunocytochemistry

HepG2 cells were fixed with 3% paraformaldehyde for 10 min. After washing, the cells were incubated in 20 mM glycine for 10 min. Next, the cells were permeabilized with 0.05% saponin for 10 min. The cells were then washed and treated with 1% bovine serum albumin in phosphate-

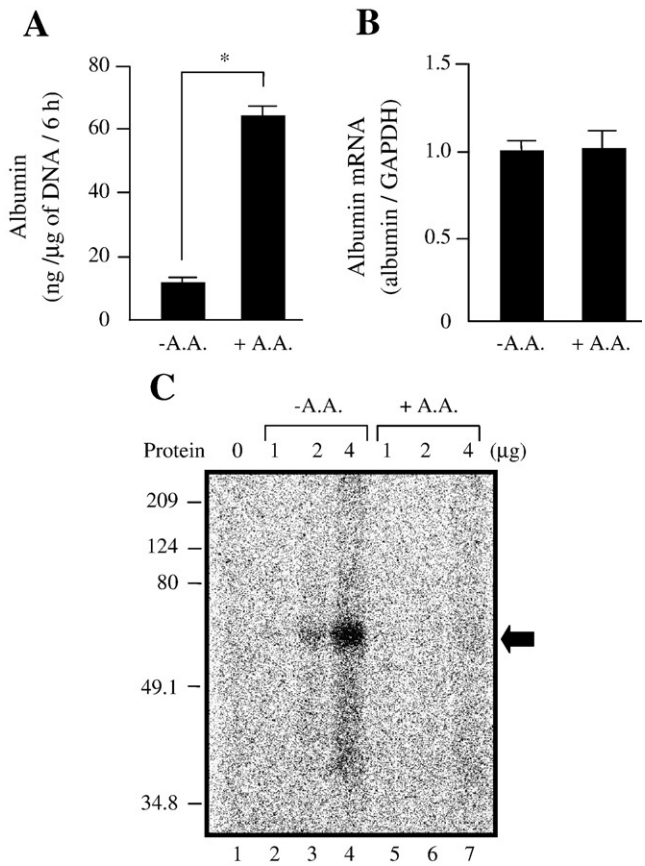

Fig. 3. Effect of amino acids on the levels of secreted albumin, albumin mRNA and albumin mRNA–PTB complex. HepG2 cells were cultured in AA-free medium (−AA) or in AA-complete medium (+AA) for 6 h. (A) Level of secreted albumin from cells cultured in −AA or in +AA. Values are presented as mean±S.E. (n=4). *P<.05. (B) Level of albumin mRNA in cells cultured in −AA or in +AA. Albumin mRNA levels were normalized to the GAPDH mRNA level. Values are presented as mean±S.E. (n=4). (C) Albumin cRNA probe was incubated alone (Lane 1) or with 1 μg (Lanes 2 and 5), 2 μg (Lanes 3 and 6) or 4 μg (Lanes 4 and 7) of cell extracts prepared from cells cultured in −AA (Lanes 2–4) or in +AA (Lanes 5–7), followed by UV cross-linking analysis. Solid arrow indicates the RNA–PTB complex. The data shown are representative of three independent experiments.

buffered saline for 30 min. Then the cells were incubated with anti-PTB or anti-PGC-1 antibody for 1 h. After washing, the cells were incubated with anti-mouse Alexa488-conjugated secondary antibody and Alexa568-conjugated phalloidin (for actin staining) for 1 h. Then the cells were examined by fluorescence microscopy. Nuclei were counterstained with DAPI (Sigma).

2.10. Statistical analysis

Data are expressed as mean±S.E. Student's *t* test was used to analyze the differences between two groups. Statistical analysis for multiple comparisons was performed using one-way analysis of variance followed by Tukey–Kramer post-hoc test. Data analysis was performed using Statcel2 software. $P<.05$ was considered statistically significant.

3. Results

3.1. Specific interaction between albumin mRNA and PTB

In UV cross-linking analysis, ^{32}P-labeled full-length albumin mRNA formed a complex when incubated with cell extracts prepared from HepG2 cells (Fig. 1, Lanes 2 and 3). This complex contained RNA-binding protein, as

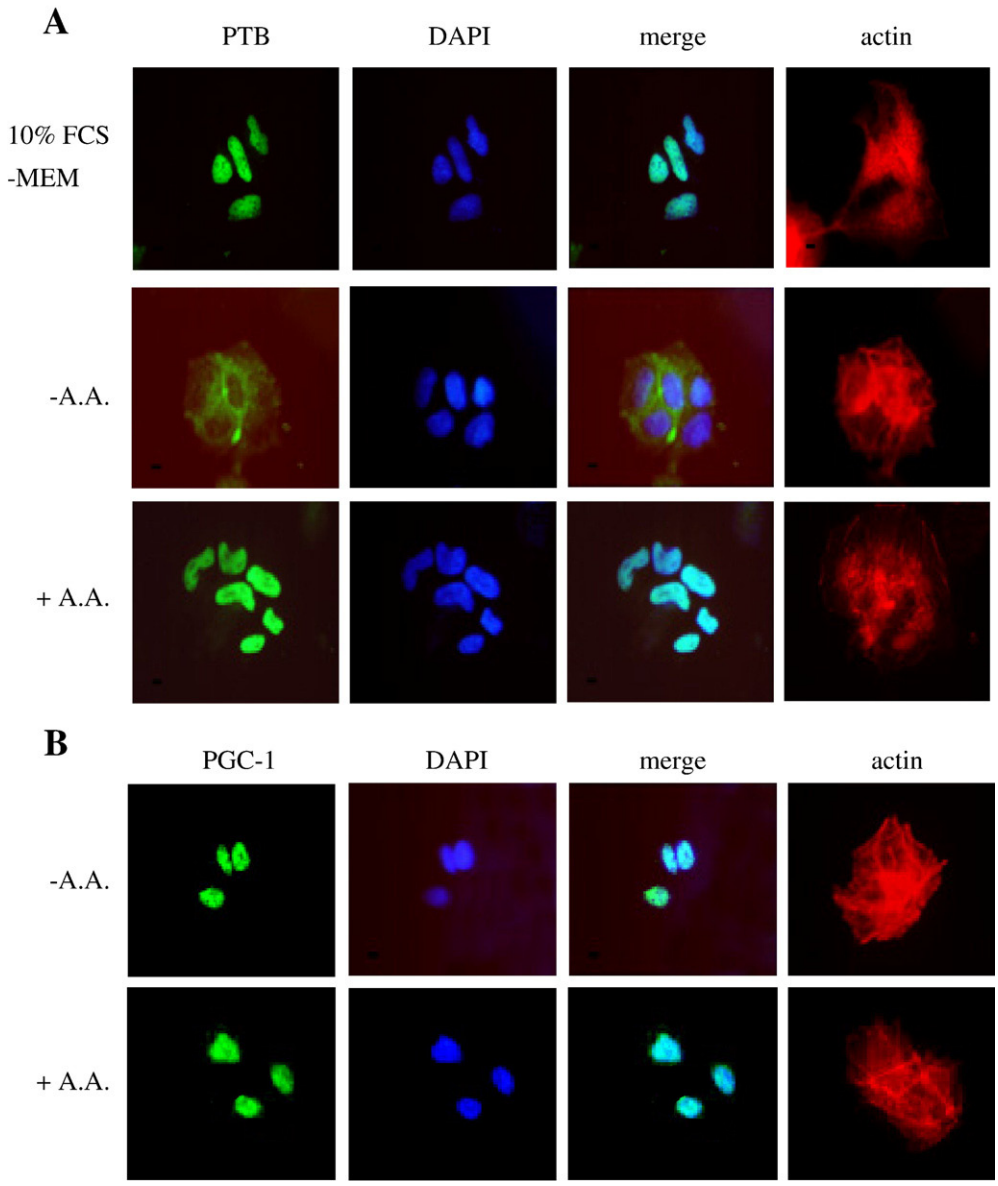

Fig. 4. Nucleocytoplasmic translocation of PTB in HepG2 cells after culture in AA-complete medium. (A) Immunofluorescence microscopy images of PTB (green) and actin (red) in cells cultured in MEM containing 10% FBS (upper panel), in AA-free medium (−AA; middle panel) or in AA-complete medium (+AA; lower panel) for 6 h. Nuclei were counterstained with DAPI (blue). (B) Immunofluorescence microscopy images of PGC-1 (green) and actin (red) in cells cultured in −AA (upper panel) or in +AA (lower panel) for 6 h. Nuclei were counterstained with DAPI (blue).

indicated by the fact that pretreatment of the extract with proteinase K completely abolished the binding signal (Fig. 1, Lane 4). This RNA-binding protein specifically bound to albumin mRNA, as indicated by the fact that formation of the mRNA–protein complex was not decreased by the addition of excess unrelated competitor RNA (Fig. 1, Lanes 5 and 6) and did not interact with CDO mRNA (Fig. 1, Lanes 8 and 9).

The signal of the RNA–protein complex was reduced by preincubation with anti-PTB antibody (Fig. 2A, Lane 4), but not by preincubation with control IgG (Fig. 2A, Lane 3). This result indicates that this albumin mRNA–protein complex contains PTB. Formation of the albumin mRNA–PTB complex was prevented by competitor transcripts 1–92 and 1–73 (Fig. 2B, Lanes 2–5), but not by competitor transcripts 1–53 (Fig. 2B, Lanes 6 and 7). The sequence of human albumin mRNA from nucleotides 62–73 is similar to that of a known PTB consensus-binding sequence (Fig. 2C).

3.2. Effect of amino acids on the levels of secreted albumin, albumin mRNA and albumin mRNA–PTB complex

HepG2 cells cultured in AA-free medium secreted significantly less albumin than cells cultured in AA-complete medium (Fig. 3A), with no difference in albumin mRNA levels (Fig. 3B). The level of the albumin mRNA–PTB complex in cell extracts was higher for cells cultured in AA-free medium than for cells cultured in AA-complete medium (Fig. 3C).

3.3. Effect of amino acids on the subcellular localization of PTB

In HepG2 cells cultured in MEM containing 10% FBS, PTB was mainly found in the nucleus of cells (Fig. 4A, upper panel). Cells cultured in AA-free medium had decreased nuclear PTB immunoreactivity and increased cytoplasmic PTB immunoreactivity (Fig. 4A, middle panel), compared to cells cultured in AA-complete medium (Fig. 4A, lower panel). There was no difference in the localization of PGC-1 between cells cultured in AA-free medium and cells cultured in AA-complete medium (Fig. 4B).

After preculture for 2 h in AA-free medium, cells were cultured in AA-complete medium for 0, 1, 2, 4 or 6 h. There were no significant differences in the total level of PTB in cell lysates among these five different durations of culture (Fig. 5A). After 2 h of culture in AA-complete medium, the level of PTB in the cytoplasmic fraction was lower than it was at 0 h of culture and remained at this reduced level until 6 h of culture (Fig. 5A). After 6 h of culture, the level of PTB in the nuclear fraction had increased (Fig. 5B) and the level of PTB in the cytoplasmic fraction had decreased (Fig. 5C), compared with cells cultured in AA-free medium.

3.4. Effect of the mTOR inhibitor rapamycin on amino-acid-induced nuclear import of PTB and albumin synthesis

Ribosomal protein S6, which is a downstream effector of mTOR, was phosphorylated in cells cultured in AA-complete medium, and the mTOR inhibitor rapamycin completely inhibited its phosphorylation (Fig. 6A). Compared with culture in AA-free medium without rapamycin, culture in AA-complete medium without rapamycin caused a 22% decrease in the level of PTB in the cytoplasmic fraction, and culture in AA-complete medium with rapamycin caused a 67% decrease in the level of PTB in the cytoplasmic fraction (Fig. 6B). In cells cultured in AA-complete medium with

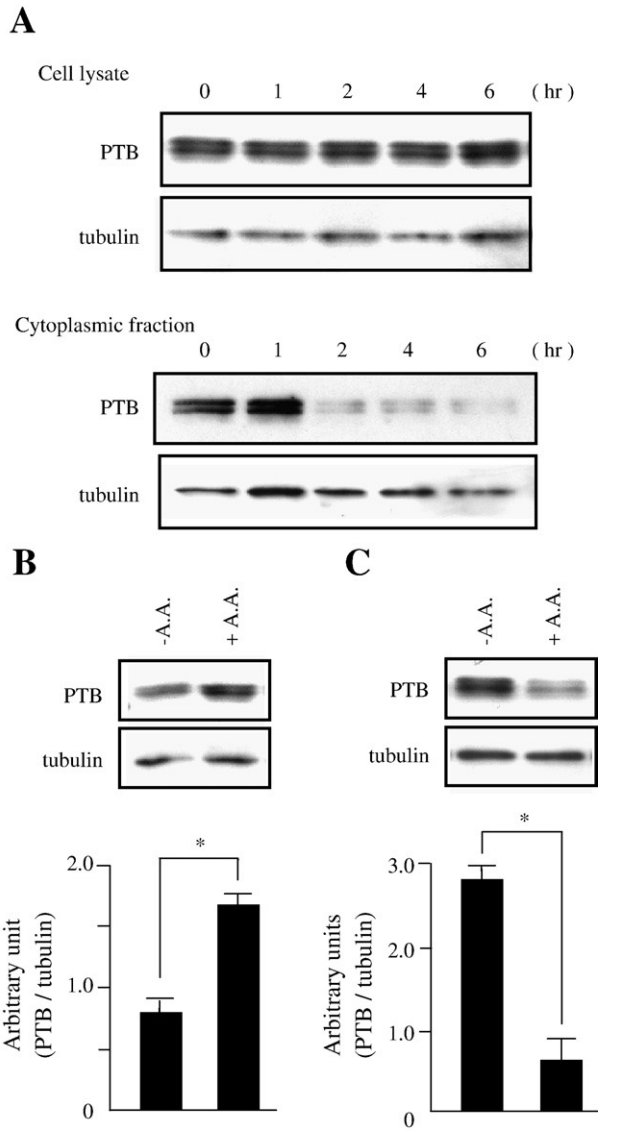

Fig. 5. Effect of culture in AA-complete medium on the level of PTB in cytoplasmic fraction. (A) After preculture for 2 h in AA-free medium (−AA), HepG2 cells were cultured in AA-complete medium (+AA) for 0–6 h. Whole-cell lysates and cytoplasmic fraction were analyzed by Western blot analysis using anti-PTB antibody. The blot was stripped and reprobed with anti-tubulin antibody to confirm equal loading. The data shown are representative of four independent experiments. (B and C) After preculture for 2 h in −AA, HepG2 cells were cultured in −AA or in +AA for 6 h. Nuclear fraction (B) and cytoplasmic fraction (C) were analyzed by Western blot analysis using anti-PTB and anti-tubulin antibodies. The corresponding densitometric analysis is shown graphically. Values are presented as mean±S.E. (n=4). *P<.05.

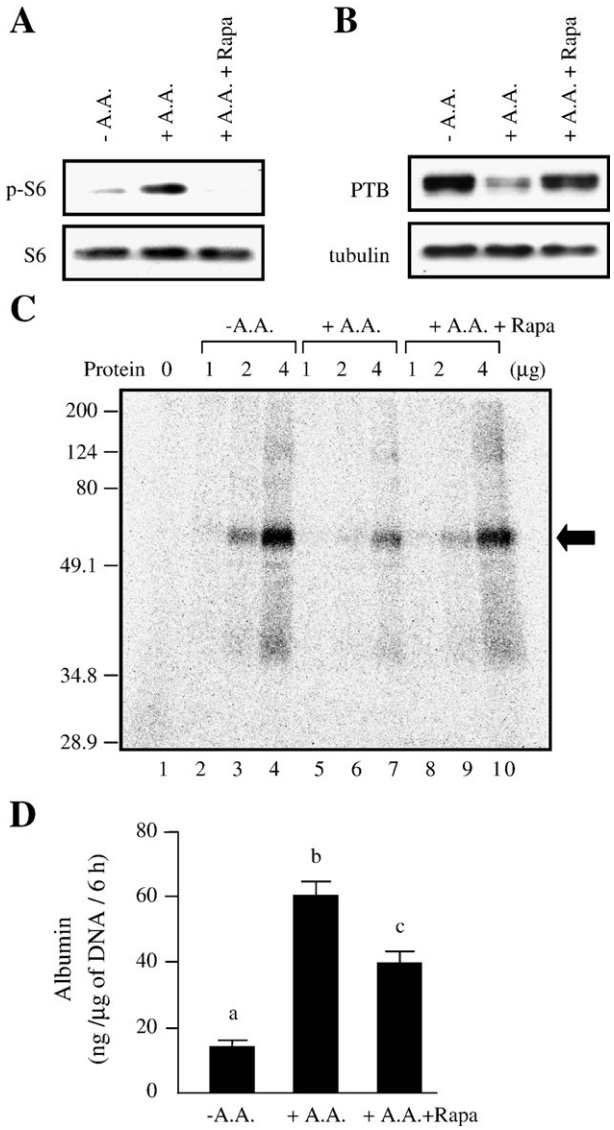

Fig. 6. Effect of rapamycin on the level of PTB in cytoplasmic fraction and the level of secreted albumin. After preculture for 2 h in AA-free medium (−AA), HepG2 cells were cultured in −AA, in AA-complete medium (+AA) or in +AA with rapamycin (+AA+Rapa). (A) HepG2 cells were cultured for 30 min. Cell extracts were analyzed by Western blot analysis using anti-S6 (S6) and anti-phospho-S6 (p-S6) antibodies. (B) HepG2 cells were cultured for 2 h. Cytoplasmic fraction was analyzed by Western blot analysis using anti-PTB and anti-tubulin antibodies. The data shown are representative of four independent experiments. (C) Albumin cRNA probe was incubated alone (Lane 1) or with 1 μg (Lanes 2, 5 and 8), 2 μg (Lanes 3, 6 and 9) or 4 μg (Lanes 4, 7 and 10) of cell extracts prepared from cells cultured in −AA (Lanes 2–4), in +AA (Lanes 5–7) or in +AA+Rapa (Lanes 8–10) for 6 h, followed by UV cross-linking analysis. Solid arrow indicates the RNA–PTB complex. The data shown are representative of three independent experiments. (D) Level of secreted albumin from cells cultured in −AA, in +AA or in +AA+Rapa for 6 h. Values are presented as mean±S.E. (n=6). Means not sharing the same letter are significantly different from each other (P<.05).

rapamycin for 6 h, the level of the albumin mRNA–PTB complex increased (Fig. 6C) and the level of secreted albumin significantly decreased (Fig. 6D), compared with cells cultured in AA-complete medium without rapamycin.

3.5. Effect of BCAA on the levels of secreted albumin and albumin mRNA–PTB complex

The addition of leucine to AA-free medium (producing a “leucine-only medium”) increased the level of secreted albumin in a dose-dependent manner as the level of leucine increased from 0.125 to 1.0 mM (Fig. 7A). Valine and isoleucine had no effect on the level of secreted albumin (Fig. 7B). There was no difference in the level of albumin mRNA among the three “BCAA-only media” (data not shown).

After 2 h of culture in the leucine-only medium, the level of PTB in the cytoplasmic fraction decreased, compared with cells cultured in AA-free medium (0 h) (Fig. 8A). However, after 4 h of culture in the leucine-only medium, the level of PTB in the cytoplasmic fraction had returned to a level similar to that of cells cultured in AA-free medium (Fig. 8A). Valine and isoleucine had no effect on the level of PTB in the cytoplasmic fraction (Fig. 8B). The level of the albumin mRNA–PTB complex decreased in cell extracts prepared from cells cultured in the leucine-only medium (Fig. 8C,

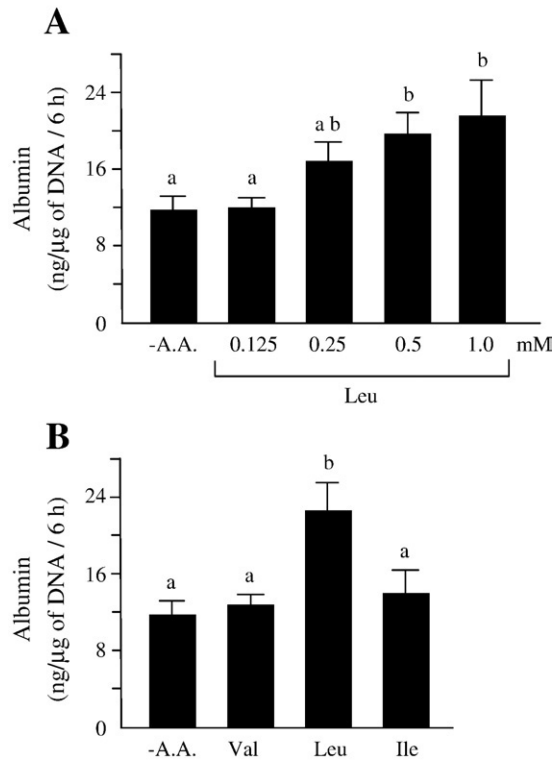

Fig. 7. Effect of each BCAA on levels of secreted albumin. (A) After preculture for 2 h in AA-free medium (−AA), HepG2 cells were cultured in −AA or in −AA with added leucine at four different concentrations for 6 h. Secreted albumin was measured. Values are presented as mean±S.E. (n=4). Means not sharing the same letter are significantly different from each other (P<.05). (B) After preculture for 2 h in −AA, HepG2 cells were cultured in −AA or in −AA with added valine, leucine or isoleucine at 1.0 mM for 6 h. Secreted albumin was measured. Values are presented as mean±S.E. (n=4). Means not sharing the same letter are significantly different from each other (P<.05).

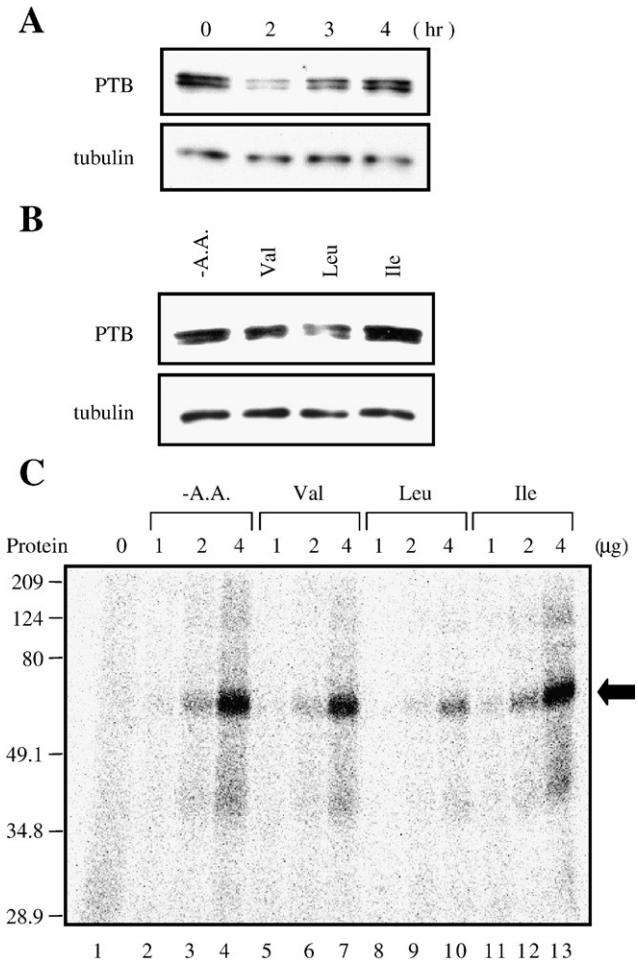

Fig. 8. Effect of leucine on the level of PTB in cytoplasmic fraction and on the level of the albumin mRNA–PTB complex. (A) After preculture for 2 h in AA-free medium (−AA), HepG2 cells were cultured in −AA with added leucine at 1.0 mM for 0–4 h. Cytoplasmic fractions were analyzed by Western blot analysis using anti-PTB and anti-tubulin antibodies. (B and C) After preculture for 2 h in −AA, HepG2 cells were cultured in −AA or in −AA with added valine, leucine or isoleucine at 1.0 mM for 2 h. (B) PTB in cytoplasmic fractions was measured by Western blot analysis using anti-PTB and anti-tubulin antibodies. (C) Albumin cRNA probe was incubated alone (Lane 1) or with 1 μg (Lanes 2, 5, 8 and 11), 2 μg (Lanes 3, 6, 9 and 12) or 4 μg (Lanes 4, 7, 10 and 13) of cell extracts prepared from cells cultured in −AA (Lanes 2–4) or in −AA with added valine (Lanes 5–7), leucine (Lanes 8–10) and isoleucine (Lanes 11–13), followed by UV cross-linking analysis. Solid arrow indicates the RNA–PTB complex. The data shown are representative of three independent experiments.

Lanes 8–10), compared with cells cultured in AA-free medium, “valine-only medium” or “isoleucine-only” medium (Fig. 8C, Lanes 2–4, 5–7 and 11–13).

4. Discussion

Malnutrition is an increasingly recognized complication of chronic liver disease that has important prognostic implications. Serum albumin is one of the most common parameters used in evaluating nutritional status. Cirrhosis patients with hypoalbuminemia have a higher rate of complications [3–5]. Previous studies have indicated that administration of BCAA improves hypoalbuminemia in patients with cirrhosis [10–12]. The present findings help to clarify the mechanism by which BCAA promotes albumin synthesis.

In a previous study, we have found that formation of the albumin mRNA–PTB complex was associated with decreased albumin synthesis [17]. In the present study, we measured albumin concentration in a cell culture medium as a marker of albumin synthesis in HepG2 cells. Albumin is secreted rapidly by cells after it is produced. In the case of HepG2 cells, the intracellular retention half-time of albumin is about 35 min [29]. Thus, changes in the concentration of albumin in a culture medium of HepG2 cells can be assumed to closely reflect changes in albumin synthesis. The results of the present study suggest that PTB is involved in the regulation of albumin synthesis that occurs in response to the exposure of HepG2 cells to amino acids.

Although PTB moves through the cytoplasm, it is predominantly detected in the nucleus at steady state, like most nuclear shuttling proteins [19,21]. The present findings suggest that nucleocytoplasmic localization of PTB is regulated at least partly via the mTOR signaling pathway because rapamycin inhibited the nuclear import of PTB in cells cultured in AA-complete medium. Leucine, but not valine or isoleucine, activates the mTOR signaling pathway [15,30]. In the present study, when HepG2 cells were cultured in the leucine-only medium, both the level of the albumin mRNA–PTB complex and the level of PTB in the cytoplasmic fraction decreased, and the level of secreted albumin increased. However, after the initial decrease in PTB observed at 2 h in cells cultured in the leucine-only medium, the level of PTB gradually increased. In contrast, in cells cultured in AA-complete medium, PTB had decreased at 2 h and remained at a low level at 6 h. Furthermore, the increase in the level of secreted albumin in cells cultured in the leucine-only medium was less than the increase in the level of secreted albumin in cells cultured in AA-complete medium. In a previous study, the mTOR signaling pathway was activated for only a short duration in cells cultured in the leucine-only medium, whereas it was activated for a long duration in cells cultured in AA-complete medium [30]. The present difference in results between the leucine-only medium and the AA-complete medium is consistent with this difference in the duration of mTOR signal activation.

Whereas activation of mTOR signaling was observed after 30 min of culture in AA-complete medium, the nuclear import of PTB was not observed until 2 h of culture in AA-complete medium. This time difference suggests that additional factors regulated by mTOR are involved in the localization of PTB. It has been reported that cAMP-dependent phosphorylation of PTB by protein kinase A induces its nucleocytoplasmic transport in neuroendocrine

PC12 cells [24]. In a study using pancreatic islet cells, culture in a glucose-containing medium induced nucleocytoplasmic transport of PTB and an increase in the level of a 27-kDa PTB species in the nucleus [21]. In a recent study, we found the same expression pattern of PTB in islets prepared from rats with acute liver failure [28]. On the other hand, in the present study, no 27-kDa PTB species was detected in the nuclear fraction, although nucleocytoplasmic transport of PTB was induced in cells cultured in AA-free medium (data not shown). Clarification of the role that the mTOR signaling pathway plays in PTB localization is important to understanding the molecular mechanisms by which albumin synthesis is promoted by BCAA.

The present results suggest that leucine promotes albumin synthesis via the regulation of PTB localization. However, it has been previously reported that administration of leucine to food-deprived rats reduced circulating concentrations of isoleucine and valine, compared with control rats [31]. This indicates that to improve hypoalbuminemia in patients with cirrhosis, it is necessary to administer a mixture of BCAA rather than leucine alone.

The cellular models used in the present study to investigate the effects of amino acids (especially BCAA) on albumin synthesis are quite different from in vivo conditions, in which serum and a mixture of amino acids are always present. Rats with carbon-tetrachloride-induced cirrhosis have been found to have lowered Fischer's ratios (the molar ratio of BCAA to aromatic amino acids) [16,32]. In studies using rats with carbon-tetrachloride-induced cirrhosis, supplementation with BCAA stimulated the mTOR signaling pathway in their livers [16] and prevented hypoalbuminemia [32]. Further study is needed to determine whether experimental induction of cirrhosis in rats leads to nucleocytoplasmic transport of PTB in the liver of those rats and whether supplementation with BCAA stimulates the nuclear import of PTB in the liver of those rats.

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