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Suppressive Role of Endogenous Regucalcin in the Enhancement of Deoxyribonucleic Acid Synthesis Activity in the Nucleus of Regenerating Rat Liver

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Abstract The role of endogenous regucalcin in the regulation of deoxyribonucleic acid (DNA) synthesis activity in the nuclei of regenerating rat liver after partial hepatectomy was investigated. The addition of regucalcin (0.25 and 0.5 μM) in the reaction mixture caused a significant decrease in the nuclear DNA synthesis activity of normal rat liver. This decrease was also seen in the presence of Ca^{2+}-chelator EGTA (0.4 mM), indicating that the effect of regucalcin is not related to nuclear Ca^{2+}. Nuclear DNA activity was significantly increased in the presence of anti-regucalcin monoclonal antibody (10–50 ng/ml) in the reaction mixture. The effect was completely abolished by the addition of regucalcin (0.5 μM). Nuclear DNA synthesis activity was significantly increased at 24, 48, and 72 h after partial heptectomy. The effect of anti-regucalcin monoclonal antibody (25 ng/ml) in increasing nuclear DNA synthesis activity was significantly enhanced at 24 and 48 h after partial hepatectomy. The presence of staurospone (10^{-6} M), trifluoperazine (2×10^{-5} M), or PD98059 (10^{-5} M) in the reaction mixture caused a significant decrease in DNA synthesis activity in the nuclei obtained at 24 after partial hepateactomy. The effect of these inhibitors in the presence of anti-regucalcin monoclonal antibody (25 ng/ml) was greater than that in the absence of the antibody. The present study suggests that endogenous regucalcin plays a suppressive role in the enhancement of nuclear DNA synthesis activity in regenerating liver with cell proliferation after partial hepatectomy in rats. J. Cell. Biochem. 85: 516–522, 2002.

Key words: regucalcin; calcium; nuclear DNA synthesis; cell proliferation; regenerating rat liver

Calcium ion (Ca^{2+}) plays an important role in the regulation of many cell functions. The Ca^{2+} effect in cells is amplified by Ca^{2+}-dependent protein kinases, which are related to a signal transduction due to hormonal stimulation [Cheung, 1980; Nishizuka, 1986; Heizman and Hunziker, 1991; Kraus-Friedman and Feng, 1996]. Regucalcin, which was found as a novel Ca^{2+}-binding protein [Yamaguchi and Yamamoto, 1978; Yamaguchi, 1988], is greatly present in liver and kidney cortex [Shimokawa and Yamaguchi, 1992; Yamaguchi and Isogai, 1993]. The expression of regucalcin mRNA is mediated through Ca^{2+}-signaling mechanism [Shimokawa and Yamaguchi, 1993a,b; Murata and Yamaguchi, 1999; Yamaguchi and Nakajima, 1999; Misawa and Yamaguchi, 2000]. Regucalcin has been demonstrated to play a multifunctional role as a regulatory protein in Ca^{2+}-signaling process [Yamaguchi, 2000a,b]. Regucalcin may play an important role in the regulation of Ca^{2+}-related cell functions.

The role of regucalcin in the regulation of proliferation of liver cells has been shown in the cloned hepatoma H4-II-E cells in vitro. Regucalcin can inhibit protein kinase [Inagaki and Yamaguchi, 2001a] and protein tyrosine phosphatase [Inagaki and Yamaguchi, 2000] activities which are increased in the proliferation of the cloned rat hepatoma cells cultured with fetal bovine serum. More recently, it has been demonstrated that endogenous regucalcin has an inhibitory effect on DNA synthesis in the nuclei of cloned rat hepatoma cells cultured with serum stimulation [Inagaki and Yamaguchi, 2001b]. Regucalcin may play an inhibitory role

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in the signaling pathway, which is related to protein kinase and protein phosphatases in proliferative cells, suggesting a suppressive role of regucalcin in cell proliferation.

The expression of regucalcin mRNA has been demonstrated to be enhanced in regenerating rat liver which induces a proliferation of liver cells after partial hepatectomy in vivo [Yamaguchi and Kanayama, 1995]. Exogenous regucalcin could inhibit DNA synthesis activity in the nucleus isolated from regenerating rat liver [Yamaguchi and Kanayama, 1996]. Recently it has been shown that regucalcin is translocated to the nucleus isolated from normal rat liver and that the protein is localized in the nucleus [Omura and Yamaguchi, 1999a; Tsurusaki et al., 2000], suggesting that endogenous regucalcin has an effect on DNA synthesis activity in the nucleus of regenerating rat liver. This, however, has not been fully clarified.

The present study was undertaken to determine the role of endogenous regucalcin in the regulation of DNA synthesis activity in the nucleus of regenerating rat liver using anti-regucalcin monoclonal antibody. We found that endogenous regucalcin plays a suppressive role in the enhancement of nuclear DNA synthesis activity in regenerating rat liver.

MATERIALS AND METHODS

Chemicals

Trifluoperazine, staurosporine, PD98059, and ethyleneglycol bis (2-aminoethylether)-N, N, N′, N′-tetraacetic acid (EGTA) were obtained from Sigma Chemical Co. (St. Louis, MO). Calcium chloride and other chemicals were reagent grade from Wako Pure Chemical Industries Ltd. (Osaka, Japan). (Methyl-^{3}H) deoxythimidine 5′triphosphate ([^{3}H]-dTTP; 2.59 TBq/mmol) was obtained from New England Nuclear (Boston, MA). The reagents were dissolved in distilled water and ethanol solution.

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 24–72 h later the animals were sacrificed by bleeding.

Isolation of Regucalcin

Regucalcin is markedly expressed in rat liver cytosol [Shimokawa and Yamaguchi, 1992; Yamaguchi and Isogai, 1993]. Regucalcin was isolated from rat liver cytosol. The livers were perfused with Tris-HCl buffer (pH 7.4), containing 100 mM Tris, 120 mM NaCl, 4 mM KCl, cooled to 4°C. The livers were removed, cut into small pieces, suspended 1:4 (w/v) in Tris-HCl buffer (pH 7.4); the homogenate was spun at 5,500*g* in a refrigerated centrifuge for 10 min, and the supernatant was spun at 5,500*g* in a refrigerated centrifuge at 38,000*g* for 20 min. Regucalcin in the supernatant was purified to electrophoretic homogeneity by gel filtration on Sephadex G-75 and G-50 followed by ion-exchange chromatography on diethylaminoethyl cellulose, as reported previously [Yamaguchi and Yamamoto, 1978]. Protein concentration was measured by the method of Lowry et al. [1951] using bovine serum albumin as a standard.

Anti-Regucalcin Antibody

Anti-regucalcin antibody was a monoclonal antibody raised against regucalcin prepared using standard methods [Omura and Yamaguchi, 1999b]. Mice (BALB/C, Japan SLC) were subcutaneously injected with 0.1 mg per animal of antigen injected with 0.1 mg per animal of antigen (rat liver regucalcin) emulsified with Freund's complete adjuvant, and 19 days later antigen (0.15 mg/animal) was intraperitoneally injected with Freund's incomplete adjuvant. Animals were killed by bleeding 3 days after the last injection. Spleen cells were prepared from immunized mouse and fused into myeloma cells. Anti-regucalcin monoclonal antibody-producing cells (hybridoma cells) were obtained by screening. The IgG from hybridoma cells was isolated through a protein A-agarose column (Sigma).

Isolation of Nucleus

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 centrifuged 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 pellets were 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 phosphate, 25 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 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].

Estimation of DNA Synthesis

DNA synthesis by the nuclear fraction of rat liver was estimated by the procedure of Lynch et al. [1970] with a minor modification. DNA synthesis was measured for 30 min at 37°C in mixtures (0.5 ml) that contained 0.18 M Tris/HCl buffer (pH 8.2), 4 mM $MgCl_2$, 2 mM ATP, dGTP, dCTP, dATP (each 0.08 mM), 0.06 mM [^{3}H] dTTP, dextran (Type 100 C, 2%, 2.5 mM cadaverine), and the suspension of nuclei (0.1 ml containing 100–150 μg DNA or 1–1.5 mg protein). Regucalcin (0.1–0.5 μM), anti-regucalcin monoclonal antibody (10–50 ng/ml), or various inhibitors (PD98059, staurosporine, and TFP) were added as indicated. Reactions were stopped with 0.5 ml of 1 M NaOH and DNA was precipitated with an addition (5 ml) of ice-cold trichloroacetic acid (10% TCA). The DNA was then dissolved (0.5 ml of 1 M NaOH) and precipitated (5 ml of 10% TCA), and the final precipitate, dissolved in 0.5 ml of 1 M NaOH, was heated at 80°C for 15 min. Finally, the DNA was precipitated with acid, ethanol, and ether. The radioactivity in nuclear DNA was measured in a hyaminetoluene liquid scintillation mixture, and all the data were corrected for the incorporation with control mixture that lacked the three unlabeled deoxynucleotides. DNA synthesis was expressed as disintegrations per minute (dpm) per milligram of nuclear DNA.

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

Effect of Regucalcin on DNA Synthesis Activity in Liver Nuclei

The effect of regucalcin on DNA synthesis activity in the nuclei of normal rat liver is shown in Figure 1. Nuclear DNA synthesis activity was significantly increased in the presence of EGTA (0.4 mM) in the reaction mixture (Fig. 1A), indicating that DNA synthesis activity may be suppressed by endogenous Ca^{2+} in the nucleus. The presence of regucalcin (0.25 and 0.5 μM) caused a significant decrease in nuclear DNA

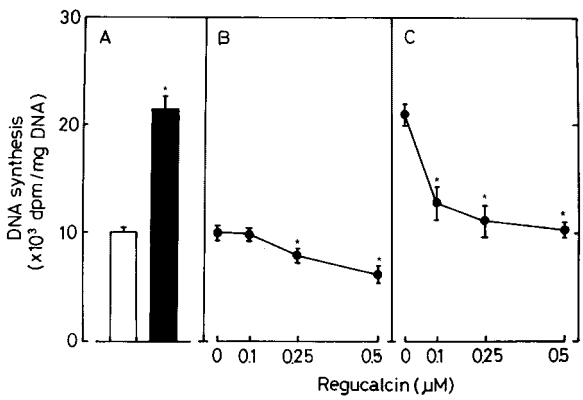

Fig. 1. Effect of regucalcin on DNA synthesis activity in the nuclei of normal rat liver. DNA synthesis activity was measured in a reaction mixture containing (**A**) either vehicle or EGTA (0.4 mM), (**B**) either vehicle or regucalcin (0.1, 0.25, and 0.5 μM) in the absence of EGTA or (**C**) either vehicle or regucalcin (0.1, 0.25, and 0.5 μM) in the presence of EGTA (0.4 mM). Each value is the mean ± SEM of five experiments with different rats. *$P < 0.01$, compared with the control value without or with EGTA. In Figure 1A, white bar, control (none); black bars, EGTA (0.4 mM) addition.

synthesis activity (Fig. 1B). The effect of regucalcin (0.1–0.5 μM) was significantly enhanced in the presence of EGTA (0.4 mM) (Fig. 1C).

The effect of anti-regucalcin monoclonal antibody on DNA synthesis activity in the nuclei of normal rat liver is shown in Figure 2. Nuclear DNA synthesis activity was significantly increased in the presence of anti-regucalcin monoclonal antibody (10, 25, and 50 ng/ml) in the reaction mixture (Fig. 2A). This effect was also seen in the presence of EGTA (0.4 mM) (Fig. 2B). The effect of anti-regucalcin monoclonal antibody (50 ng/ml) in increasing nuclear DNA synthesis activity was completely abolished by the addition of exogenous regucalcin (0.5 μM) (Fig. 2C).

The effect of $CaCl_2$ addition on DNA synthesis activity in the nuclei of normal rat liver is shown in Figure 3. The presence of $CaCl_2$ (5–50 μM) in the reaction mixture caused a significant decrease in the nuclear DNA synthesis activity (Fig. 3A). The effect of anti-regucalcin monoclonal antibody (25 ng/ml) in increasing nuclear DNA synthesis activity was seen in the presence of $CaCl_2$ (10 μM) (Fig. 3B).

Role of Endogenous Regucalcin in the Enhancement of Nuclear DNA Synthesis Activity in Regenerating Rat Liver

The change in DNA synthesis activity in the nuclei of regenerating rat liver is shown in Figure 4. Rats were killed 24, 48, and 72 h after partial hepatectomy. DNA synthesis activity was significantly increased in the nuclei of regenerating rat liver after partial hepatectomy as compared with that of rats killed immediately after sham operation. Nuclear DNA

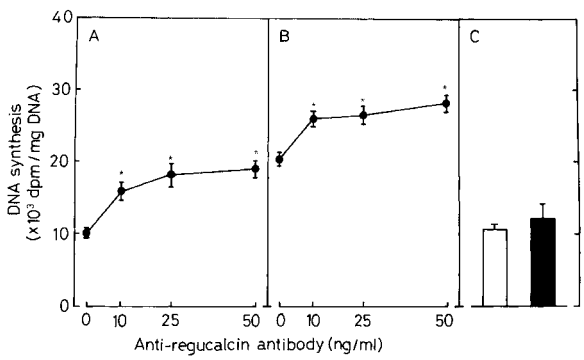

Fig. 2. Effect of anti-regucalcin monoclonal antibody on DNA synthesis activity in the nuclei of normal rat liver. DNA synthesis activity was measured in a reaction mixture containing (**A**) either vehicle or anti-regucalcin monoclonal antibody (10, 25, and 50 ng/ml), (**B**) either vehicle or the antibody in the presence of EGTA (0.4 mM), or (**C**) either vehicle or the antibody (50 ng/ml) in the presence of regucalcin (0.5 μM) using the nuclear fraction. Each value is the mean ± SEM of five experiments with different rats. $*P < 0.01$, compared with the control value without the antibody. White bars, regucalcin; black bars, regucalcin plus antibody.

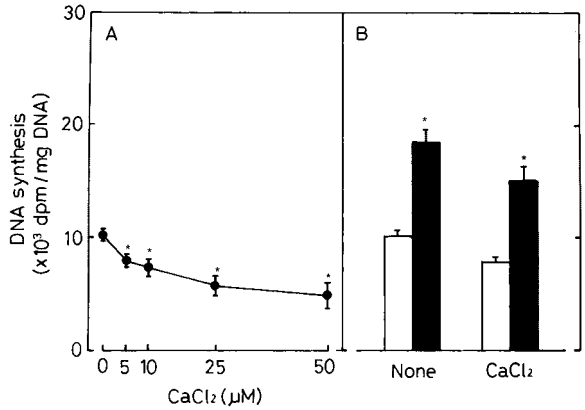

Fig. 3. Effect of calcium chloride addition on anti-regucalcin monoclonal antibody-increased DNA synthesis activity in the nuclei of normal rat liver. DNA synthesis activity was measured in a reaction mixture containing (**A**) either vehicle or $CaCl_2$ (5, 10, 25, and 50 μM), or (**B**) either vehicle or anti-regucalcin monoclonal antibody (25 ng/ml) in the absence or presence of $CaCl_2$ (10 μM) using the nuclear fraction. Each value is the mean ± SEM of five experiments with different rats. $*P < 0.01$, compared with the control value. White bars, control; black bars, anti-regucalcin antibody.

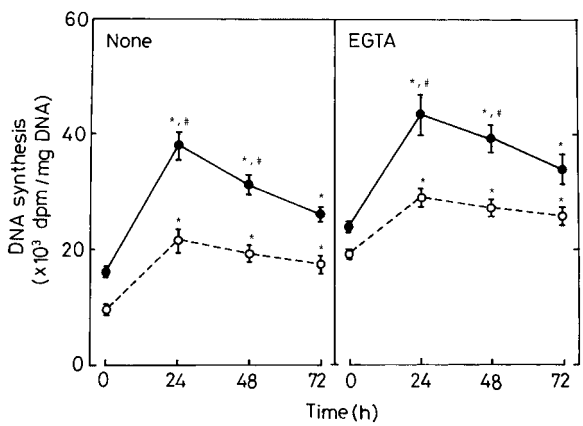

Fig. 4. Change in DNA synthesis activity in the nuclei of rats after partial hepatectomy. Rats were killed by bleeding at 24, 48, and 72 h after partial hepatectomy. DNA synthesis activity was measured in a reaction mixture containing either vehicle or anti-regucalcin monoclonal antibody (25 ng/ml) in the absence or presence of EGTA (0.4 mM) using the nuclear fraction. Each value is the mean ± SEM of five experiments with separate rats. $*P < 0.01$, compared with the control value obtained at zero time. $\#P < 0.01$, compared with the net increase with regucalcin antibody addition obtained at the zero time. Open circles, none; closed circles, anti-regucalcin antibody addition.

synthesis activity in the liver obtained from normal rats was not significantly altered by sham operation (data not shown). This increase with partial hepatectomy was also seen in the presence of EGTA (0.4 mM) in the reaction mixture. The presence of anti-regucalcin monoclonal antibody (25 ng/ml) in the reaction mixture significantly enhanced the increase in DNA synthesis activity in the nuclei of regenerating liver obtained at 24 and 48 h after partial hetapectomy. This enhancement was also in the presence of EGTA (0.4 mM) in the reaction mixture.

The effect of protein kinase inhibitors on DNA synthesis activity in the nuclei of regenerating rat liver is shown in Figure 5. Liver nuclei were obtained 24 h after sham operation or partial hepatectomy. Nuclear DNA synthesis activity was significantly decreased in the presence of staurosporine (10^{-6} M), an inhibitor of protein kinase C [Tamaoki et al., 1986], trifluoperazine (2×10^{-5} M), an inhibitor of Ca^{2+}/calmodulin-dependent protein kinase [Vincenzi, 1982], or PD98059 (10^{-5} M), an inhibitor of MAPK kinase [Zhang et al., 1999], in the reaction mixture. The effect of these inhibitors was also seen in the presence of anti-regucalcin monoclonal antibody (25 ng/ml). The effect of staurosporine, trofluoperazine, or PD98059 in decreasing nuclear DNA synthesis activity was remarkable in the presence of anti-regucalcin monoclonal antibody.

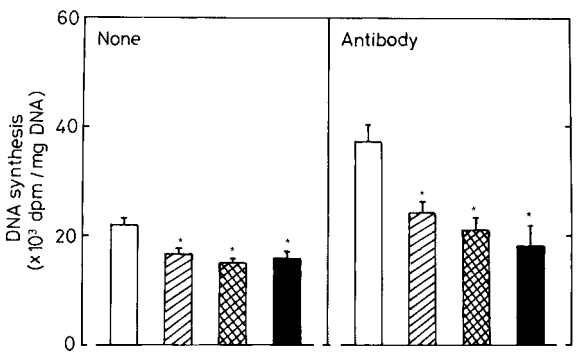

Fig. 5. Effect of protein kinase inhibitors on DNA synthesis activity in the nuclei of regenerating rat liver. Rats were killed by bleeding at 24 h after partial hepatectomy. DNA synthesis activity was measured in a reaction mixture containing either vehicle, staurosporine (10^{-6} M), trifluoperazine (2×10^{-5} M), or PD98059 (10^{-5} M) in the absence or presence of anti-regucalcin monoclonal antibody (25 ng/ml) without EGTA addition using the nuclear fraction. Each value is the mean ± SEM of five experiments with separate rats. $^*P < 0.01$, compared with the control value without or with the antibody addition. White bars, control; hatched bars, staurosporine; double hatched bars, trifluoperazine; black bars, PD98059.

DISCUSSION

This study demonstrates that regucalcin has an inhibitory effect on DNA synthesis activity in the nucleus of normal rat liver. The regucalcin effect was also seen in the presence of EGTA, a chelator of Ca^{2+}, in the reaction mixture. Ca^{2+} is present in liver nucleus [Yamaguchi and Oishi, 1993; Allbritton et al., 1994]. Liver nuclear DNA synthesis activity was significantly increased in the presence of EGTA in the reaction mixture, suggesting that nuclear Ca^{2+} regulates DNA synthesis activity. The effect of regucalcin in inhibiting nuclear DNA synthesis may be not related to Ca^{2+} in the liver nucleus, although the mechanism of Ca^{2+} effect on nuclear DNA synthesis activity is not determined.

Regucalcin is present in the nucleus of rat liver [Omura and Yamaguchi, 1999a; Tsurusaki et al., 2000]. The role of endogenous regucalcin in the regulation of DNA synthesis activity in the liver nucleus is not clarified. The presence of anti-regucalcin monoclonal antibody in the reaction mixture caused a significant increase in DNA synthesis activity in the nucleus of normal rat liver. This increase was completely abolished by the addition of regucalcin. These results suggest that endogenous regucalcin in liver nucleus regulates nuclear DNA synthesis activity. The effect of anti-regucalcin monoclonal antibody in increasing nuclear DNA synthesis activity was also seen in the presence of EGTA. Presumably, endogenous regucalcin regulates DNA synthesis activity independently of Ca^{2+} in the nucleus of liver cells DNA synthesis activity has been shown to be increased in the nucleus of regenerating rat liver after partial hepatectomy [Yamaguchi and Kanayama, 1996]. This was also seen in the present study.

DNA synthesis activity in the nucleus of regenerating rat liver was found to be significantly enhanced in the presence of anti-regucalcin monoclonal antibody in the reaction mixture. The expression of regucalcin mRNA has been demonstrated to be increased in regenerating rat liver after partial hepatectomy [Yamaguchi and Kanayama, 1995]. The endogenous regucalcin in liver nucleus may play a suppressive role in the enhancement of nuclear DNA synthesis activity of regenerating liver.

Nuclear functions in regenerating liver may be stimulated by many signaling factors [Csermely et al., 1995]. The increase in nuclear

DNA synthesis activity in regenerating rat liver was significantly prevented in the presence of staurosporine, trifluoperazine, and PD 98059, which are various protein kinase inhibitors, in the reaction mixture. This result suggests that the nuclear DNA synthesis activity in regenerating liver with proliferative cells is partly stimulated through signaling pathway, which is related to protein kinase C, Ca^{2+}/calmodulin-dependent protein kinase, and MAPK kinase in the nucleus. The effect of anti-regucalcin monoclonal antibody in enhancing nuclear DNA synthesis in regenerating liver was significantly blocked in the presence of staurosporine, trifluoperazine, and PD98059. The suppressive effect of regucalcin on nuclear DNA synthesis activity in regenerating liver may be partly mediated through the inhibitory action of regucalcin on various protein kinases. Regucalcin has been shown to inhibit Ca^{2+}-dependent protein kinases and protein tyrosine kinases in liver nucleus [Katsumata and Yamaguchi, 1998]. It is possible, however, that regucalcin binds to DNA molecules and that the protein inhibits nuclear DNA synthesis activity in liver cells. This, however, remains to be elucidated.

Regucalcin has also been shown to inhibit DNA synthesis in the nuclei of cloned rat hepatoma cells with proliferation after serum stimulation [Inagaki and Yamaguchi, 2001b]. Regucalcin may play a suppressive role in the proliferation of liver cells, because of regulating overexpression of cell proliferation. The present study demonstrates that endogenous regucalcin in the nucleus suppresses an enhancement of nuclear DNA synthesis activity with cell proliferation of regenerating rat liver in vivo. This finding further supports the view that regucalcin may play a physiologic role in the suppression for the enhancing proliferation of liver cells.

In conclusion, it has been demonstrated that endogenous regucalcin can suppress the enhancement of nuclear DNA synthesis activity in regenerating liver with cell proliferation after partial hepatectomy in rats.

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