Journal of Cellular Biochemistry 57:185–190 (1995)

Enhanced Expression of Calcium-Binding Protein Regucalcin mRNA in Regenerating Rat Liver

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Abstract The expression of hepatic calcium-binding protein regucalcin mRNA was investigated in regenerating rat liver. The change of regucalcin mRNA levels was analyzed by Northern blotting, using liver regucalcin cDNA (0.9 kb with complete open reading frame). The reduced liver weight by partial hepatectomy (about 70%) was completely restored at 3 days after surgery. Regenerating liver significantly increased calcium content. Liver regucalcin mRNA levels clearly increased 1–5 days after hepatectomy, in comparison with that of sham-operated rats, although the increase was not seen 12 hr after the surgery. Increased regucalcin mRNA levels in regenerating liver were appreciably reduced by single intraperitoneal administration of actinomycin D (100 μg/100 g body weight), an inhibitor of transcriptional process. Moreover, the increased regucalcin mRNA levels by hepatectomy was weakened by a single intraperitoneal administration of trifluoperazine (2.5 mg/100 g), an inhibitor of Ca^{2+}/calmodulin. These findings demonstrate that the expression of hepatic regucalcin mRNA is enhanced in regenerating rat liver.

Key words: regucalcin, calcium-binding protein, gene expression, Northern blot analysis, regenerating rat liver

Calcium ion (Ca^{2+}) plays an important role in regulating of many cell functions. Liver metabolism is regulated by increased Ca^{2+} in the cytosol of liver cells due to hormonal stimulation [Cheung, 1980; Williamson et al., 1981; Kraus-Friedman, 1990]. The Ca^{2+} effect is amplified by calmodulin and protein kinase C. It was recently reported that a novel Ca^{2+}-binding protein (regucalcin) has a reversible effect on the activation and inhibition of various enzymes by Ca^{2+} in liver cells [Yamaguchi and Tai, 1991; Yamaguchi and Mori, 1990; Yamaguchi and Sakurai, 1991, 1992]. Regucalcin can inhibit activation of Ca^{2+}/calmodulin-dependent cAMP phosphodiesterase [Yamaguchi and Tai, 1991], protein kinase C [Yamaguchi and Mori, 1990] and Ca^{2+}-activated DNA fragmentation [Yamaguchi and Sakurai, 1991], due to binding of Ca^{2+}. Regucalcin probably plays an important role in the regulation of liver cell functions related to Ca^{2+}.

More recently, we have identified a sequencing of the complementary deoxyribonucleic acid (cDNA) and the determination of a complete amino acid sequence for regucalcin in rat liver [Shimokawa and Yamaguchi, 1993b]. Regucalcin has been demonstrated to be specifically synthesized in the liver of rats from the observations of Northern blot analyses by using liver regucalcin cDNA as a probe [Shimokawa and Yamaguchi, 1992] and of enzyme-linked immunoadsorbent assay (ELISA) with rabbit anti-regucalcin IgG [Yamaguchi and Isogai, 1993]. The expression of hepatic regucalcin mRNA may be mediated through Ca^{2+}/calmodulin [Shimokawa and Yamaguchi, 1993a]. The expression of regucalcin mRNA under a physiological condition is not fully clarified, however. The present investigation was undertaken to clarify whether the expression of regucalcin mRNA is demonstrated in regenerating rat liver, which induces a proliferation of liver cells by hepatectomy. It was found that regucalcin mRNA enhances in regenerating rat liver.

MATERIALS AND METHODS

Chemicals

Deoxycytidine 5′-[α-^{32}P]triphosphate ([^{32}P] dCTP); 110 Tbq/mmol and nylon membranes (Hybond N^+) for Northern hybridization were obtained from Amersham (Buckingham-

Received January 24, 1994; revised April 5, 1994; accepted June 14, 1994.

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shire, England). A human β-actin gene fragment (0.43 kb) as an internal standard was obtained from Wako Pure Chemical Co. (Osaka, Japan). Molecular-size standards (0.24–9.5-kb RNA ladder) for electrophoresis of RNA was purchased from Bethesda Research Laboratories (Gaithersburg, MD). Trifluoperazine dimaleate (TFP) and actinomycin D were obtained from Sigma Chemical Co. (St. Louis, MO). Other reagents were purchased from Wako Pure Chemical Co. Any water and solutions used for RNA preparation were treated with chemical diethylpyrocarbonate (DEPC, Sigma) to inhibit RNase activity.

Animals and Hepatectomy

Male Wistar rats, weighing 80–100 g, purchased from Japan SLC, Inc. (Hamamatsu, Japan), were fed commercial laboratory chow (solid, Oriental Yeast Co., Ltd., Tokyo) containing 57.5% carbohydrate, 1.1% calcium, and 1.1% phosphorus, and distilled water, ad libitum. Partial hepatectomy was carried out by excision of two-thirds of the liver (the medium and left lateral lobes), as described by Higgins and Anderson [1931]. Rats were anesthetized with ether before surgery. In sham-operated rats, an incision was made, and the liver was manipulated, but not ligated. Surgery was routinely performed at 12:00–14:00.

Administration Procedure

Actinomycin D or TFP was administered at 3 days after being sham-operated and after hepatectomy. Actinomycin D was dissolved in 99.5% ethanol at a concentration of 1 mg/ml. This solution (0.1 ml/100 g body weight) was intraperitoneally administered to rats; 30, 60, and 120 min later, the animals were sacrificed by bleeding. TFP (2.5 mg/ml) was dissolved in sterile distilled water. This solution (1.0 ml/100 g body weight) was administered intraperitoneally to rats. At 18 hr after administration, rats were sacrificed by bleeding. The livers were perfused with ice cold 0.25 M sucrose solution and immediately removed and frozen at −80°C. Control animals received vehicle solution.

Isolation of RNA

Hepatic total RNAs were prepared as described [Chomczynski and Sacchi, 1987]. Liver was quickly removed, rinsed with ice cold 0.25 M sucrose solution, and homogenized in buffer solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% sarcosyl, 0.1 M 2-mercaptoethanol, and 2 M sodium acetate. Total RNAs were extracted by vigorous shaking in a mixture of phenol, chloroform, and isoamylalcohol, and the phases were separated by centrifugation at 10,000*g* for 20 min at 4°C. RNA located in the aqueous phase was precipitated with isopropanol at −20°C. RNA precipitates were pelleted by centrifugation, and the pellets were redissolved in 50 μl of DEPC-treated 0.5% sodiumdodecyl sulfate (SDS).

Northern Blotting

Ten μg of total RNAs extracted from liver was electrophoresed in 1.2% agarose denaturing gels containing 2.2 M formaldehyde in MOPS buffer (pH 7, containing 20 mM 3-*N*-morpholinopropanesulfonic acid, 5 mM sodium acetate, and 1 mM EDTA) with 3 V/cm^3 for 3 hr [Sambrook et al., 1987]. The electrophoresed gels were transferred to nylon membranes by blotting [Shimokawa and Yamaguchi, 1992]. Part of regucalcin cDNA (the 0.6-kb, *Kpn*I–*Pst*I insert) was labeled with [^{32}P]dCTP by random primers with the DNA polymerase Klenow fragment [Shimokawa and Yamaguchi, 1992]. This radioactive probe was used for hybridization detection of RNAs on blots. The membranes were prehybridized and hybridized in buffer solution containing 50% formamide, 5× SSPE (1× SSPE; 1.15 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA), 5× Denhardt's reagent (1× Denhardt's reagent; 0.02% w/v each of bovine serum albumin (BSA), Ficoll, polyvinylpyrrolidine) and 0.5% SDS with ^{32}P-labeled regucalcin cDNA in a sealed plastic bag at 42°C for 16 hr. After hybridization, the membranes were washed as follows: 2× SSPE and 0.1% SDS at 42°C (twice, each for 15 min), followed by 0.1× SSPE and 0.1% SDS at room temperature (twice, each for 15 min), and then the membranes were exposed to X-ray film for 12 hr.

The quantity and integrity of mRNA were monitored by rehybridizing with a radioactive cDNA probe from human β-actin gene fragment under identical conditions. No noticeable change in the level of RNA hybridized with the β-actin probe was observed throughout the present experiments (data not shown). The size of the hybridizing RNA was determined by running the standard RNA molecules of known sizes in parallel. The density of the autoradiographic data was quantified by densitometer scanning

(Dual-wavelength Flying-spot Scanner, CS-9000, Shimadzu Co., Japan).

Calcium Determination

The animals were bled by cardiac puncture under light anesthesia with ether. Liver was perfused with a cold 0.25 M sucrose solution after bleeding and removed immediately. The amount of calcium in liver tissues (about 0.2 g) was determined by atomic absorption spectrophotometry, after digestion with nitric acid [Yamaguchi et al., 1975]. The calcium content was expressed as μg/g wet tissue.

Statistical Methods

The significance of the difference between the values was estimated by Student's *t*-test or by analysis of variance (ANOVA) for comparing multiple groups. *P*-values of < 0.05 were considered to indicate a statistically significant difference.

RESULTS

Altered weight of the liver in rats after partial hepatectomy is shown in Table I. Liver was surgically removed about 70% of that of sham-operated rats. At 1 day after hepatectomy, the liver weight was increased about 50% of that of sham-operated rats, and it reached to the same levels as sham operation at 3 days after hepatectomy. The alteration of regucalcin mRNA level in regenerating rat liver was then examined; the result is shown in Figures 1 and 2. Northern hybridization of rat liver mRNA with the regucalcin cDNA probe obtained from rat liver regucalcin cDNA cloning gave a definite band of about 1.7 kb, as reported previously [Shimokawa and Yamaguchi, 1992, 1993b]. Liver regucalcin mRNA levels were slightly reduced during 12 h after hepatectomy in comparison with that of sham-operated rats (Fig. 1). At 24 hr after hepatectomy, a remarkable increase of hepatic regucalcin mRNA levels was seen. This increase was significant ($P < 0.01$) as compared with the densitometric data obtained from the 3-hr group with hepatectomy. At 48 hr after hepatectomy, the increase in regucalcin mRNA levels were slightly. This slight increase was seen at 5 days after hepatectomy. Meanwhile, no noticeable change in the level of RNA hybridized with the β-actin was observed in regenerating rat liver, as shown in the legends of Figures 1 and 2.

TABLE I. Change in Liver Weights After Partial Hepatectomy in Rats†

Treatment	Liver weight (g) 1 day	3 days	5 days
Sham operation	3.79 ± 0.20	3.97 ± 0.10	4.72 ± 0.15
Hepatectomy	2.03 ± 0.15*	3.70 ± 0.17	4.41 ± 0.18

†Rats were partially hepatectomized; 1, 3, and 5 days later the animals were sacrificed by bleeding. Livers were surgically removed, about 70% of total liver in comparison with that of sham-operated rats. Each value represents the mean ±SEM of five rats.
*$P < 0.01$, as compared with the control (sham-operated rats) value.

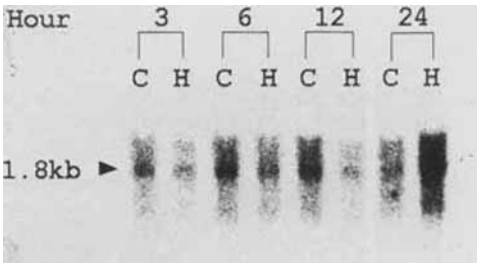

Fig. 1. Alteration of regucalcin mRNA levels in regenerating rat liver. The animals were sacrificed by bleeding at 3, 6, 12, and 24 hr after sham operation (C) or hepatectomy (H). Total RNA (10 μg) isolated from the liver was subjected to Northern blot analysis. The pattern of hybridization obtained with rat liver regucalcin cDNA is shown. Arrowhead, hybridizing bands correspond to mRNA encoding the regucalcin, showing one of four experiments with separate rats. Densitometric data (% of control; mean ±SEM) for each of the time points with hepatectomy were 86.1 ± 2.4, 85.4 ± 3.7, 70.1 ± 5.5, and 170.2 ± 10.6, respectively. Likewise, the densitometric data of β-actin mRNA levels in the same samples were 101.9 ± 4.7, 97.2 ± 3.9, 96.9 ± 4.6, and 106.3 ± 7.5 (% of control; mean ±SEM of four rats), respectively.

The effect of actinomycin D, an of transcriptional process, on regucz NA levels in regenerating rat liver is sl 'ig- ure 3. Actinomycin D (100 μg/100 g l ht) was administered intraperitoneally to rats at 3 days after partial hepatectomy; 30, 60, and 120 min later, the animals were sacrificed. The administration of actinomycin D caused an appreciable decrease of regucalcin mRNA levels in regenerating rat liver. In sham-operated rats, the hepatic mRNA levels were not decreased at 30–120 min after actinomycin D administration. However, the decrease in hepatic mRNA levels was seen at 15 min after the administration; the densitometric data showed 68 ± 5.1%

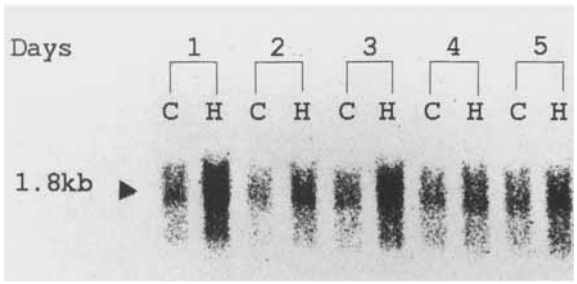

Fig. 2. Alteration of regucalcin mRNA levels in regenerating rat liver. The animals were sacrificed by bleeding at 1, 2, 3, 4, and 5 days after sham operation (C) or hepatectomy (H). Total RNA (10 μg) isolated from the liver was subjected to Northern blot analysis. The pattern of hybridization obtained with rat liver regucalcin cDNA is shown. Arrowhead, hybridizing bands corresponding to mRNA encoding the regucalcin, showing one of four experiments with separate rats. The densitometric data (% of control; mean ±SEM of four rats) for each of the time points with hepatectomy were 175.4 ± 11.2, 129.3 ± 6.5, 138.0 ± 6.9, 119.2 ± 5.8, and 123.4 ± 4.9, respectively. Likewise, the densitometric data of β-actin mRNA levels in the same samples were 105.8 ± 6.9, 101.3 ± 5.7, 102.9 ± 4.1, 98.9 ± 6.3, and 102.0 ± 7.8 (% of control; mean ±SEM of four rats), respectively.

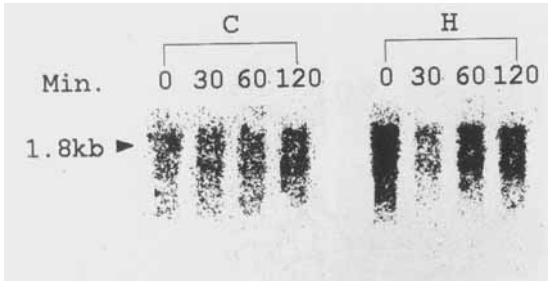

Fig. 3. Effect of the actinomycin D administration on regucalcin mRNA levels in regenerating rat liver. Animals received a single intraperitoneal administration of actinomycin D (100 μg/100 g body weight) at 3 days after sham operation (C) or hepatectomy (H); 30, 60, and 120 min later, they were sacrificed by bleeding. Control rats (C) received an equivalent volume of ethanol solution. Total RNA (10 μg) isolated from the liver were subjected to Northern blot analysis. The pattern of hybridization obtained with rat liver regucalcin cDNA is shown. Arrowhead, hybridizing bands corresponding to mRNA encoding the regucalcin, showing one of four experiments with separate rats. The densitometric data (% of value from zero time; mean ±SEM of four rats) for each of the time points with hepatectomy were 67.2 ± 4.7, 83.9 ± 5.3, and 85.2 ± 7.6, respectively. Likewise, the densitometric data of β-actin mRNA levels in the same samples were 98.3 ± 6.1, 103.1 ± 7.5 and 101.0 ± 6.8 (% of value from zero time; mean ±SEM of four rats), respectively.

(mean ±SEM of four experiments) of control level, demonstrating an effect as the inhibitor.

Meanwhile, hepatectomy produced a remarkable increase of calcium content in regenerating liver (Fig. 4). This increase was seen during 5 days after partial hepatectomy. The effect of calmodulin antagonist (trifluoperazine) on the enhanced regucalcin mRNA levels in regenerating liver was then examined, because it has been demonstrated that the expression of hepatic regucalcin mRNA is mediated through Ca^{2+}/calmodulin [Shimokawa and Yamaguchi, 1993a]. Trifluoperazine (2.5 mg/100 g body weight) was administered intraperitoneally to rats at 3 days after hepatectomy, and the increased hepatic regucalcin mRNA level and calcium content was clearly observed. The animals were sacrificed 18 hr after the administration. Administration of trifluoperazine caused a decrease of regucalcin mRNA level in the liver of sham-operated rats (Fig. 5). The increased regucalcin mRNA levels in regenerating liver were also significantly weakened by administration.

DISCUSSION

A novel calcium-binding protein regucalcin, which can regulate the Ca^{2+} effect on liver cell function, differs from other calcium-binding proteins, such as calmodulin [Cheung, 1980]. The expression of the regucalcin gene, however, is not fully clarified. Recent investigations demonstrate that regucalcin mRNA level is clearly increased at the early time point (within 30 min) of calcium administration [Shimokawa and Yamaguchi, 1992, 1993a] and that this expression may be mediated through Ca^{2+}/calmodulin [Shimokawa and Yamaguchi, 1993a]. By con-

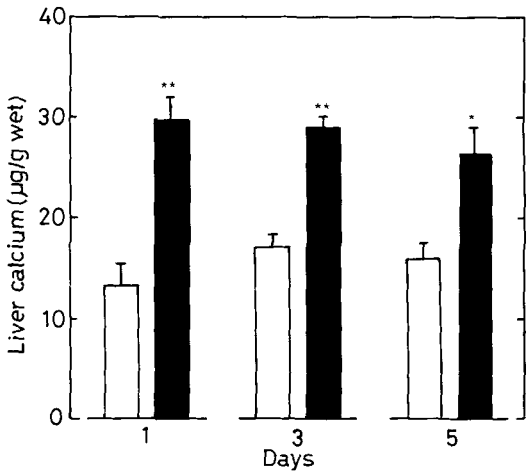

Fig. 4. Alteration of calcium content in regenerating rat liver. The animals were sacrificed by bleeding at 1, 3, and 5 days after hepatectomy. Each value represents the mean ±SEM of five animals. *P <0.05 and **P <0.01, as compared with the control (sham operation) value. □, control (sham operation); ■, hepatectomy.

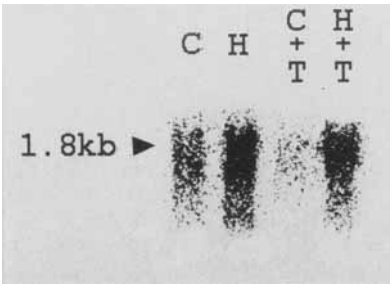

Fig. 5. Effect of the calmodulin antagonist (trifluoperazine) administration on regucalcin mRNA levels in regenerating rat liver. Animals received a single intraperitoneal administration of trifluoperazine (2.5 mg/100 g body weight) at 3 days after sham operation (C) or hepatectomy (H); 18 hr later, they were sacrificed by bleeding. Control rats (C) received an equivalent volume of distilled water. Total RNA (10 μg) isolated from the liver was subjected to Northern blot analysis. The pattern of hybridization obtained with rat liver regucalcin cDNA is shown. Arrowhead, hybridizing bands corresponding to mRNA encoding the regucalcin, showing one of four experiments with separate rats. The densitometric data (% of control; mean ±SEM of four rats) for each treatment group were 132.2 ± 3.5, 81.1 ± 6.3, and 112.4 ± 3.3, respectively. Likewise, the densitometric data of β-actin mRNA level in the same samples were 102.6 ± 4.1, 100.9 ± 5.3, and 104.0 ± 6.2 (% of control; mean ±SEM of four rats), respectively.

trast, it has been reported that increased calmodulin during proliferative liver cell activation with partial hepatectomy [Pinol et al., 1988; Serratosa et al., 1988; Pujades et al., 1990]. The present study was undertaken to clarify whether the expression of regucalcin mRNA is altered in regenerating rat liver, which induces the proliferation of liver cells after hepatectomy in vivo [Higgins and Anderson, 1931].

Adult rat hepatocytes are normally quiescent in vivo; however, 20–30 hr after partial hepatectomy (two-thirds), they undergo a synchronous wave of DNA synthesis and cell division and continue to divide until the original mass of the liver is regenerated 5–7 days later [Higgins and Anderson, 1931]. The present results show that the expression of regucalcin mRNA is clearly increased in regenerating rat liver 5 days after hepatectomy. This increase was remarkable at 24 hr after hepatectomy, which is at the time point during liver proliferative activation; with a longer time (5 days), increased expression was also seen.

However, hepatic regucalcin mRNA levels decreased slightly 12 hr after hepatectomy, which probably DNA synthesis and cell division do not synchronize. The increased expression of regucalcin mRNA in regenerating liver suggests a correlation in the promotion of proliferation of liver cells. By the way, the increase of calmodulin synthesis in regenerating rat liver is observed at 8 hr after hepatectomy [Pinol et al., 1988]. Ca^{2+}/calmodulin may play a role in the proliferation of liver cells [Boyton et al., 1980]. It is possible that the increased expression of regucalcin mRNA levels may have an inhibitory effect in the development of liver cell proliferation, since regucalcin can inhibit the Ca^{2+}/calmodulin effects in liver cells [Yamaguchi and tai, 1991; Yamaguchi and Mori, 1990; Yamaguchi and Sakurai, 1991, 1992].

The increased regucalcin mRNA levels after hepatectomy was reduced by the administration of actinomycin D, an inhibitor of transcriptional process, although the decrease was not seen in sham-operated rat liver. This result indicates that the increase of regucalcin mRNA levels in regenerating liver is not related to the decomposition of mRNA. Presumably, the process that transcripts to regucalcin mRNA is stimulated in regenerating liver. Furthermore, the expression of regucalcin mRNA may be mediated through Ca^{2+}/calmodulin in rat liver [Shimokawa and Yamaguchi, 1993a]. The administration of trifluoperazine, an antagonist of calmodulin effect, weakened the expression of regucalcin mRNA in regenerating rat liver, suggesting a possible role of Ca^{2+}/calmodulin. In fact, hepatic calcium content markedly increased in regenerating rat liver. It was recently reported that hepatocyte growth factor, which can promote liver regenerating after partial hepatectomy [Higuchi and Nakamura, 1991], increases calcium concentration in rat hepatocytes [Baffy et al., 1992].

In conclusion, it has been demonstrated that the expression of regucalcin mRNA is enhanced in regenerating rat liver after partial hepatectomy in vivo, suggesting a correlation in the proliferation of liver cells.

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