Polypyrimidine Tract-Binding Protein Is Involved in Regulation of Albumin Synthesis in Response to Food Intake

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Summary Our recent study demonstrates that polypyrimidine tract-binding protein (PTB), which is a sequence specific RNA-binding protein, attenuates albumin synthesis in a cell-free translation system. In this study, the effects of food intake on regulation of albumin synthesis through binding of PTB to albumin messenger RNA (mRNA) were investigated. Rats were divided into 1 of 3 groups: fed; fasted for 36 h; or fasted for 36 h and then refed for 24 h. No significant differences in albumin mRNA levels were found among fed, fasted and refed rats. However, a decrease in the proportion of albumin mRNA associated with polysomes was identified in fasted rats. Furthermore, UV-cross linking analysis demonstrated that levels of albumin mRNA-PTB complex were increased in liver extracts from fasted rats. No significant differences in PTB levels in liver homogenate were found among the experimental groups. However, PTB level in the cytoplasmic fraction was higher in fasted rats than in fed rats. In refed rats, PTB level in the cytoplasmic fraction returned to a level comparable to that in fed rats, but was inhibited by treatment with rapamycin, a mammalian target of rapamycin (mTOR) inhibitor. These results suggest that localization of PTB is regulated by food intake through mTOR signaling, and alterations in level of albumin mRNA-PTB complex play a role in mediating the effects of food intake on albumin synthesis in the rat liver.

Key Words albumin synthesis, food intake, polypyrimidine tract-binding protein, mammalian target of rapamycin, rats

Plasma albumin has become an indispensable factor for evaluating nutritional status. In the regulation of albumin synthesis, meal stimulation is one of the most important factors. For example, albumin synthesis is activated by meal feeding in healthy subjects, whereas fibrinogen synthesis remains unchanged (1). Furthermore, albumin synthesis is reportedly influenced by varying the relative contribution of protein from animal and vegetable sources in the meal (2), and consumption of only the protein component of a meal is sufficient to stimulate albumin synthesis (3).

In rats under conditions of protein malnutrition, fractional and absolute synthesis rates of total protein in liver are decreased, and the rate of hepatic synthesis of secreted proteins, particularly albumin, is greatly reduced compared with the rate of synthesis of liver domestic proteins (4). As with protein deficiency, a

reduction in the rate of albumin synthesis is observed under fasting (5–7). Level of albumin messenger RNA (mRNA) is unchanged by fasting and refeeding (8), and reduced albumin synthesis in fasted rats is associated with disaggregation of membrane-bound polysomes and a shift of albumin mRNA to free polysomes and to the post-ribosomal supernatant fraction (9, 10). Translational regulation thus plays an important role in reduced albumin synthesis under fasting.

Polypyrimidine tract-binding protein (PTB) is an abundant eukaryotic RNA-binding protein implicated in several aspects of mRNA metabolism, including splicing regulation (11), RNA nuclear export (12), internal ribosome entry site-mediated translation initiation (13), mRNA stability (14), and cytoplasmic RNA localization (15). Our recent study demonstrated that 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 (16). These results suggest that binding of PTB to albumin mRNA would suppress translation.

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Molecular mechanisms underlying the reduction in albumin synthesis under conditions of malnutrition remain unclear. The objective for this study was to determine whether PTB is involved in the regulation of albumin synthesis in fasted rats.

MATERIALS AND METHODS

Animals and feeding protocol. The animal facilities and protocol were reviewed and approved by the Institutional Animal Care and Use Committee of Tokushima University. Male Sprague-Dawley rats (weight, 200 g) were purchased from Charles River Japan, Inc. (Yokohama, Japan). Rats were housed individually in a temperature- and humidity-controlled room with 12-h light and dark exposure, and were fed a commercial diet (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and given water ad libitum. After an acclimatization period of 5 d, rats were randomly separated into different groups: fed (ad libitum); fasted (for 36 h, starting at 20:00); and refed (fed for 24 h after fasting for 36 h). Some refed rats were injected intraperitoneally with rapamycin (0.4 mg/kg; Wako Pure Chemical Industries, Ltd., Osaka, Japan) or vehicle (dimethyl sulfoxide) before refeeding. Each rat was anesthetized with sodium pentobarbitol (60 mg/kg). Blood was drawn and centrifuged to separate plasma. The liver was rapidly removed and weighed. Plasma and liver samples were stored at -70°C until analysis.

Measurement of plasma albumin and insulin. Plasma concentrations of albumin and insulin were determined using the albumin B test (Wako, Osaka, Japan) and ELISA Insulin Kit (Morinaga, Yokohama, Japan), respectively.

Northern blot analysis. Total RNA was extracted from rat livers using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Northern blot was performed as described previously (17). Filters were exposed and analyzed with a Fuji BAS-1500 system.

Polysome preparation and slot blot analysis. Polysomes were prepared from livers and analyzed by sucrose density gradient centrifugation as described previously (17). After centrifugation, gradient fractions (1 mL each) were collected from the top, and absorbance at 254 nm was measured. Each of the fractions was extracted with phenol:chloroform, and RNA was

precipitated with ethanol. The RNA pellet was dissolved in water and denatured with 25% formaldehyde, $5\times\text{SSC}$ at 65°C for 15 min. Denatured RNA was blotted directly onto a nylon filter in a slot blot apparatus. Blotted RNA was covalently cross-linked by exposure to UV light. Hybridization was performed as described previously (17).

Preparation of ribosome salt wash (RSW) fraction and RNA-binding analysis. Preparation of RSW fraction

Fig. 1. Effect of food intake on albumin mRNA levels in rat liver. (A) Levels of albumin mRNA and GAPDH mRNA were measured in rats fasted for 0 h (Fed) or 36 h (Fasted) or fasted for 36 h and then refed for 24 h (Refed). The corresponding densitometric analysis is shown. All values were corrected for differences in blotting using GAPDH signals. Values represent mean $\pm$ SE ($n=6$). (B) Polysomes were resolved by centrifugation in 0.5–1.5 mol/L linear sucrose density gradients. After centrifugation, sucrose gradients were separated 1–7 from top to bottom of gradients. Absorbance at 254 nm of fractions was measured. The study was repeated twice and 2 animals per condition were analyzed. Data shown are representative of 2 independent experiments. (C) RNA was isolated from each fraction and subjected to slot blot analysis using albumin and GAPDH cDNA probes. The corresponding densitometric analysis is shown. All values were corrected for differences in blotting using GAPDH signals. Values represent mean $\pm$ SE ($n=4$).

Table 1. Effect of fasting and refeeding on body weight, liver weight, plasma albumin and insulin.¹

	Fed	Fasted	Refed
Body weight, g	263.3 $\pm$ 6.2	218 $\pm$ 3.5*	248.6 $\pm$ 8.1
Liver weight, g	10.3 $\pm$ 0.7	6.5 $\pm$ 0.6*	10.3 $\pm$ 0.6
Plasma			
Albumin, g/dL	3.6 $\pm$ 0.1	3.5 $\pm$ 0.2	3.6 $\pm$ 0.2
Insulin, ng/mL	1.4 $\pm$ 0.3	0.3 $\pm$ 0.1*	2.0 $\pm$ 0.4

¹ Values represent mean $\pm$ SE ($n=6$).

* Significantly different from fed group, $p<0.05$.

Fig. 2. Effect of food intake on formation of albumin mRNA-PTB complex. (A) RSW extracts from livers of refed rats were incubated with 0.5 μ g of control IgG (lane 3) or anti-PTB antibody (lane 4) for 30 min. A full-length albumin cRNA probe was then added. Lane 2 shows a control reaction with no antibody. Lane 1 contains only the probe. Complexes were separated by 4% PAGE. Solid arrow indicates albumin mRNA-PTB complex. (B) The full-length albumin cRNA probe was incubated alone (lane 1) or with 5 μ g (lanes 2, 4 and 6) or 10 μ g (lanes 3, 5 and 7) of RSW extracts prepared from liver of fed (lanes 2, 3), fasted (lanes 4, 5) or refed (lanes 6, 7) rats, and subjected to UV-cross linking analysis. Solid arrow indicates albumin mRNA-PTB complex. The study was repeated 3 times and 2 animals per condition were analyzed. Data shown are representative of 3 independent experiments. (C) RSW extracts were analyzed by Western blot using anti-PTB antibody. The corresponding densitometric analysis is shown. Values represent mean $\pm$ SE ($n=4$). Means not sharing the same letter are significantly different from each other, $p<0.05$.

from livers and subsequent RNA mobility shift analysis and UV-cross linking analysis were performed as described previously (16).

Western blot analysis. For Western blot analysis using anti-PTB (Zymed Laboratories Inc., CA) and anti-actin (Chemicon International, CA), liver was homogenized in 0.35 mol/L sucrose buffer containing 0.2 mol/L Tris-Cl (pH 8.5), 50 mmol/L KCl and 10 mmol/L Mg-acetate. Part of the homogenate was centrifuged at 10,000 $\times g$ for 30 min at 4°C. This supernatant fraction was used as the cytoplasmic fraction.

For Western blot analysis using anti-S6 and anti-phospho-S6 (Ser235/236) antibodies (Cell Signaling Technology, MA), liver was homogenized in a buffer containing 20 mmol/L HEPES (pH 7.4), 100 mmol/L KCl, 0.2 mmol/L EDTA, 2 mmol/L EGTA, 1 mmol/L dithiothreitol, 50 mmol/L NaF, 50 mmol/L β -glycerophosphate, 0.1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L benzamidine and 0.5 mmol/L sodium vanadate. Homogenates were centrifuged at 8,000 $\times g$ for 30 min at 4°C. The resulting supernatant fraction was

subjected to Western blot analysis.

Western blot analysis was performed as described previously (18).

Statistical analysis. Data are expressed as mean $\pm$ SE. Student's *t* test was used to analyze differences between two groups. Statistical analysis for multiple comparisons was performed using one-way analysis of variance followed by a Tukey-Kramer post hoc test. Data analysis was performed using Statcel2 software (Oms Publishing Inc., Tokyo, Japan) and values of $p<0.05$ were considered statistically significant.

RESULTS

Characteristics of experimental animals

To study the mechanisms of albumin synthesis in response to food intake, rats were divided into 3 groups (Table 1). Body and liver weights were reduced after 36 h of fasting to approximately 80% and 60% of fed rats, respectively. After refeeding for 24 h, no significant differences in body or liver weights were seen compared to fed rats. Concentration of plasma albumin did not dif-

Fig. 3. Effect of food intake on localization of PTB protein in rat liver. Liver homogenate (A) and cytoplasmic fraction (B) were analyzed by Western blot using anti-PTB and anti-actin antibodies. The corresponding densitometric analysis is shown. Values represent mean $\pm$ SE ($n=6$). Means not sharing the same letter are significantly different from each other, $p<0.05$.

fer significantly among groups, probably because the experimental period was short. Concentration of plasma insulin was significantly decreased in fasted rats compared to the other 2 groups.

Effect of food intake on albumin gene expression

Level of albumin mRNA did not show any significant differences in any of the experimental groups (Fig. 1A). To determine whether the decrease in albumin synthesis was related to a modification of translation efficiency for albumin mRNA, we analyzed the polysome distribution of albumin mRNA after centrifugation in sucrose density gradient. Mean size of polysomes was slightly decreased in fasted rats compared to fed and refed rats (Fig. 1B). Furthermore, in fasted rats, the level of albumin mRNA was increased in non-polysomal fractions (Fig. 1C, fractions 1 and 2) and was decreased in polysomes (Fig. 1C, fractions 4–7). Conversely, refed rats displayed distribution patterns of albumin mRNA similar to those of fed rats.

Effect of food intake on level of albumin mRNA-PTB complex

In RNA mobility shift analysis, 32 P-labeled full-length albumin mRNA formed a complex when incubated with RSW extracts (Fig. 2A, lane 2). This complex contained PTB because the signal of the complex was

Fig. 4. Effect of rapamycin on level of PTB in cytoplasmic fraction. Refed rats were treated with rapamycin or vehicle before refeeding. (A) Phosphorylation of S6 was analyzed by Western blot using anti-phospho-S6 (P-S6) and anti-S6 (S6) antibodies. Liver homogenate (B) and cytoplasmic fraction (C) were analyzed by Western blot using anti-PTB and anti-actin antibodies. The corresponding densitometric analysis is shown. Values represent mean $\pm$ SE ($n=4$). * $p<0.05$.

reduced by preincubation with anti-PTB antibody (Fig. 2A, lane 4). The level of albumin mRNA-PTB complex was markedly higher in RSW extracts from fasted rats (Fig. 2B, lanes 4 and 5) than in RSW extracts from fed rats (Fig. 2B, lanes 2 and 3). In refed rats (Fig. 2B, lanes 6 and 7), level of albumin mRNA-PTB complex decreased to almost the level of fed rats. The level of PTB protein was markedly increased in RSW extracts from fasted rats, compared with levels in RSW extracts from fed and refed rats (Fig. 2C).

Effect of food intake on subcellular localization of PTB

The abundance of PTB protein in liver homogenate did not differ significantly in any of the experimental groups (Fig. 3A). However, the level of PTB was

increased in the cytoplasmic fraction in fasted rats (Fig. 3B), compared with levels in fed and refed rats.

Effect of rapamycin on subcellular localization of PTB in liver of refed rats

Ribosomal protein S6, a downstream effector of the mammalian target of rapamycin (mTOR), was phosphorylated in livers of refed rats, and treatment with the mTOR inhibitor rapamycin completely inhibited phosphorylation (Fig. 4A). Rapamycin had no effect on hepatic PTB abundance (Fig. 4B). However, in refed rats administered rapamycin, the level of PTB was increased in the cytoplasmic fraction, compared with the level in refed rats not administered rapamycin (Fig. 4C).

DISCUSSION

Nutritional state is known to be important in regulating albumin synthesis (1–7). Our recent findings (16) demonstrated that PTB interacts with a pyrimidine-rich region of albumin mRNA from nucleotides 41 to 70 (position relative to transcription start site) and attenuates albumin synthesis. The present results indicate that alterations in the level of albumin mRNA-PTB complex play a role in mediating the effects of food intake on translation efficiency of albumin mRNA.

PTB is predominantly detected in the nucleus at steady state (12, 14, 18). The present findings suggest that food intake modulates the cellular distribution of PTB. The localization of PTB is regulated via the mTOR signaling pathway, as the refeeding-induced decrement of PTB in the cytoplasmic fraction was inhibited by rapamycin treatment. The effect of rapamycin on the level of albumin mRNA-PTB complex was not examined in the present study. However, this result supports our recent findings that amino acid-induced nuclear import of PTB and amino acid-induced decrement of albumin mRNA-PTB complex are inhibited by rapamycin treatment in human hepatoma HepG2 cells (18). Previous studies reported that food intake was not affected by an intraperitoneal injection of rapamycin (19, 20). However, in the present study, we can not exclude the possibility that the food intake influences the results obtained from rapamycin-treated rats.

Activation of the mTOR signaling pathway by feeding leads to increased translation of mRNAs containing a terminal oligopyrimidine (TOP) sequence at the 5'-end of the transcript (21, 22). The mRNAs encoding most ribosomal proteins contain a 5'-TOP sequence (23). Rapamycin treatment before a refeeding for 24 h clearly inhibited recruitment of ribosomal protein mRNA into polysomes (22). On the other hand, the results of microarray analysis showed that 78 different mRNAs had increased polysome association after feeding in the liver (21). However, 36 of the 78 mRNAs lack a 5'-TOP sequence. These mRNAs may be regulated by feeding through unique mechanisms. Regulation of translation through the binding of PTB may be involved in one of such mechanisms. On the other hand, activation of mTOR signaling was observed at 1 h after refeeding in livers of 18 h-starved rats (24). However, nuclear

import of PTB was not seen for 8 h after the meal in refed rats (data not shown). This time difference suggests that additional factors regulated by mTOR are involved in regulation of the localization of PTB. Furthermore, circulating concentrations of branched-chain amino acids are known to be increased in the fasted state, primarily as a result of skeletal muscle catabolism (25, 26). This increase might induce activation of the mTOR signaling pathway. As in the previous study (22), phosphorylation of S6 was increased in fasted rats compared with fed rats (data not shown). However, the level of PTB in the cytoplasmic fraction is increased in fasted rats. Activation of other signaling, such as insulin signaling, might be required for nuclear import of PTB with activation of the mTOR signaling pathway.

PTB plays an important role in the nuclear export of hepatitis B virus RNA (12). Our data showed that the level of albumin mRNA-PTB complex was increased in RSW extracts from fasted rats. This result raises the possibilities that PTB may serve as a nuclear export factor of albumin mRNA, and dissociation of PTB from albumin mRNA may be attenuated in the fasting condition. Further study is needed to determine whether the albumin mRNA-PTB complex is formed in nuclei or outside of nuclei.

A recent genome-wide analysis using HeLa cell extracts has shown that PTB can associate with 20% of mRNA in the cytoplasmic fraction, and that PTB-associated transcripts are enriched in mRNA species that encode proteins implicated in intracellular transport, vesicle trafficking, and apoptosis (27). The present study focused on posttranscriptional regulation of albumin mRNA under fasting conditions. However, in the fasting liver, the possibility exists that not only albumin mRNA, but also numerous mRNAs are regulated through PTB binding. To clarify the influence of localization changes of PTB in response to food intake on liver physiology, identifying the target mRNAs of PTB in the liver will be important.

In summary, food deprivation decreases albumin synthesis in rat liver. This effect is mediated primarily by a posttranscriptional mechanism. Clarification of the regulation of PTB localization is thus important for understanding the molecular mechanisms of albumin synthesis under the condition of malnutrition. The present findings suggest that diet-induced changes in the abundance of albumin mRNA-PTB complex in rat liver may regulate the translation efficiency of albumin mRNA. To establish this hypothesis, it will be important to evaluate the amount of albumin mRNA in RNA-PTB complex immunoprecipitated by anti-PTB antibody. Furthermore, investigations are needed to elucidate the dietary composition important for reducing levels of albumin mRNA-PTB complex.

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