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Enhancement of Neutral Phosphatase Activity in the Cytosol and Nuclei of Regenerating Rat Liver: Role of Endogenous Regucalcin

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Abstract The role of endogenous regucalcin (RC) in the regulation of neutral phosphatase activity in regenerating rat liver was investigated. The liver weight reduced by a partial hepatectomy (about 70%) was completely restored at 72 h after surgery. Phosphotyrosine, phosphoserine, and phosphothreonine were used as the substrate for the assay of phosphatase activity. Phosphatase activity toward phosphotyrosine in the hepatic cytosol and nuclei was significantly increased at 24–72 h after hepatectomy. Such an increase was not seen in the case of phosphoserine and phosphothreonine. However, the presence of anti-RC monoclonal antibody (200 ng/ml) in the enzyme reaction mixture caused a remarkable elevation of phosphatase activity toward three phosphoaminoacids in the hepatic cytosol at 24 and 48 h after hepatectomy. In the liver nuclei after sham operation or hepatectomy, phosphatase activity toward three phosphoaminoacids was significantly raised by the addition of anti-RC antibody (150 ng/ml). The nuclear phosphatase activity toward phosphothreonine in regenerating liver was significantly enhanced in the presence of anti-RC antibody (100 and 150 ng/ml). The effect of anti-RC antibody to increase phosphatase activity toward three phosphoaminoacids in the cytosol and nuclei of regenerating liver was completely blocked by the addition of exogenous RC (1.0 μM). The present study demonstrates that protein phosphatase activity in the cytoplasm and nuclei is enhanced in regenerating rat liver. This enhancement may be suppressed by endogenous RC. J. Cell. Biochem. 73:332–341, 1999.

Key words: regucalcin; Ca^{2+}-binding protein; protein phosphatase; anti-regucalcin antibody; regenerating liver

Calcium ion (Ca^{2+}) plays a role as an important second messenger signal in a variety of pathways to produce a Ca^{2+}-mediated physiological response in many cells [Bygrave and Benedetti, 1993; Kraus-Friedman and Feng, 1996]. The Ca^{2+} signal is transmitted to an intracellular response partly via a family of calcium-binding proteins [Heizman and Hunziker, 1991]. A calcium-binding protein regucalcin (RC) has been mainly found to be expressed in the cytoplasm of liver and renal cortex of rats [Shimokawa and Yamaguchi, 1992, 1993a; Yamaguchi and Isogai, 1993]. The RC gene is localized on the proximal end of rat chromosome Xq11.1–12 [Shimokawa et al., 1995], and the gene consists of seven exons and six introns [Yamaguchi et al., 1996]. The expression of RC mRNA is partly mediated through a Ca^{2+}-dependent mechanism in liver cells [Shimokawa and Yamaguchi, 1993b]. RC has an inhibitory effect on the activation of Ca^{2+}/calmodulin-dependent enzymes and protein kinase C in cells [Yamaguchi, 1992a; Yamaguchi and Tai, 1991; Yamaguchi and Mori, 1990]. Thus, RC may play a regulatory role in cell functions related to Ca^{2+}.

The expression of RC mRNA has been demonstrated to be enhanced in regenerating rat liver, which induces a proliferation of liver cells by hepatectomy [Yamaguchi and Kanayama, 1996]. RC has an inhibitory effect on deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) syntheses in the nuclei of regenerating rat liver [Yamaguchi and Kanayama, 1996; Yamaguchi and Ueoka, 1997]. Presumably, RC plays a regulatory role in the proliferation of liver cells. However, the role of RC in regenerating rat liver is not fully clarified.

By contrast, protein phosphorylation-dephosphorylation is a universal mechanism by which

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numerous cellular events are regulated. The precise physiological role of phosphatases is to dephosphorylate a wide variety of phosphoproteins, and it may serve an important cellular function [Wang et al., 1995]. RC has been shown to have an inhibitory effect on Ca^{2+}/calmodulin-dependent protein kinase and protein kinase C [Yamaguchi and Mori, 1990; Mori and Yamaguchi, 1990], suggesting a role in the regulation of protein phosphorylation in liver cells. However, the regulatory role of RC in liver protein phosphatase activity has not been fully clarified.

The present study was undertaken to clarify the effect of RC on protein phosphatase in the cytosol and nuclei in the proliferative cells of regenerating rat liver. It was found that neutral phosphatase activity toward phosphoaminoacids is enhanced in the cytosol and nuclei of regenerating liver and that endogenous RC may play an inhibitory role in the enhancement of the enzyme activity.

MATERIALS AND METHODS

Chemicals

o-Phospho-L-tyrosine, o-phospho-L-serine, o-phospho-L-threonine, and ethyleneglycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), were obtained from Sigma Chemical Co. (St. Louis, MO). Calcium chloride, vanadate, sodium fluoride (NaF), 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, 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.

Isolation of Regucalcin

Regucalcin (RC) is markedly expressed in rat liver cytosol [Yamaguchi and Isogai, 1993]. Regucalcin 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 (PAGE) [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, 1999]. Mice (BALB/C, Japan SLC) were subcutaneously injected with 0.1 mg per animal of antigen (rat liver regucalcin) emulsified with Freund's complete adjuvant, and 19 days later antigen (0.25 mg/animal) was injected intraperitoneally with Freund's incomplete adjuvant. Animals were killed by bleeding 3 days after the last injection. 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).

Preparation of Cytosol

Rats were sacrificed by cardiac puncture, and the livers perfused with ice-cold 0.25 M sucrose solution were removed, frozen immediately, cut into small pieces, suspended 1:4 in 0.25 M sucrose solution containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 µg/ml leupeptin, and homogenized in Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was spun at 5,500*g* in a refrigerated centrifuge for 10 min to remove mitochondria. The 5,500*g* supernatant was spun at 105,000*g* for 60 min, and the supernatant fraction (cytosol) was pooled to assay phosphatase activity.

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 nuclei were pelleted 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 gently mixed, and a 6-ml cushion (TKM containing 2.3 M sucrose) was carefully layered on the bottom of each tube. 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 phosphate, 25 mM Hepes, 4 mM $MgCl_2$, pH 7.0) by hand homogenization. Assay of marker enzymes, as reported previously [Yamaguchi, 1992b], showed that there was less than 5% contamination by microsomes, plasma membranes, or mitochondria. DNA content in the nuclei was determined by 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 ml of reaction mixture containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 6 mM $MgCl_2$, 0.5 mM dithiothreiol, 9 mM phosphoaminoacid either liver cytosol (1.5 mg protein/ml), or nuclei (5.0 mg protein/ml; 0.5–0.8 mg DNA/ml) as reported elsewhere [Pallen and Wang, 1983; Fruman et al., 1992]. In separate experiments, the above reaction mixture contained either vehicle, anti-regucalcin (RC) antibody (50–200 ng/ml), RC (1.0 μM), vanadate (10^{-5}–10^{-3} M) or sodium fluoride (10^{-5}–10^{-3} M). The enzyme reaction was terminated after 15 min by the addition of 1.0 ml of ice-cold 10% trichloroacetic acid (TCA) and centrifuged to precipitate protein. Released inorganic phosphate in the supernatant was quantified by the method of Nakamura and Mori [1958]. Results were expressed as nmoles of inorganic phosphate liberatated per minutes (min) per milligram (mg) of cytosolic or nuclear protein. Protein concentration was determined by the method of Lowry et al. [1951] using bovine serum albumin (BSA) as the standard.

Statistical Analysis

Data were expressed as the mean ±SEM. 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. A *P*-value of <0.05 was considered to indicate a statistically significant difference.

RESULTS

Enhancement of Phosphatase Activity in Regenerating Liver Cytosol

The alteration in neutral phosphatase activity in the hepatic cytosol after a partial hepatectomy of rats is shown in Figure 1. Rats were sacrificed by bleeding at 24, 48, and 72 h after a sham operation or a partial hepatectomy. The liver weight reduced by partial hepatectomy was restored at 72 h after surgery. Phosphotyrosine, phosphoserine, and phosphothreonine was used as the substrate for phosphatase activity. Phosphatase activity toward three phosphoaminoacids in the hepatic cytosol was not significantly altered by sham operation. In the hepatic cytosol of partially hepatectomized rats, phosphatase activity toward phosphotyrosine was significantly increased at 24–72 h after a partial hepatectomy (Fig. 1A), whereas the enzyme activity toward phosphoserine or phosphothreonine was not appreciably changed (Fig.

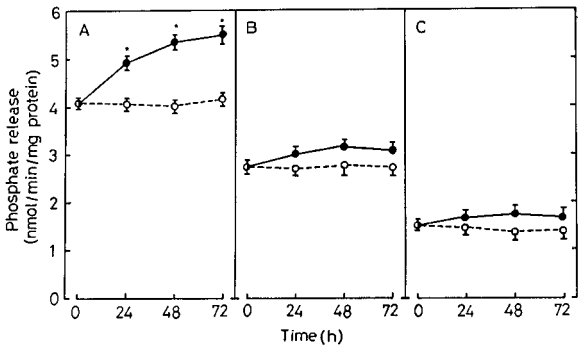

Fig. 1. Alteration in neutral phosphatase activity in the cytosol of regenerating rat liver. Rats were sacrificed by bleeding at 24, 48, and 72 h after sham operation or partial hepatectomy. Phosphotyrosine (**A**), phosphoserine (**B**), and phosphothreonine (**C**) were used as the substrate for the assay of phosphatase activity. Each value is the mean ±SEM of five rats with different experiments. **P* < 0.01, as compared with the control value at each time point. ○, Normal liver; ●, regenerating liver.

1B,C). The cytosolic phosphotyrosine phosphatase activity raised in regenerating liver was not altered in the presence of EGTA (10^{-3} M) (data not shown), indicating that the enhancement is independent on Ca^{2+}.

The effect of endogenous regucalcin (RC) on neutral phosphatase activity toward phosphotyrosine in the cytosol of regenerating rat liver was examined by using anti-RC monoclonal antibody, and the result is shown in Figure 2. The presence of anti-RC antibody (100 and 200 ng/ml) in the enzyme reaction mixture caused a significant increase in phosphatase activity toward phosphotyrosine in the hepatic cytosol of normal rat liver. Meanwhile, an increase in the liver cytosolic phosphatase activity at 24 and 48 h after a partial hepatectomy was markedly enhanced in the presence of anti-RC monoclonal antibody (200 ng/ml) in the enzyme reaction mixture. This enhancement was not seen in the presence of nonimmune IgG (data not shown).

The effect of the endogenous RC on neutral phosphatase activity toward phosphoserine and phosphothreonine in the cytosol of regenerating rat liver is shown in Figures 3 and 4. The presence of anti-RC monoclonal antibody (100 and 200 ng/ml) in the enzyme reaction mixture caused a significant increase in the cytosolic phosphatase activity toward phosphoserine (Fig. 3) and phosphothreonine (Fig. 4) of normal rat liver. At 24 and 48 h after a partial hepatectomy, phosphatase activity toward phosphoserine and phosphothreonine in the cytosol of regenerating liver was markedly enhanced in the presence of anti-RC antibody (100 and

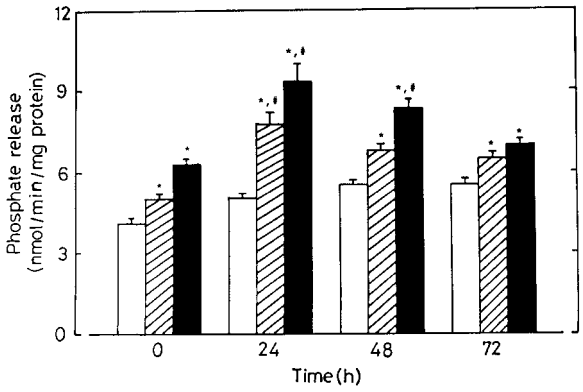

Fig. 2. Effect of anti-regucalcin (RC) monoclonal antibody on neutral phosphatase activity toward phosphotyrosine in the cytosol of regenerating rat liver. Rats were sacrificed by bleeding at 24, 48, and 72 h after partial hepatectomy. The enzyme reaction mixture contained either vehicle or anti-RC monoclonal antibody (100 and 200 ng/ml). Each value is the mean ±SEM five rats with different experiments. $*P < 0.01$, as compared with the control value without RC antibody addition at each time point. $\#P < 0.01$, as compared with the value of net increase in phosphatase activity with RC antibody addition obtained at zero time point. □, Control (none); ▨, anti-RC antibody (100 ng/ml); ■, anti-RC antibody (200 ng/ml).

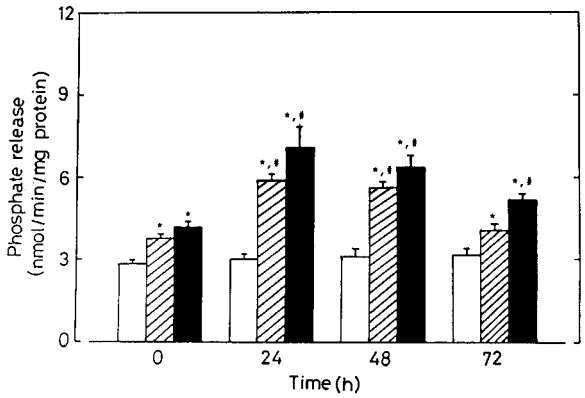

Fig. 3. Effect of anti-regucalcin (RC) monoclomal antibody on neutral phosphatase activity toward phosphoserine in the cytosol of regenerating rat liver. Rats were sacrificed by bleeding at 24, 48, and 72 h after partial hepatectomy. The enzyme reaction mixture contained either vehicle or anti-RC monoclonal antibody (100 and 200 ng/ml). Each value is the mean ±SEM of five rats with different experiments. $*P < 0.01$, as compared with the control value without RC antibody addition at each time point. $\#P < 0.01$, as compared with the value of net increase in phosphatase activity with RC antibody addition obtained at zero time point. □, Control (none); ▨, anti-RC antibody (100 ng/ml); ■, anti-RC antibody (200 ng/ml).

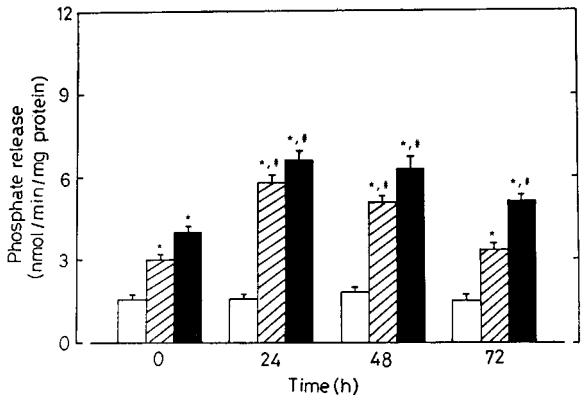

Fig. 4. Effect of anti-regucalcin (RC) monoclonal antibody on neutral phosphatase activity toward phosphothreonine in the cytosol of regenerating rat liver. Rats were sacrificed by bleeding at 24, 48, and 72 h after partial hepatectomy. The enzyme reaction mixture contained either vehicle or anti-RC monoclonal antibody (100 and 200 ng/ml). Each value is the mean ±SEM of five rats with different experiments. $*P < 0.01$, as compared with the control value without RC antibody addition at each time point. $\#P < 0.01$, as compared with the value of net increase in phosphatase activity with RC antibody addition obtained at zero time point. □, Control (none); ▨, anti-RC antibody (100 ng/ml); ■, anti-RC antibody (200 ng/ml).

200 ng/ml). Such an enhancement was also seen in the liver cytosolic phosphatase activity toward phosphoserine and phosphothreonine at 72 h after a partial hepatectomy in the presence of anti-RC antibody (200 ng/ml). Meanwhile, nonimmune IgG (200 ng/ml) had no effect on phosphatase activity toward phosphoserine and phosphothreonine in the liver cytosol obtained at 24, 48, and 72 h after a partial hepatectomy (data not shown).

Anti-RC monoclonal antibody (200 ng/ml)-enhanced phosphatase acticity toward phosphotyrosine (Fig. 5A), phosphoserine (Fig. 5B), and phosphothreonine (Fig. 5C) in the liver cytosol obtained at 24 h after a partial hepatectomy was completely blocked in the presence of the exogenous RC (1.0 μM) in the enzyme reaction mixture (Fig. 5). Thus, the endogenous RC had a suppressive effect on the enhancement of neutral phosphatase activity in regenerating rat liver.

Enhancement of Phosphatase Activity in Regenerating Liver Nuclei

Phosphatase activity toward phosphotyrosine, phosphothreonine was present in the nuclei of rat liver. The enzyme activity toward phosphoserine and phosphothreonine in the nuclei was a lower level in comparison with that of the cytosol. However, liver nuclei contained a comparatively higher level of phosphotyrosine phosphatase activity, as compared with that of phosphoserine and phosphothreonine phosphatase activities. The alteration in phosphatase activity in the liver nuclei after partial hepatectomy is shown in Figure 6. Phosphatase activity toward phosphotyrosine (Fig. 6A), phosphoserine (Fig. 6B), and phosphothreonine (Fig. 6C) in the hepatic nuclei was not significantly altered by sham operation of rats. The enzyme activity toward phosphotyrosine was significantly increased by the hepatectomy (Fig. 6A). An appreciable elevation of phosphatase activity toward phosphoserine and phosphothreonine in the liver nuclei was not occured by hepatectomy (Fig. 6B,C).

The effect of the endogenous RC on protein phosphatase activity in the nuclei of regenerating rat liver was examined by using anti-RC monoclonal antibody (Fig. 7). Liver nuclei were obtained at 24 h after a sham operation or a partial hepatectomy. Phosphatase activity toward phosphotyrsine in the nuclei of normal and regenerating livers was significantly increased by the presence of anti-RC antibody (150 ng/ml) in the enzyme reaction mixture (Fig. 7A). The enzyme activity toward phosphoserine in the nuclei of normal and regenerating livers was significantly elevated by the presence of anti-RC antibody (50–150 ng/ml) (Fig. 7B). Meanwhile, the effect of anti-RC antibody to increase phosphatase activity toward phosphothreonine of normal liver nuclei was significantly enhanced in regenerating liver (Fig. 7C). The presence of nonimmune IgG (150 ng/ml) did not have an appreciable effect on phospha-

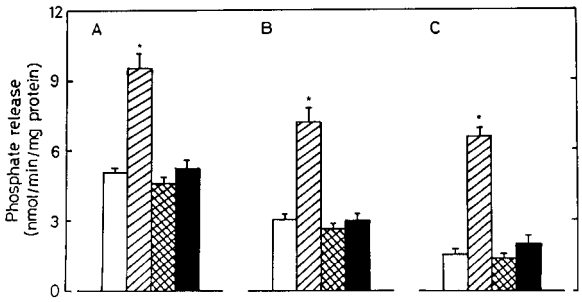

Fig. 5. Effect of regucalcin (RC) on anti-RC monoclonal antibody-increased neutral phosphatase activity in the cytosol of regenerating rat liver. Rats were sacrificed by bleeding at 24 h after partial hepatectomy. Phosphotyrosine (**A**), phosphoserine (**B**), and phosphothreonine (**C**) were used as the substrate for the assay of phosphatase activity. The enzyme reaction mixture contained either vehicle, anti-RC antibody (200 ng/ml), RC (1.0 μM) or anti-RC antibody (200 ng/ml) plus RC (1.0 μM). Each value is the mean ±SEM of five rats with different experiments. *$P < 0.01$, as compared with the control (none) value. □, Control (none); ▨, anti-RC antibody; ▩, RC; ■, anti-RC antibody plus RC.

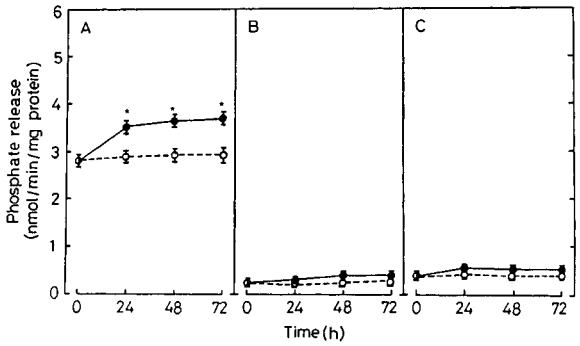

Fig. 6. Alteration in neutral phosphatase activity in the nuclei of regenerating rat liver. Rats were sacrificed by bleeding at 24, 48, and 72 h after sham operation or partial hepatectomy. Phosphotyrosine (**A**), phosphoserine (**B**), and phosphothreonine (**C**) were used as the substrate for the assay of phosphatase activity. Each value is the mean ±SEM of five rats with different experiments. *$P < 0.01$, as compared with the control value at each time point. ○, Normal liver; ●, regenerating liver.

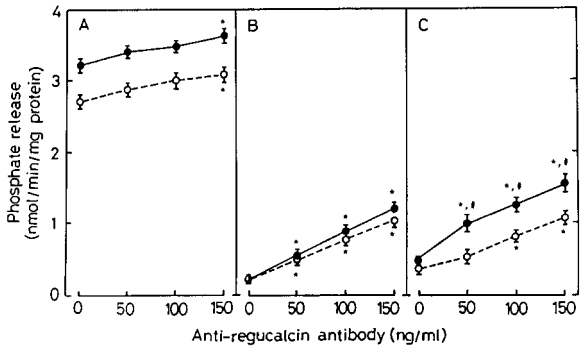

Fig. 7. Effect of anti-regucalcin (RC) monoclonal antibody on neutral phosphatase activity in the nuclei of regenerating rat liver. Rats were sacrificed by bleeding at 24 h after sham operation or partial hepetectomy. Phosphotyrosine (**A**), phosphoserine (**B**), and phosphothreonine (**C**) were used as the substrate for the assay of phosphatase activity. The enzyme reaction mixture contained either vehicle or anti-RC monoclonal antibody (50, 100, and 150 ng/ml). Each value is the mean ±SEM of five rats with different experiments. $*P < 0.01$, as compared with the control value without RC antibody addition. $\#P < 0.01$, as compared with the control value with RC antibody addition obtained from sham-operated rats. ○, normal liver; ●, regenerating liver.

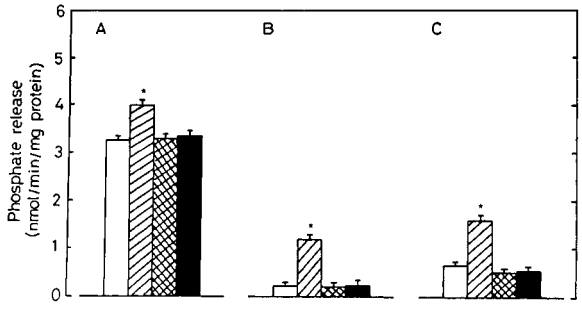

Fig. 8. Effect of regucalcin (RC) on anti-RC monoclonal antibody-increased neutral phosphatase activity in the nuclei of regenerating rat liver. Rats were sacrificed by bleeding at 24 h after partial hepatectomy. Phosphotyrosine (**A**), phosphoserine (**B**), and phosphothreonine (**C**) were used as the substrate for the assay of phosphatase activity. The enzyme reaction mixture contained either vehicle, anti-RC antibody (150 ng/ml), RC (1.0 μM) or anti-RC antibody (150 ng/ml) plus RC (1.0 μM). Each value is the mean ±SEM of five rats with different experiments. $*P <$ 0.01, as compared with the control value. □, Control (none); ▨, anti-RC antibody; ▩, RC; ■, anti-RC antibody plus RC.

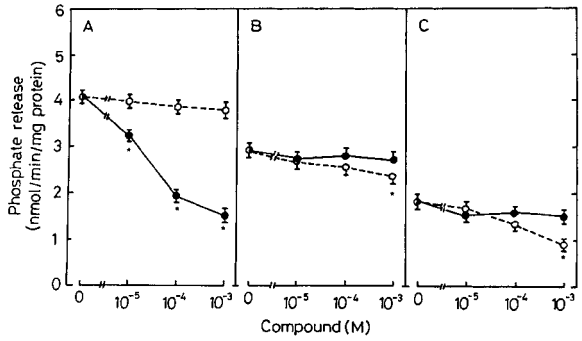

Fig. 9. Effect of vanadate or sodium fluoride (NaF) on neutral phosphatase activity in the cytosol of normal rat liver. Phosphotyrosine (**A**), phosphoserine (**B**), and phosphothreonine (**C**) were used as the substrate for the assay of phosphatase activity. The enzyme reaction mixture contained either vehicle, vanadate (10^{-5}–10^{-3} M) or NaF (10^{-5}–10^{-3} M). Each value is the mean ±SEM of five rats with different experiments. $*P < 0.01$, as compared with the control (none) value. ○, NaF, ●, vanadate.

tase activity toward three phosphoaminoacids in the nuclei of normal rat liver (data not shown).

The effect of the exogenous RC addition on anti-RC antibody-increased phosphatase activity in the nuclei of regeneration rat liver is shown in Figure 8. The addition of the exogenous RC (1.0 μM) in the enzyme reaction mixture caused a complete prevention of the phosphatase activity toward phosphotyrosine, phosphoserine, and phosphothreonine raised by anti-RC antibody (150 ng/ml) in the liver nuclei obtained at 24 h after a partial hepatectomy. The addition of RC (1.0 μM) had no effect on the enzyme activity in the nuclei of regenerating rat liver in the absence of anti-RC antibody.

Effect of Inhibitors on Phosphatase Activity in Regenerating Liver

The effect of vanadate or NaF on phosphatase activity toward phosphotyrosine, phosphoserine, and phosphothreonine in the cytosol of normal rat liver is shown in Figure 9. Phosphatase activity toward phosphotyrosine was significantly decreased by the addition of vanadate (10^{-5}–10^{-3} M) in the enzyme reaction mixture, while NaF (10^{-5}–10^{-3} M) had no effect (Fig. 9A). Phosphatase activity toward phosphoserine and phosphothreonine was significantly reduced by the addition of NaF (10^{-3} M), while vanadate (10^{-5}–10^{-3} M) did not have an effect (Fig. 9B,C).

The effect of vanadate or NaF on the anti-RC antibody-increased phosphotase activity toward phosphotyrosine, phosphoserine, and phosphothreonine in the cytosol of normal and regenerating rat livers is shown in Table I. Rats were sacrificed by bleeding at 24 h after a sham operation (normal) and a partial hepetectomy. In regenerating liver, the cytosolic phosphatase activity toward phosphotyrosine, but not phosphoserine and phosphothreonine, was markedly inhibited by vanadate (10^{-3} M). NaF

(10^{-3} M) significantly decreased phosphatase activity toward phosphothreonine. The effect of anti-RC monoclonal antibody (200 ng/ml) to increase phosphatase activity toward three phosphoaminoacids in the nuclei of normal and regenerating livers was also seen in the presence of vanadate (10^{-3} M) or NaF (10^{-3} M).

The effect of vanadate or NaF on phosphatase activity in the nuclei of normal and regenerating rat livers is shown in Table II. Rats were sacrificed by bleeding at 24 h after a sham operation or a partial hepatectomy. Vanadate (10^{-3} M) had an inhibitory effect on phosphatase activity toward phosphotyrosine, but not

TABLE I. Effect of Vanadate or NaF on Phosphatase Activity in the Cytosol of Normal and Regenerating Rat Livers†

Treatment	Phosphatase (nmol/min/mg protein)		
	Phosphotyrosine	Phosphoserine	Phosphothreonine
Normal			
Control	4.02 ± 0.18	2.91 ± 0.14	1.68 ± 0.13
RC antibody	6.03 ± 0.31*	4.21 ± 0.18*	4.01 ± 0.21*
Vanadate	1.51 ± 0.02*	2.40 ± 0.16	1.32 ± 0.18
RC antibody + vanadate	3.98 ± 0.22#	3.52 ± 0.11#	3.56 ± 0.18#
NaF	3.99 ± 0.03	2.20 ± 0.05*	0.82 ± 0.08*
RC antibody + NaF	5.68 ± 0.11#	4.12 ± 0.14#	3.20 ± 0.21#
Regenerating			
Control	4.90 ± 0.23	2.94 ± 0.18	1.55 ± 0.07
RC antibody	9.35 ± 0.77*	7.13 ± 0.66*	6.41 ± 0.51*
Vanadate	2.19 ± 0.05*	2.98 ± 0.20	1.11 ± 0.18
RC antibody + vanadate	7.73 ± 0.82#	5.96 ± 0.40#	5.69 ± 0.19#
NaF	4.71 ± 0.27	2.27 ± 0.15	0.53 ± 0.34*
RC antibody + NaF	5.99 ± 0.29*	5.29 ± 0.06#	5.13 ± 0.35#

†Rats were sacrificed by bleeding at 24 h after partial hepatectomy. The enzyme reaction mixture contained either vehicle, anti-RC antibody (200 ng/ml), vanadate (10^{-3} M), or NaF (10^{-3} M). Each value is the mean ± SEM of five rats.
*$P < 0.01$, as compared with the control value.
# $P < 0.01$, as compared with the value of vanadate or NaF alone.

TABLE II. Effect of Vanadate or NaF on Phosphatase Activity in the Nuclei of Normal and Regenerating Rat Livers†

Treatment	Phosphatase (nmol/min/mg protein)		
	Phosphotyrosine	Phosphoserine	Phosphothreonine
Normal			
Control	2.70 ± 0.11	0.35 ± 0.02	0.36 ± 0.04
RC antibody	3.76 ± 0.23*	1.19 ± 0.01*	1.02 ± 0.15*
Vanadate	0.22 ± 0.03*	0.25 ± 0.12	0.21 ± 0.08
RC antibody + vanadate	1.05 ± 0.07*	0.83 ± 0.04#	0.77 ± 0.02#
NaF	2.21 ± 0.12	0.26 ± 0.05	0.32 ± 0.03
RC antibody + NaF	3.23 ± 0.05#	0.87 ± 0.04#	0.81 ± 0.02#
Regenerating			
Control	3.15 ± 0.05	0.36 ± 0.06	0.38 ± 0.10
RC antibody	3.71 ± 0.11*	1.28 ± 0.09*	1.65 ± 0.15*
Vanadate	0.30 ± 0.09*	0.35 ± 0.01	0.40 ± 0.12
RC antibody + vanadate	2.09 ± 0.23#	0.77 ± 0.03#	1.01 ± 0.05#
NaF	2.78 ± 0.06	0.33 ± 0.05	0.50 ± 0.07
RC antibody + NaF	3.34 ± 0.10#	0.99 ± 0.17#	1.14 ± 0.06#

†Rats were sacrificed by bleeding at 24 h after partial hepatectomy. The enzyme reaction mixture contained either vehicle, anti-RC antibody (150 ng/ml), vanadate (10^{-3} M) or NaF (10^{-3} M). Each value is the mean ± SEM of five rats.
*$P < 0.01$, as compared with the control value.
#$P < 0.01$, as compared with the value of vanadate or NaF alone.

phosphoserine and phosphothreonine, in the nuclei of normal and regenerating livers, whereas NaF (10^{-3} M) did not cause a significant alteration in the nuclear phosphatase activity toward three phosphoaminoacids. The effect of anti-RC antibody (150 ng/ml) to increase the nuclear phosphatase activity toward three phosphoaminoacids in normal and regenerating livers was also seen in the presence of vanadate (10^{-3} M) or NaF (10^{-3} M).

DISCUSSION

Protein phosphorylation plays a cardinal role in regulating many cellular processes in eukaryotes. Processes that are reversibly controlled by protein phosphorylation require not only a protein kinase but a protein phosphatase as well [Hunter, 1995]. Target proteins are phosphorylated at specific sites by one or more protein kinases, and these phosphates are removed by specific protein phosphatases [Cohen and Cohen, 1989].

There may be many protein phosphatases in liver cells. Neutral phosphatase activity toward phosphotyrosine, phosphoserine, and phosphothreonine was present in the cytosol of normal rat liver. Phosphatase activity toward phosphotyrosine was also found in the liver nuclei, whereas the nuclear enzyme activity toward phosphoserine and phosphothreonine was only slightly. Thus, the distribution of phosphatases toward phosphotyrosine may differ from that of the enzymes toward phosphoserine and phosphothreonine in the cytoplasm and nuclei of rat liver. Phosphotyrosine phosphatase activity in the cytoplasm and nuclei of liver cells was clearly enhanced in regenerating liver after a partial hepatectomy in rats.

Phosphatase activity toward phosphotyrosine, phosphoserine, and phosphothreonine in the cytosol of normal rat liver was significantly increased by the presence of anti-regucalcin (RC) monoclonal antibody in the enzyme reaction mixture, indicating that the endogenous RC exhibits an inhibitory effect on the enzyme activity. Moreover, the cytosolic enzyme activity in regenerating liver after a partial hepatectomy. These findings suggest that the endogenous RC in the cytoplasm has a suppressive role in the enhancement of protein phosphatase activity induced by the proliferation of liver cells. Hepatic RC mRNA expression has been demonstrated to stimulate in regenerating rat liver [Yamaguchi and Kanayama, 1995]. RC is mainly expressed in liver tissues with a comparatively higher concentration [Yamaguchi and Isogai, 1993; Shimokawa and Yamaguchi, 1992]; the tissue concentration is about 50 μM. The enzyme reaction mixture contained about 0.074 μM of RC in the used liver cytosol. The effect of anti-RC antibody to increase phosphatase activity in the liver cytosol was completely abolished by the presence of exogenous RC (1.0 μM). Presumably, regucalcin (RC) plays a physiological role in the inhibition of protein phosphatases toward phosphotyrosine, phosphoserine, and phosphothreonine in the hepatic cytoplasm.

Protein phosphatases are present in the nucleus of mitotic cells [Fernandez et al., 1992; Galaktinov and Beach, 1991]. Anti-RC antibody significantly increased phosphatase activity toward three phosphoaminoacids in the nuclei of normal rat liver. Nuclear phosphatase activity toward phosphotyrosine was significantly enhanced in regenerating rat liver. Such an effect was not seen in the case of phosphotyrosine and phosphoserine. These results suggest that the endogenous RC can inhibit phosphatase activity in the liver nuclei and that RC has a potent inhibitory effect on phosphothreonine phosphatase activity in the liver nuclei. Liver cytoplasmic RC may be transported into the nuclear matrix, although this mechanism is unknown. Presumably, RC participates in the regulation of liver nuclear functions. Previous investigations demonstrated that RC could inhibit DNA and RNA syntheses in the nuclei of normal and regenerating rat livers [Yamaguchi and Kanayama, 1996; Yamaguchi and Ueoka, 1997]. Thus, RC may play a suppressive role in the enhancement of nuclear functions in the proliferation of liver cells.

Many phosphatases toward phosphotyrosine, phosphoserine, and phosphothreonine may exist in the cytosol and nuclei of rat liver. The molecule of phosphatases that are inhibited by RC is unknown. This was examined by using an inhibitor of phosphatases. Protein phosphatases is known to be inhibited by vanadate and other inhibitors [MacKintosh and MacKintosh, 1994]. Vanadate can inhibit protein-tyrosine phosphatases. The present result shows that vanadate has an inhibitory effect on phosphotyrosine phosphatase activity in the liver cytosol and nuclei, although it has no effect on the enzyme activity toward phosphoserine and phosphothreonine. Meanwhile, NaF inhibited phosphoserine phosphatase activity in the liver

cytosol but not nuclei. Thus, vanadate and NaF had a specific inhibitory effect on phosphatase activity. The effect of anti-RC antibody in increasing phosphatase activity in the liver cytosol and nuclei was also seen in the presence of vanadate or NaF. From these results, it is assumed that the endogenous RC may act on phosphatases that are insensitive to vanadate or NaF. Moreover, RC may have a more potent inhibitory effect than vanadate or NaF on phosphatases. RC may act on various phosphatases in liver cells.

The physiological significance that RC inhibits protein phosphatase activity in liver cells is not clarified. RC has been demonstrated to inhibit protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase [Yamaguchi and Mori, 1990; Mori and Yamaguchi, 1990]. Thus, RC can regulate both protein kinases and protein phosphatases. Presumably, RC participates in the regulation of cellular functions due to inhibiting processes of phosphorylation and dephosphorylation of various proteins in liver cells. In particular, this role may be intensively exhibited in the control of proliferation of liver cells.

In conclusion, it has been found that neutral phosphatase activity toward phosphoamino acids is enhanced in the cytoplasm and nuclei of regenerating rat liver, and that this enhancement is suppressively regulated by the endogenous regucalcin.

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