Journal of Cellular Biochemistry 75:437–445 (1999)

Regulation of Protein Phosphatase Activity by Regucalcin Localization in Rat Liver Nuclei

Masami Omura and Masayoshi Yamaguchi*

Laboratory of Endocrinology and Molecular Metabolism, Graduate School of Nutritional Sciences, University of Shizuoka, Shizuoka 422-8526, Japan

Abstract The regulatory role of regucalcin on protein phosphatase activity in isolated rat liver nuclei was investigated. Phosphatase activity toward phosphotyrosine and phosphoserine was significantly increased by the addition of $CaCl_2$ (10^{-5} and 10^{-4} M) in the enzyme reaction mixture. Trifluoperazine (25 and 50 μM), an antagonist of calmodulin, significantly inhibited protein phosphatase activity toward phosphoserine, while it had no effect on the enzyme activity toward phosphotysine and phosphothreonine. Cyclosporin A (10^{-6}–10^{-4} M), an inhibitor of Ca^{2+}/calmodulin-dependent protein phosphatase activity toward phosphoserine, but not phosphotyrosine and phosphoserine. Thus, Ca^{2+}/calmodulin-dependent phosphatases were present in liver nuclei. Regucalcin (0.25 and 0.5 μM) had an inhibitory effect on liver nuclear phosphatase activity toward phosphotyrosine, phosphoserine, and phosphothreonine. The presence of anti-regucalcin monoclonal antibody (25 and 50 ng/ml) in the enzyme reaction mixture caused a significant elevation of nuclear phosphatase activity toward three phosphoaminoacids. An analysis with sodium sulfate-polyacrylamide gel electrophoresis suggested a possibility of localization of regucalcin in liver nuclei. Moreover, regucalcin was determined in liver nuclei using enzyme-linked immunoadsorbent assay. The present study demonstrates that the endogenous regucalcin inhibits phosphatase activity in the liver nuclei. J. Cell. Biochem. 75:437–445, 1999.

Key words: regucalcin; protein phosphatase; calcium-binding protein; liver nuclei

Calcium ion (Ca^{2+}) plays an important role in the regulation of many cell functions. The Ca^{2+} effect in cells is amplified by calmodulin and protein kinase C, which is related to a signal transduction due to hormonal stimulation [Cheung, 1980; Nishizuka, 1986; Heizmann and Hunziker, 1991; Kraus-Friedman and Feng, 1996]. There has been growing evidence that Ca^{2+} plays a role in liver nuclear function [Jones et al., 1989; Backs et al., 1990; Allbritton et al., 1994]. The existence of an adenosine 5′-triphosphatase (ATP)-stimulated Ca^{2+} sequestration system in rat liver nuclei that generates a net increase in nuclear matrix-free Ca^{2+} concentration has been reported [Nicotera et al., 1989; Yamaguchi and Oishi, 1993]. Calmodulin exists in rat liver nuclei [Backs and Carafoli, 1987], and the protein stimulates deoxyribonucleic acid (DNA) synthesis by liver cells [Backs et al., 1990].

A novel Ca^{2+}-binding protein, regucalcin, has been demonstrated to regulate Ca^{2+}-dependent signaling system; the protein inhibits Ca^{2+}/calmodulin-dependent protein kinase and protein kinase C [Mori and Yamaguchi, 1990; Yamaguchi and Mori, 1990; Yamaguchi, 1992]. The regucalcin gene is localized on rat chromosome Xq 11.1–12 proximal end, and regucalcin messenger ribonucleic acid (mRNA) is mainly expressed in liver [Shimokawa et al., 1995; Murata and Yamaguchi, 1999]. The expression of hepatic regucalcin mRNA is clearly stimulated by the administration of calcium chloride to rats; the expression may be mediated through Ca^{2+} signaling factors [Shimokawa and Yamaguchi, 1993a; Murata and Yamaguchi, 1998]. Moreover, regucalcin mRNA has been demonstrated to be enhanced in regenerating rat liver, which induces a proliferation of liver cells after a partial hepatectomy [Yamaguchi and Kanayama, 1995]. Regucalcin has an inhibitory effect on deoxyribonucleic acid (DNA) and RNA syntheses in the nuclei isolated from regenerating rat liver, suggesting that the protein plays a role in the regulation of nuclear func-

*Correspondence to: Masayoshi Yamaguchi, Ph.D., Laboratory of Endocrinology and Molecular Metabolism, Graduate School of Nutritional Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan.

Received 12 March 1999; Accepted 29 April 1999

tion in proliferative liver cells [Yamaguchi and Kanayama, 1996; Yamaguchi and Ueoka, 1997]. The action of regucalcin on liver nuclear function, however, remains to be elucidated.

It has been reported that Ca^{2+}/calmodulin-dependent protein kinase and protein kinase C exist in liver nuclei [Backs and Carafoli, 1987; Block et al., 1992]. Many phosphorylated proteins may be present in liver nuclei, and protein phosphatases may play an important role in the regulation of liver nuclei, and protein phosphatases may play an important role in the regulation of liver nuclear functions [Hunter, 1995]. The present study, therefore, was undertaken to clarify the effect of regucalcin in protein phosphatase activity in isolated rat liver nuclei. It was found that endogenous regucalcin plays an inhibitory role in the regulation of protein phosphatase activity in liver nuclei.

MATERIALS AND METHODS

Chemicals

o-Phospho-L-tyrosine, *o*-phospho-L-serine, *o*-phospho-L-threonine, cyclosporin A, trifluoperazine, and ethyleneglycol bis (2-aminoethylether)-N, N, N′, N′-tetraacetic acid (EGTA), were obtained from Sigma Chemical Co. (St. Louis, MO). Calcium chloride, vanadate, and all other chemicals were reagent grade from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All reagents used were dissolved in distilled water, and then passed through an ion-exchange resin to remove metal ions.

Animals and Hepatectomy

Male Wistar rats (80–100 g, 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 at a room temperature of 25°C, and distilled water, ad libitum. A partial hepatectomy was carried out by excision of two-thirds of the liver (the medium and left laternal 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 10:00–12:00, and 24 h later the animals were sacrificed by bleeding.

Isolation of Regucalcin

Regucalcin (RC) is markedly expressed in rat liver cytosol [Yamaguchi and Isogai, 1993]. RC was isolated from rat liver cytosol. RC in the cytosol fraction (the supernatant of 105,000*g*) of rat liver homogenate was purified by gel filtration on Sephadex G-75 and G-50 followed by ion-exchange chromatography on diethylaminoethyl (DEAE)-cellulose, as reported previously [Yamaguchi and Yamamoto, 1978]. The homogeneity of RC was established by polyacrylamide gel electrophoresis [Yamaguchi and Yamamoto, 1978].

Anti-RC Antibody

Anti-RC antibody was a monoclonal antibody raised against RC prepared by using standard methods [Omura and Yamaguchi, 1998]. Mice (BALB/C, Japan SLC) were subcutaneously injected with 0.1 mg per animals of antigen (rat liver regucalcin) emulsified with Freund's complete adjuvant, and 19 days later antigen (0.25 mg/animal) was intraperitoneally injected with Ferund's incomplete adjuvant. Spleen cells were prepared from immuned mouse, and fused into myeloma cells. Anti-RC monoclonal antibody-producing cells (hybridoma cells) were obtained by screening. The IgG from hybridoma cells was isolated through protein A-agarose column (Sigma).

Isolation of Nuclei

Liver nuclei were isolated by the procedure of Jones et al. [1989] with a minor modification. Rats were killed by cardiac puncture, and the livers were perfused with approximately 10 ml of ice-cold TKM solution (50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$) to remove blood. Livers were then removed, cut into small pieces, and homogenized in a Potter-Elvehjem homogenizer with a Teflon pestle in 40 ml of the same solution containing 0.25 M sucrose and 1.0 mM EGTA. The homogenate was filtered through three layers of cheesecloth. The homogenate was filtered by centrifugation at 700*g* for 10 min. The pellets were homogenized (five strokes) in 40 ml of the same solution and centrifuged again at 700*g* for 10 min. The pellet was resuspended in 24 ml of the same solution by homogenization (five strokes), and 6 ml was added to each of four tubes containing 12 ml of TKM including 2.3 M sucrose. The tubes were centrifuged at 37,000*g* for 30 min. The upper layer and the sucrose cushion were removed with an aspirator. The resulting pellet of highly purified nuclei was resuspended in the incubation medium (125 mM KCl, 2 mM potassium phos-

phate, 25 mM Hepes, 4 mM $MgCl_2$, pH 7.0) by hand homogenization. Assay of marker enzymes, as reported previously [Yamaguchi and Oishi, 1993], showed that there was less than 5% contamination by microsomes, plasma membranes, or mitochondria. DNA content in the nuclei was determined using the diphenylamine reaction [Burton, 1956].

Assay of Phosphatase Activity

Phosphatase activity toward phosphotyrosine, phosphoserine, and phosphothreonine was assayed at 30°C in 1.0 mM NaCl, 6 mM $MgCl_2$, 0.5 mM dithiothreiol, 9 mM phosphoaminoacid and nuclei (5.0–6.0 mg protein/ml; 0.3–0.4 mg DNA/ml) as reported elsewhere [Pallen and Wang, 1983; Fruman et al., 1992]. In the separate experiments, the above reaction mixture contained either vehicle, calcium chloride (10^{-6}–10^{-4} M), anti-regucalcin (RC) antibody (10–50 ng/ml), RC (0.1–0.5 μM), trifluoperazine (10–50 μM), cyclosporin A (10^{-6}–10^{-4} M), or vanadate (10^{-6}–10^{-4} M). The enzyme reaction was terminated after 15 min by the addition of 1.0 ml of ice-cold 10% trichloroacetic acid and centrifuged to precipiate protein. Inorganic phosphate released in the supernatant was quantified by the method of Nakamura and Mori [1958]. Results were expressed as nanomoles of inorganic phosphate liberatated per minutes (min) per milligram (mg) of nuclear protein. Protein concentration was determined by the method of Lowry et al. [1951] using bovine serum albumin as standard.

Calmodulin-Agarose Binding Assay

Calmodulin was insolubilized on 4% beaded agarose (containing 1.5 mg calmodulin per milliliter of packed gel). Calmodulin-agarose beads were preactivated by washing three times in buffer (10 mM Tris, pH 8.0, 150 mM NaCl, and 1 mM $CaCl_2$). The beads were aliquoted into individual microcentrifuge tubes and spun, and all of the supernatant was removed. The reaction mixture (1.0 ml) containing packed beads (60 μl; 90 μg of calmodulin) in the above buffer was incubated in the presence of regucalcin (10 μg/ml) and/or liver nuclei (500 μg of protein/ml) for 1 h at 4°C [Loh et al., 1996]. After centrifugation, the beads were then washed three times in wash buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, and protease inhibitors supplemented with 1 mM $CaCl_2$ or 5 mM EGTA) and once in 10 mM Hepes, pH 7.4, and then boiled in Laemmli reducing buffer. The samples were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Nuclear Regucalcin Transport Assay

The reaction mixture (1.0 ml) containing liver nuclei (500 μg of protein) in the buffer (10 mM Tris, pH 8.0, 150 mM NaCl, and 1 mM $CaCl_2$) was incubated at 37°C for 10, 30, and 60 min in the absence or presence of regucalcin (40 μg/ml). At the time point indicated, the nuclei were precipitated by centrifugation at 20,000*g* for 15 min. After centrifugation, the precipitates were then washed two times in the above buffer, and then boiled in Laemmli reducing buffer. The samples were analyzed by 12% SDS-PAGE.

Electrophoresis

SDS-PAGE was performed by the method of Laemmli [1970] with minor modifications. The electrophoresis was carried out using 12% polyacrylamide resolving gel and the discontinuous Tris-glycine buffer system. Twenty microliters of samples were dissolved in each same volume of SDS-loading buffer containing 4% SDS, 10% β-mercaptoethanol, and bromphenol bule (BPB) marker. The protein mixture was denatured by heating at 90°C for 2 min and applied to individual wells. Electrophoresis voltage was applied (8 V/cm for 3 h at room temperature) to the gel. After separation, proteins were simultaneously fixed with methanol-acetic acid and stained with Coomassie Brilliant Blue R250. Then, the gels were destained with methanol-acetic acid and stored in water containing 20% glycerol.

Determination of Regucalcin

Detection of regucalcin in isolated rat liver nuclei was performed using an enzyme-linked immunoadsorbent assay (ELISA) [Yamaguchi and Isogai, 1993]. This assay sestem was specific for regucalcin [Yamaguchi and Isogai, 1993]. Ninety-six-well assay plates (Nunc-Imuno, Naperville, IL) were coated with 50 μl of anti-regucalcin IgG diluted in 0.1 M carbonate buffer (pH 9.7) to a final concentration of 10 μg/ml for 2 h at 37°C. Wells were then blocked with 5% Tween 20. The plates were incubated with standard amounts of regucalcin (0.5–10 ng/ml, 50 μl) or samples (50 μl) for 18 h at 4°C. After wash, biotinylated anti-regucalcin IgG

with NHS-LC-Biotin were added. Plates were then incubated with streptavidin-peroxidase conjugate (1/40,000) for 2 h at 37°C, washed and finally incubated with *o*-phenylendiamine (3 mg/ml, 100 µl) for 15 min at room temperature. The reaction was stopped with 100 µl 4 N H_2PO_4 and the plate read at 450 nm. Nuclear regucalcin was expressed as nanogram or microgram per milligrams of nuclear protein or DNA, respectively.

Statistical Analysis

Data were expressed as the mean ± SEM. Statistical differences were analyzed using Student's *t*-test. A P value of less than 0.05 was considered to indicate statistically significant differences.

RESULTS

Characterization of Phosphatase Activity in Liver Nuclei

The effect of $CaCl_2$ addition on phosphatase activity toward phosphotyrosine, phosphoserine, and phosphothreonine in isolated rat liver nuclei is shown in Figure 1. The enzyme reaction mixture contained either vehicle or $CaCl_2$ (10^{-6}–10^{-4} M). The addition of $CaCl_2$ (10^{-5} and 10^{-4} M) caused a significant increase in nuclear phosphatase activity toward phosphotyrosine (Fig. 1A) and phosphoserine (Fig. 1B), while it had no effect on phospohthreonine (Fig. 1C). The effect of Ca^{2+}/calmodulin addition on phosphatase activity toward three phosphoaminoacids in rat liver nuclei was examined. The addition of $CaCl_2$ (10 µM) and calmodulin (5 µg/ml) in the enzyme reaction mixture did not cause a significant elevation of Ca^{2+}-increased phosphatase activity toward phosphotyrosine and phosphothreonine (data not shown).

The effect of various inhibitors on phosphatase activity in isolated rat liver nuclei is shown in Figure 2. Phosphatase activity toward phosphoserine was significantly decreased by the addition of trifluoperazine (25 and 50 µM), an inhibitor of calmodulin, in the enzyme reaction mixture. Such an effect was not seen in the enzyme activity toward phosphotyrosine and phosphothreonine. The presence of cyclosporin A (10^{-6}–10^{-4} M), an inhibitor of Ca^{2+}/calmodulin-dependent phosphatase calcineurin, in the enzyme reaction mixture caused a significant decrease in liver nuclear phosphatase activity toward phosphoserine, while the enzyme activity toward phosphotyrosine and phosphothreonine was not altered by cyclosporin A. Thus, phosphoserine phosphatase activity in liver nuclei may be dependent on Ca^{2+}/calmodulin. Meanwhile, nuclear phosphatase activity toward phosphotyrosine and phosphoserine was significantly decreased by the addition of vanadate (10^{-6}–10^{-4} M) in the enzyme reaction mix-

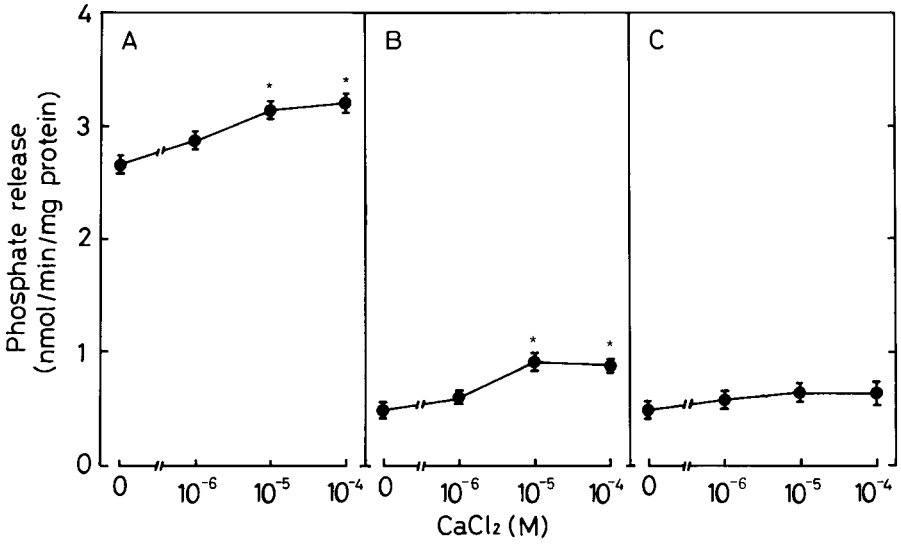

Fig. 1. Effect of calcium chloride ($CaCl_2$) on phosphatase activity toward phosphotyrosine (**A**), phosphoserine (**B**), and phosphothreonine (**C**) in rat liver nuclei. The enzyme reaction mixture contained either vehicle or $CaCl_2$ (10^{-6}–10^{-4} M) in the presence of each phosphoaminoacid. Each value represents the mean ± SEM of five experiments with different rats. *$P < 0.01$, compared with the control value without $CaCl_2$ addition.

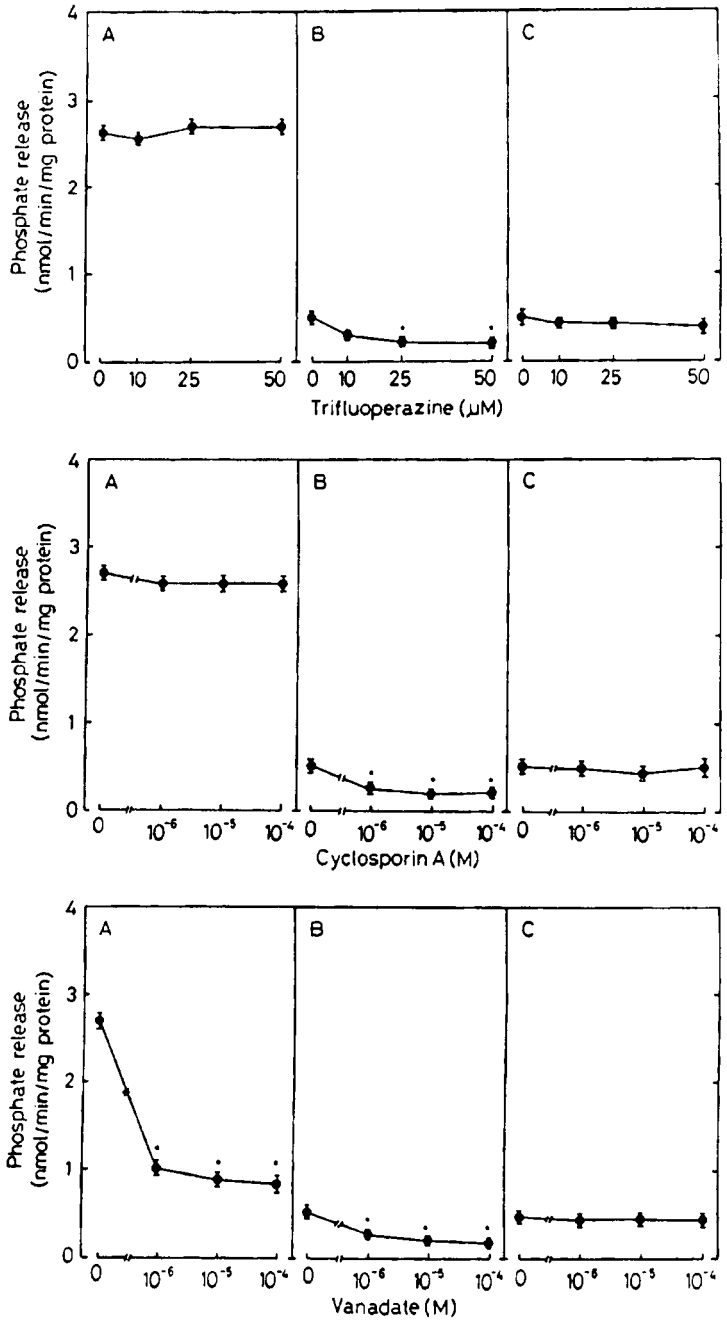

Fig. 2. Effect of various inhibitors on phosphatase activity toward phosphotyrosine (**A**), phosphoserine (**B**), and phosphothreonine (**C**) in rat liver nuclei. The enzyme reaction mixture contained either vehicle, trifluoperazine (10, 25, and 50 μM), cyclosporin A (10^{-6}–10^{-4} M), or vanadate (10^{-6}–10^{-4} M). Each value represents the mean ± SEM of five experiments with different rats. $^*P < 0.01$, compared with the control value without trifluoperazine addition.

ture, while the enzyme activity toward phosphothreonine was not altered.

Effect of Regucalcin on Phosphatase Activity in Liver Nuclei

The effect of regucalcin on phosphatase activity toward phosphoaminoacids in isolated rat liver nuclei is shown in Figure 3. The enzyme reaction mixture contained either vehicle or regucalcin (0.1, 0.25, and 0.5 μM). The addition of regucalcin (0.25 and 0.5 μM) caused a significant decrease in nuclear phosphatase activity toward phosphotyrosine (Fig. 3A), phosphoserine (Fig. 3B), and phosphothreonine (Fig. 3C).

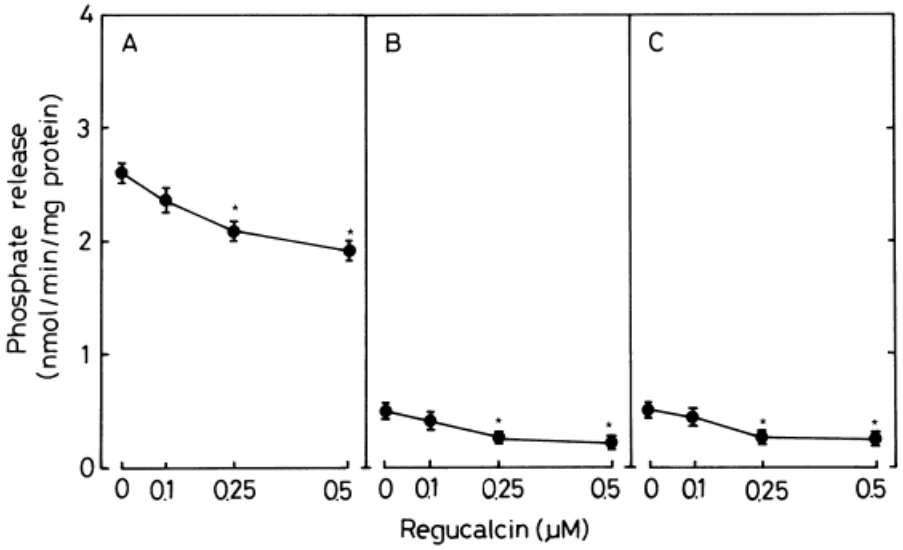

Fig. 3. Effect of regucalcin on phosphatase activity toward phosphotyrosine (**A**), phosphoserine (**B**), and phosphothreonine (**C**) in rat liver nuclei. The enzyme reaction mixture contained either vehicle or regucalcin (0.1, 0.25, and 0.5 μM) without Ca^{2+} and calmodulin addition. Each represents the mean ± SEM of five experiments with different rats. $*P < 0.01$, compared with the control value without regucalcin addition.

The presence of anti-regucalcin monoclonal antibody (25 and 50 ng/ml) in the enzyme reaction mixture caused a significant elevation of nuclear phosphatase activity toward phosphotyrosine (Fig. 4A), phosphoserine (Fig. 4B), and phosphothreonine (Fig. 4C). Anti-regucalcin monoclonal antibody (50 ng/ml)-induced elevation of nuclear phosphatase activity was completely prevented by the addition of regucalcin (0.1 and 0.5 μM) (data not shown).

Localization of Regucalcin in Rat Liver Nuclei

Regucalcin has been demonstrated to bind on calmodulin agarose beads [Omura and Yamaguchi, 1998]. Liver nuclear extracts (500 μg of protein/ml) were incubated in the reaction mixture containing calmodulin-agarose beads (90 μg of calmodulin). Incubated calmodulin-agarose beads were applied to SDS-PAGE. There were many bands on SDS-PAGE (Fig. 5; lane 2). Regucalcin has been found to be 33,388 daltons [Shimokawa and Yamaguchi, 1993b]. The band coincided with regucalcin, a marker (lane 4), was seen on SDS-PAGE (lane 3). This band was also seen on SDS-PAGE with nuclear extracts (20 μg of protein; lane 3).

Regucalcin (40 μg/ml) was incubated for 10, 30, and 60 min in the reaction mixture containing liver nuclei (500 μg of protein/ml). Nuclear particulates incubated were applied to SDS-PAGE. The band coincided with regucalcin was seen on lanes 2, 3, and 4 (Fig. 6).

Regucalcin content in isolated rat liver nuclei was determined by using ELISA. Regucalcin was present in liver nuclei (Fig. 7). Nuclear regucalcin content of regenerating rat liver was significantly increased at 24 h after a partial hepatectomy (Fig. 7).

DISCUSSION

Ca^{2+}-binding protein regucalcin has been shown to have an inhibitory effect on DNA and RNA syntheses in the nuclei of normal (sham-operated) and regenerating rat livers [Yamaguchi and Kanayama, 1996; Yamaguchi and Ueoka, 1997], and the protein can inhibit Ca^{2+}-dependent protein kinase activity in rat liver nuclei [Katsumata and Yamaguchi, 1998]. Regucalcin may play an inhibitory role in the regulation of liver nuclear function. The present study, furthermore, was undertaken to clarify the effect of regucalcin on protein phosphatase activity in isolated rat liver nuclei.

Phosphatase activity toward phosphotyrosine, phosphoserine, and phosphothreonine was found in the liver nuclei. Nuclear phosphotyrosine phosphatase activity was significantly increased by Ca^{2+} addition in the enzyme reaction mixture, although the enzyme activity was not altered by trifluoperazine, an inhibitor of

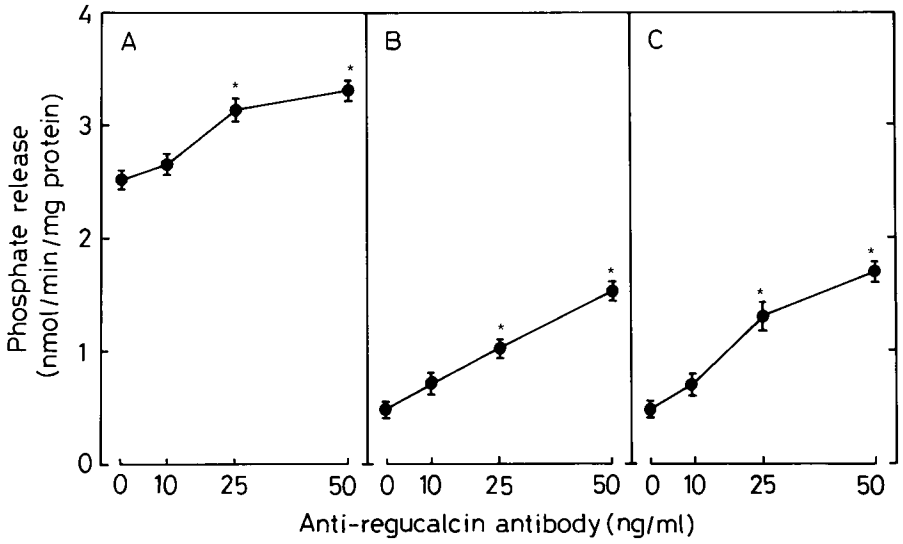

Fig. 4. Effect of anti-regucalcin monoclonal antibody on phosphatase activity toward phosphotyrosine (**A**), phosphoserine (**B**), and phosphothreonine (**C**) in rat liver nuclei. The enzyme reaction mixture contained either vehicle or anti-regucalcin antibody (10, 25, and 50 ng/ml) without regucalcin addition. Each value represents the mean ± SEM of five experiments with different rats. $*P < 0.01$, compared with the control value without antibody addition.

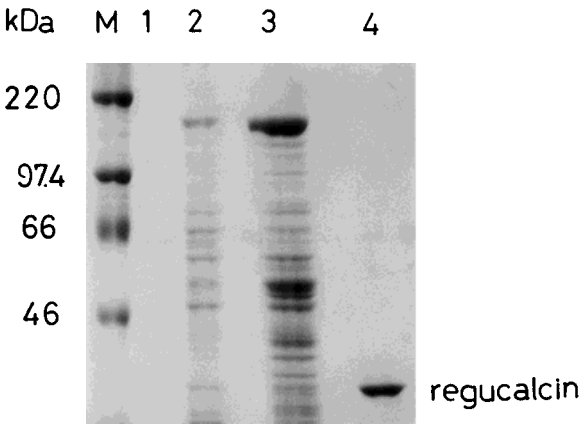

Fig. 5. Pattern of SDS-PAGE of rat liver nuclear proteins bound to calmodulin-agarose beads. The reaction mixture containing either vehicle or nuclear proteins (500 μg/ml) in the presence of calmodulin-agarose beads (90 μg calmodulin/ml) was incubated for 60 min at 4°C. Samples for SDS-PAGE were pretreated with SDS in the presence of β-mercaptoethanol. The protein bands were visualized using Coomassie Blue staining. **Lane 1,** control (beads); **lane 2,** nuclear proteins bound to beads; **lane 3,** nuclear extracts (20 μg); **lane 4,** regucalcin (10 μg) as the marker. The result shows one of four experiments with separate samples.

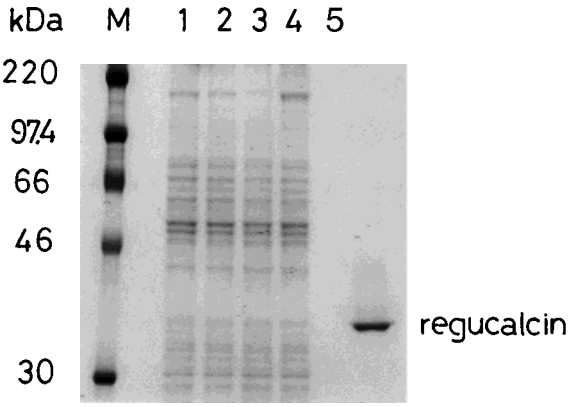

Fig. 6. Pattern of SDS-PAGE of rat liver nuclei incubated with regucalcin addition. The reaction mixture containing either vehicle or regucalcin (40 μg/ml) in the presence of liver nuclei (500 μg protein/ml) was incubated for 10, 30, and 60 min at 37°C. Samples for SDS-PAGE were pretreated with SDS in the presence of β-mercaptoethanol. The protein bands were visualized using Coomassie Blue staining. **Lane 1,** nuclei samples (10 min; 50 μg protein); **lane 2,** nuclei samples (10 min; 50 μg protein); **lane 3,** nuclei samples (30 min; 50 μg protein); **lane 4,** nuclear samples (60 min; 50 μg protein); **lane 5,** regucalcin (10 μg) as the marker. The result shows one of four experiments with separate samples.

calcineurin [MacKintosh and MacKintosh, 1994]. Nuclear phosphatase activity toward phosphotyrosine may be independent of calmodulin. Meanwhile, nuclear phosphatase activity toward phosphoserine was significantly elevated by the addition of Ca^{2+}, while the enzyme activity was appreciably decreased by trifluoperazine and cyclosporin A, suggesting that the enzyme activity is partly involved in Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin. Nuclear phosphatase activity toward phosphothreonine was not significantly

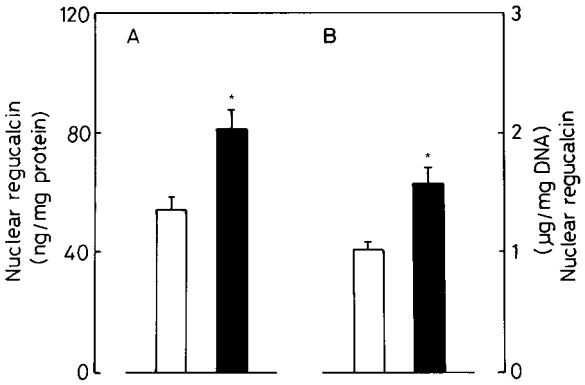

Fig. 7. Determination of regucalcin in rat liver nuclei by enzyme-linked immunoadsorbent assay (ELISA). Nuclei were isolated from normal and regenerating rat livers. Regenerating livers were obtained at 24 h after a partial hepatectomy of rats. Each value represents the mean ± SEM of five experiments with different rats. $*P < 0.01$, compared with the value of normal rats. Open bar, normal liver nuclei; solid bar, regenerating rat liver nuclei.

altered by the addition of Ca^{2+}, trifluoperazine, and cyclosporin A in the enzyme reaction mixture. Meanwhile, vanadate caused a significant inhibition of nuclear phosphatase activity toward phosphotyrosine and phosphoserine but not phosphothreonine. Thus, different protein phosphatases toward phosphotyrosine, phosphoserine, and phosphothreonine may be present in liver nuclei.

The addition of regucalcin in the enzyme reaction mixture caused a significant decrease in phosphatase activity toward phosphotyrosine, phosphoserine, and phosphothreonine in the liver nuclei. Moreover, nuclear phosphatase activity toward three phosphoaminoacids was significantly elevated by the presence of anti-regucalcin monoclonal antibody in the enzyme reaction mixture. This elevation was completely abolished by regucalcin addition. These results suggest that the endogenous regucalcin in liver nuclei regulates protein phosphatase activity toward phosphotyroisine, phosphoserine, and phosphothreonine. Thus, regucalcin has a unique inhibitory effect on phosphatase activity in the liver nuclei. The finding may be first time.

Exogenous regucalcin has been shown to regulate liver nuclear functions [Yamaguchi and Kanayama, 1996; Yamaguchi and Ueoka, 1997; Katsumata and Yamaguchi, 1998]. The localization of regucalcin in the liver nuclei, however, is unknown. Regucalcin can bind on calmodulin-agarose beads [Omura and Yamaguchi, 1998]. Liver nuclear extracts were incubated with calmodulin-agarose beads, and calmodulin-agarose beads were applied to SDS-PAGE. Band that coincides with regucalcin was found on SDS-PAGE. When liver nuclei were incubated in the presence of exogenous regucalcin, the band coincided with regucalcin on SDS-PAGE was seen. These results suggest that regucalcin is present in the nuclei. Moreover, endogenous regucalcin in isolated rat liver nuclei was determined using ELISA. Thus, regucalcin seems to be localized in liver nuclei. This, however, remains to be confirmed using immunocytochemistry and Western blot analysis.

Liver nuclear phosphatase activity toward phosphoaminoacids was assayed using 5–6 mg of nuclear protein per milliliter of reaction mixture; it contained 275–330 ng of nuclear regucalcin. The concentration of endogenous nuclear regucalcin was estimated about 82.4–98.8 nM. Further addition of exogenous regucalcin (0.25 μM) caused a significant decrease in nuclear phosphatase activity, although the effect was saturated by increasing concentrations of regucalcin (0.5 μM). Meanwhile, nuclear phosphatase activity was significantly elevated by the addition of anti-regucalcin monoclonal antibody (25 and 50 ng/ml of reaction mixture). From these results, it is assumed that the endogenous regucalcin in liver nuclei has an inhibitory effect on phosphatase activity.

The expression of regucalcin mRNA has been demonstrated to be enhanced in regenerating liver after a partial hepatectomy of rats [Yamaguchi and Kanayama, 1995]. The nuclear regucalcin content was significantly elevated in regenerating rat liver. Presumably, regucalcin plays a role in the regulation of nuclear functions in the proliferative cells of regenerating liver.

Interestingly, many nuclear proteins were found to be bound on calmodulin-agarose beads. It has been reported that calmodulin and its binding proteins are present in liver nuclei [Backs and Carafoli, 1987; Backs et al., 1990; Csermely et al., 1995]. If regucalcin can bind to calmodulin [Omura and Yamaguchi, 1998], regucalcin may have a role in the regulation of liver nuclear functions related to calmodulin. Whether regucalcin can bind other proteins in liver nuclei remains to be elucidated.

In conclusion, it has been demonstrated that endogenous regucalcin in liver nuclei has an inhibitory effect on protein phosphatase activity, supporting a regulatory role of regucalcin in nuclear functions.

REFERENCES

Allbritton NL, Oancea E, Kuhn MA, Meyer T. 1994. Source of nuclear calcium signals. Proc Natl Acad Sci USA 91: 12458–12462.

Backs O, Carafoli E. 1987. Calmodulin and calmodulin-binding proteins in liver cell nuclei. J Biol Chem 262: 10786–10790.

Backs O, Lanini L, Serrratosa J, Coll MJ, Bastors R, Aline R, Ruis E, Carofoli E. 1990. Calmodulin-binding proteins in the nuclei of quiescent and proliferatively activated rat liver cells. J Biol Chem 265:18595–18600.

Burton A. 1956. Study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem J 62:315–323.

Cheung WY. 1980. Calmodulin plays a pivotal role in cellular regulation. Science 202:19–27.

Csermely P, Schnaider T, Szanto I. 1995. Signalling and transport through the nuclear membrane. Biochim Biophys Acta 1241:425–452.

Fruman DA, Klee CB, Bierer BE, Burakoff SJ. 1992. Calcineurin phosphatase activity in T lymphocytes is inhibited by FK 506 and cyclosporin A. Proc Natl Acad Sci USA 89:3686–3690.

Heizman CW, Hunziker W. 1991. Intracellular calcium-binding proteins: More sites than in sights. Trends Biochem Sci 16:98–103.

Higgins GH, Anderson RM. 1931. Experimental pathology of the liver. Restoration of the liver of the white rat following partial surgical removal. Arch Pathol 12:186–202.

Hunter T. 1995. Protein kinases and phosphatases: the Yin and Yang of protein phosphorylation and signaling. Cell 80:225–236.

Jones DP, McConkey DJ, Nicotera P, Orrenius S. 1989. Calcium activated DNA fragmentation in rat liver nuclei. J Biol Chem 264:6398–6403.

Katsumata T, Yamaguchi M. 1998. Inhibitory effect of calcium-binding protein regucalcin on protein kinase activity in the nuclei of regenerating rat liver. J Cell Biochem 71:569–576.

Kraus-Friedman N, Feng L. 1996. The role of intracellular Ca^{2+} in the regulation of gluconeogenesis. Metabolism 42:389–403.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 224:680–685.

Loh C, Shaw KT-Y, Carew J, Viola JPB, Luo C, Perrino BA, Rao A. 1996. Calcineurin binds the transcription factor NFAT1 and reversibly regulates its activity. J Biol Chem 271:10884–10891.

Lowry OH, Rosebrough MJ, Farr AL, Randall FJ. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–273.

Mackintosh C, Mackintosh RW. 1997. Inhibitors of protein kinase and phosphatases. Trends Biochem Sci 19:444–448.

Mori S, Yamaguchi M. 1990. Hepatic calcium-binding protein regucalcin decreases Ca^{2+}/calmodulin-dependent protein kinase activity in rat liver cytosol. Chem Pharm Bull 38:2216–2218.

Murata T, Yamaguchi M. 1999. Promoter characterization of the rat gene for Ca^{2+}-binding protein regucalcin. Transcriptional regulation by signaling factors. J Biol Chem 274:1277–1285.

Murata T, Yamaguchi M. 1998. Ca^{2+} administration stimulates the binding of AP-1 factor to the 5′-flanking region of the rat gene for the Ca^{2+}-binding protein regucalcin. Biochem J 329:157–163.

Nakamura M, Mori K. 1958. Calorimetric determination of inorganic phosphorus in the presence of glucose-1-phosphatase and adenosine triphosphate. Nature 182: 1441–1442.

Nicotera P, MaConkey Dj, Jones DP, Orrenis S. 1989. ATP stimulates Ca^{2+} uptake and increases the free Ca^{2+} concentration in isolated rat liver nuclei. Proc Natl Acad Sci USA 86:453–457.

Nishizuka WY. 1986. Studies and perspectives of protein kinase C. Science 233:305–312.

Omura M, Yamaguchi M. 1998. Inhibition of Ca^{2+}/calmodulin-dependent phosphatase activity by regucalcin in rat cytosol: involvement of calmodulin binding. J Cell Biochem 71:140–148.

Pallen CJ, Wang JH. 1983. Calmodulin-stimulated dephosphorylation of *p*-nitrophenylphosphate and free phosphotyrosine by calcineurin. J Biol Chem 258:8550–8553.

Shimokawa N, Yamaguchi M. 1993a. Expression of hepatic calcium-binding protein regucalcin mRNA is mediated through Ca^{2+}/calmodulin in rat liver. FEBS Lett 316:79–84.

Shimokawa N, Yamaguchi M. 1993b. Molecular cloning and sequencing of the cDNA coding for a calcium-binding protein regucalcin from rat liver. FEBS Lett 327:251–255.

Shimokawa N, Matsuda Y, Yamaguchi M. 1995. Genomic cloning and chromosomal assignment of rat regucalcin gene. Mol Cell Biochem 151:157–163.

Vincenzi FF. 1982. Pharmacology of calmodulin antagonism. In: Godfraind T, Albertini A, Paoletti R, editors. Calcium modulators. Amsterdam: Elsevier Biomedical Press. p 67–80.

Yamaguchi M. 1992. A novel Ca^{2+}-binding protein regucalcin and calcium inhibition: regulatory role in liver cell function. In: Kohama K, editor. Calcium inhibition. Tokyo: Japan Scientific Society Press; Boca Raton, FL: CRC Press. p 19–41.

Yamaguchi M, Isogai M. 1993. Tissue concentration of calcium-binding protein regucalcin in rats by enzyme-linked immunoadsorbent assay. Mol Cell Biochem 122:65–68.

Yamaguchi M, Kanayama Y. 1995. Enhanced expression of calcium-binding protein regucalcin mRNA in regenerating rat liver. J Cell Biochem 57:185–190.

Yamaguchi M, Kanayama Y. 1996. Calcium-binding protein regucalcin inhibits deoxyribonucleic acid synthesis in the nuclei of regenerating rat liver. Mol Cell Biochem 162: 121–126.

Yamaguchi M, Mori S. 1990. Inhibitory effect of calcium-binding protein regucalcin on protein kinase C activity in rat liver cytosol. Biochem Med Metab Biol 43:140–146.

Yamaguchi M, Oishi K. 1993. Characterization of Ca^{2+}-stimulated adenosine 5′-triphosphatase and Ca^{2+} sequestering in rat liver nuclei. Mol Cell Biochem 125:43–49.

Yamaguchi M, Ueoka S. 1997. Inhibitory effect of calcium-binding protein regucalcin on ribonucleic acid synthesis in isolated rat liver nuclei. Mol Cell Biochem 173:169–175.

Yamaguchi M, Yamatomo T. 1978. Purification of calcium binding substance from soluble fraction of normal rat liver. Chem Pharm Bull 26:1915–1918.