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Role of Endogenous Regucalcin in Transgenic Rats: Suppression of Kidney Cortex Cytosolic Protein Phosphatase Activity and Enhancement of Heart Muscle Microsomal Ca^{2+}-ATPase Activity

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Abstract Rats were generated by pronuclear injection of the transgene with a cDNA construct encoding rat regucalcin that is a regulatory protein of Ca^{2+} signaling. Transgenic (TG) founders were fertile, transmitted the transgene at the expected frequency, and bred to homozygote. Western analysis of the cytosol prepared from the tissue of TG female rats (5-week-old) showed a remarkable expression of regucalcin (3.3 kDa) protein in the liver, kidney cortex, heart, lung, stomach, brain, spleen, muscle, colon, and duodenum. Regucalcin expression of TG male rats was seen in the liver, kidney cortex, heart, and lung. In wild-type (wt) male and female rats, regucalcin was mainly present in the liver and kidney cortex. Regucalcin inhibited protein phosphatase activity in rat kidney cortex cytosol and activated Ca^{2+}-ATPase activity in rat heart muscle microsomes. The suppressive effect of regucalcin on protein phosphatase activity was significantly enhanced in the cytosol of kidney cortex of TG male and female rats as compared with those of wt rats. Likewise, heart muscle microsomal Ca^{2+}-ATPase activity was significantly enhanced in TG rats. The changes in their enzyme's activities in TG rats were completely abolished in the presence of anti-regucalcin monoclonal antibody (100 ng/ml) in the enzyme reaction mixture. Moreover, the body weight of TG female rats was significantly lowered as compared with that of wt rats. Serum inorganic phosphorus concentration was significantly increased in TG male and female rats, while serum calcium, glucose, triglyceride, free cholesterol, albumin, and urea nitrogen concentrations were not significantly altered in TG rats. Regucalcin TG rats should be a useful model to define a regulatory role of endogenous regucalcin in the tissues in vivo. J. Cell. Biochem. 86:520–529, 2002.

Key words: regucalcin; protein phosphatase; Ca^{2+}-ATPase; transgenic rats

Calcium ion (Ca^{2+}) plays an important role in the regulation of many cell functions. The Ca^{2+} effect 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], 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 cell function.

The regucalcin gene is localized on chromosome Xq11.1-12 proximal end [Shimokawa et al., 1995a], and the gene has been demonstrated in human, mouse, bovine, monkey, dog, rabbit, and chicken, but not yeast [Shimokawa et al., 1995b; Misawa and Yamaguchi, 2000a]. Comparison of the nucleotide sequences of regucalcin from vertebrate species was highly conserved in their coding region, and they were conserved throughout evolution [Shimokawa and Yamaguchi, 1993; Misawa and Yamaguchi, 2000a]. The organization of rat regucalcin gene consists of seven exons and six introns, and the several consensus regulatory elements are

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present in the upstream of the 5′-flanking region of the gene [Yamaguchi et al., 1996].

Rat regucalcin mRNA is greatly expressed in the liver and only to a small extent in the kidney as assayed by Northern blot analysis [Shimokawa and Yamaguchi, 1992], suggesting that it is expressed in a highly tissue-specific manner. The expression of hepatic regucalcin mRNA has been shown to be stimulated by various factors; the expression is raised by the administration of calcium chloride [Shimokawa and Yamaguchi, 1992], insulin [Yamaguchi et al., 1995], and 17β-estradial [Yamaguchi and Oishi, 1995] to rats. The promoter characterization of the 5′-flanking region in the regulation of the rat regucalcin gene and its transcriptional regulation by various signaling factors has been shown [Murata and Yamaguchi, 1999; Misawa and Yamaguchi, 2000b]. AP-1 [Murata and Yamaguchi, 1998] and NF1-A1 [Misawa and Yamaguchi, 2002] have been found to be a transcription factor for the enhancement of regucalcin gene promoter activity.

Regucalcin is prominently expressed in liver cells [Shimokawa and Yamaguchi, 1993; Yamaguchi and Isogai, 1993]. A lower expression of regucalcin is found in rat brain [Yamaguchi et al., 1999] and heart muscle [Yamaguchi and Nakajima, 2002], suggesting that the protein has a role in the regulation of their tissues. The role of regucalcin in various tissues remains to be elucidated.

The present study was undertaken to determine a regulatory role of endogenous regucalcin in vivo using a transgenic (TG) rat model. Here, we generated regucalcin TG rats. The regulatory effect of endogenous regucalcin on kidney cortex cytosolic protein phosphatase and heart muscle microsomal Ca^{2+}-ATPase activities, which are regulated by exogenous regucalcin in normal rat tissues in vitro, is also demonstrated in regucalcin TG rats in vivo.

MATERIALS AND METHODS

Chemicals

o-Phospho-L-tyrosine, *o*-phospho-L-serine, *o*-phospho-L-threonine, and adenosine-5′-triphosphate (ATP) were purchased from Sigma (St. Louis, MO). Other chemicals were obtained from Wako Pure Chemical Co. (Osaka, Japan).

Regucalcin Expression Construct

The cDNA encoding rat regucalcin was isolated and cloned into the pBluescript vector [Shimokawa and Yamaguchi, 1993]. The regucalcin cDNA contains *Pst*I site downstream of the translational stop codon, and a *Pst*I site and an *Eco*RI upstream of the regucalcin cDNA. The *Pst*I-*Pst* fragment was isolated (including the *Eco*RI site) and ligated into the cloning vector pBluscript II KS (+) in the corresponding restriction sites in order to introduce an extra *Eco*RI site downstream of the regucalcin cDNA. The resulting *Eco*RI fragment (containing the complete coding cDNA) was cloned into the *Eco*RI site of the pCXN2 expression vector [Niwa et al., 1991]. A construct was selected with the cDNA encoding regucalcin in the sense direction. The resultant plasmid was designated as regucalcin (RC)/pCXN2. Transgene expression was monitored in the stably-transfected rat hepatoma (H4-II-E) cell lines [Misawa et al., 2002].

Generation of TG Rats

A 3.6 liner DNA fragment containing the regucalcin (RC)/pCXN2 was used for pronuclear microinjection of rats (Sprague–Dawley, SD) embryos to generate TG rats. The embryos were implanted into pseudopregnant female rats. The founder rats were mated with SD rats to produce F1 litters. To identify founder rats and determine transgene copy number, genomic DNA was isolated from tails and amplified by reverse transcription-polymerase chain reaction (RT-PCR) using primer sets that recognized two different regions of the regucalcin cDNA [Shimokawa and Yamaguchi, 1993]. Primers huRC-1 (5′-GGAGGCTATGTTGCCACCATTGGA-3′) and huRC-2 (5′-CCCTCCAAAGCAGCATGAAGTTG-3′) amplified a fragment containing the regucalcin cDNA that was present in the transgene sequence, but absent in the wild-type (wt). The amplified products were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining. Two TG founder rats, one male and one female, carrying the regucalcin fusion gene were obtained. Both founders were fertile, transmitted the transgene at the expected frequency, and bred to homozygote.

Isolation of Regucalcin

Male Wistar rats, weighing 100–120 g, were obtained commercially from Japan SLC (Hamamatsu, Japan). 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 (weight:volume) 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 105,000*g* for 60 min. The resulting supernatant was heated at 60°C for 10 min and re-centrifuged 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 (DEAE)-cellulose, as reported previous [Yamaguchi and Yamamoto, 1978].

Anti-Regucalcin Antibody

Anti-regucalcin antibody was a monoclonal antibody raised against regucalcin prepared using standard methods [Omura and Yamaguchi, 1998]. 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 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).

Preparation of Cytosol and Microsomes in Tissues

Male and female SD rats (wt) or regucalcin TG rats (5–6 weeks old) were killed by bleeding. Various tissues were removed, rinsed with ice-cold 250 mM sucrose solution, cut into small pieces, suspended 1:9 in the homogenization medium containing 250 mM sucrose, 10 mM Tris-HCl, 1.0 mM EGTA, and 1 mM dithiothreitol (DTT), pH 7.0, and homogenized in a Potter–Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged at 1,000*g* for 10 min to remove nuclei, unbroken cells, and cell debris. The resultant supernatant was centrifuged at 7,700*g* for 20 min to remove the mitochondrial fraction. The post-mitochondrial supernatant was then centrifuged at 110,000*g* for 60 min to sediment the microsomal fraction, and the supernatant (cytosol) was pooled. The microsomal (sarcoplasmic reticulum) fraction in heart muscle was resuspended in buffer containing 50 mM Tris-HCl, 5 mM $MgCl_2$, and 120 mM KCl, pH 6.8, to a final protein concentration of 1.0–2.0 mg/ml. Protein concentration was determined by the method of Lowry et al. [1951].

Western Blot Analysis

The cytosol obtained from various tissues or the microsomes prepared from heart muscle were used for Western blot analysis [Wessendorf et al., 1993]. Aliquots of cytosol (10 μg of protein) or (25 μg of protein) were mixed with 5 × Laemmli sample buffer, boiled for 5 min, and SDS–PAGE was performed by the method of Laemmli [1970] using 125 polyacrylamide resolving gel. After SDS–PAGE, the proteins were then transferred onto a polyvinylidene difluoride membrane at 100 mA for 4 h. The membranes were incubated with a polyclonal rabbit anti-regucalcin antibody [Yamaguchi and Isogai, 1993], which was diluted 1:2,000 in 10 mM Tris-HCl, pH 8, containing 150 mM NaCl, 0.1% w/v Tween-20 (washing buffer), and 5% (w/v) skim milk for 1 h. The membranes incubated with antibody were washed four times with washing buffer. Then membranes were incubated for 1 h with horse-radish peroxidase linked anti-rabbit IgG, which was diluted 1:5,000 with washing buffer containing 5% (w/v) skim milk, and again they were washed. Detection of the protein bands was performed using a enhanced chemiluminescent kit following the manufacturer's instructions. The molecular size of the detecting protein was determined by running the standard protein molecules of known sizes in parallel. The membranes obtained by using cytosol or microsomes were exposed for 5 or 1 min on the film, respectively.

Assay of Protein Phosphatase Activity

Protein phosphatase activity toward various phosphoamino acids 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 DTT, 9 mM phosphoamino acid (neutralized with NaOH solution) in kidney cortex cytosol (0.7–0.8 mg protein/ml) as reported elsewhere [Pallen and Wang, 1983]. In separated experiments, the reaction mixture contained either vehicle, anti-regucalcin antibody (100 ng/ml) or regucalcin (1 μM). The enzyme reaction was terminated after 15 min by the

addition of 1.0 ml of ice-cold 10% trichloracetic acid, 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 nanomoles (nmol) of inorganic phosphate liberated per minute per milligram of cytosolic protein.

Assay of Ca^{2+}-ATPase

Ca^{2+}-ATPase activity in the microsomes of heart muscle was assayed by the procedure of Narayanan et al. [1982]. Mg^{2+}-ATPase activity was determined for 10 min at 37°C in a medium (1.0 ml) containing 50 mM Tris-HCl buffer (pH 6.8), 5 mM $MgCl_2$, 120 mM KCl, 5 mM NaN_3, 0.2 mM EGTA, 2.5 mM Mg-ATP (neutralized with KOH), and the microsomes (100–200 μg of protein) in the absence of $CaCl_2$. $(Ca^{2+} + Mg^{2+})$-ATPase activity was measured in the same medium containing 10 μM $CaCl_2$ without EGTA addition. The enzyme reaction was stooped by the addition of 10 % trichloroacetic acid (1.0 ml). The amount of inorganic phosphate released from ATP by enzyme reaction was measured according to the method of Nakamura and Mori [1958]. Ca^{2+}-ATPase activity was calculated as the difference between $(Ca^{2+} + Mg^{2+})$-ATPase and Mg^{2+}-ATPase. Enzyme activity was expressed as nmol of inorganic phosphate released per minute per milligram (mg) protein.

Serum Biochemical Determination

Blood samples obtained by cardiac puncture were centrifuged 30 min after collection, and the serum was separated. Serum was frozen at −80°C until assay. Serum calcium, inorganic phosphorus, glucose, triglyceride, free cholesterol, albumin, and urea nitrogen concentrations were determined by using the assay Kit (Wako Pure Chemical Co.).

Statistical Analysis

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

RESULTS

Tissue Expression of Regucalcin

Two TG founder rats, one male and one female, carrying the regucalcin fusion gene were obtained. Both founders were fertile, transmitted the transgene at expected frequency, and bred to homozygote. Western analysis of the cytosol prepared from tissues of 5-week-old regucalcin homogeneous TG male and female rats showed a prominent expression of regucalcin (3.3 kDa) protein in the tissues (Fig. 1). In male rats, regucalcin expression was markedly seen in the heart, lung, and stomach of homogeneous animals, while there were no detectable in their tissues of wt animals. Regucalcin was mainly present in the liver and kidney cortex of wt male rats. Regucalcin levels in the liver and kidney cortex were slightly increased in homogeneous TG male rats.

Meanwhile, regucalcin levels in the kidney cortex, heart, brain, spleen, lung, muscle, stomach, colon, and duodenum of homogeneous TG female rats were markedly expressed as compared with those of wt female rats (Fig. 1B). In liver tissue of homogeneous TG female rats, an enhancement of regucalcin expression was slight, since the protein was prominently expressed in the liver of wt male and female rats. Thus, sexual difference in tissue expression of regucalcin was obtained in the TG rats. Four TG male and female rats used gave the same results.

Change in Protein Phosphatase Activity in Liver and Kidney Cortex of TG Rats

Regucalcin has been shown to have a suppressive effect on protein phosphatase activity toward phosphotyrosine, phosphoserine, and phosphothreonine in the cytosol of liver and kidney cortex of normal (wt) rats [Omura and Yamaguchi, 1998; Morooka and Yamaguchi, 2001]. Whether protein phosphatase activity is changed in homozygote TG rats was examined. The change in protein phosphatase activity toward three phosphoamino acids in the liver cytosol of regucalcin TG rats is shown in Figure 2. Protein phosphatase activity toward phosphotyrosine, phosphoserine, and phosphothreonine was not difference in the liver cytosol of TG male and female rats as compared with that of wt rats. This result may be associated with an only slight increase in regucalcin expression in the liver cytosol of TG male and female rats.

The change in protein phosphatase activity in the kidney cortex cytosol of TG male and female rats is shown in Figure 3. Protein phosphatase activity toward phosphothreonine

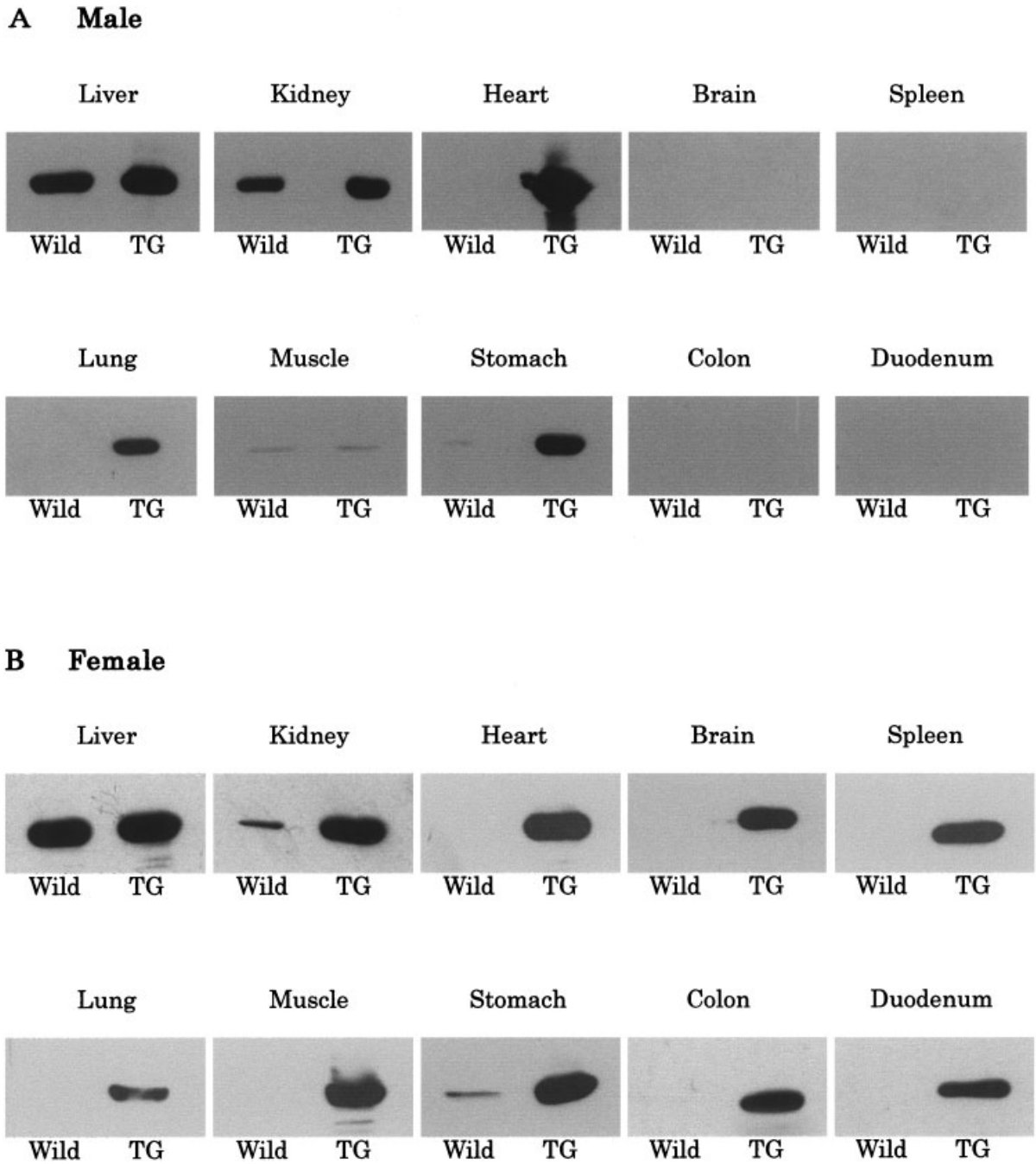

Fig. 1. Analysis of regucalcin protein in the cytosol of various tissues of regucalcin TG rats. Western blot analysis was carried out on the extracts (10 μg of cytosolic proteins) obtained from the tissues of normal (wt) or TG rats. The figure shows one of four experiments with separate rats.

was significantly suppressed in TG male rats as compared with that of wt rats. The enzyme activity toward phosphotyrosine and phosphoserine was not significantly changed in the kidney cortex cytosol of TG male rats. In TG female rats, protein phosphatase activity toward phosphotyrosine, phosphoserine, and phosphothreonine was significantly suppressed in the kidney cortex cytosol as compared with those of wt rats. The expression of regucalcin in the kidney cortex cytosol of TG female rats was remarkable as compared with that of wt female rats

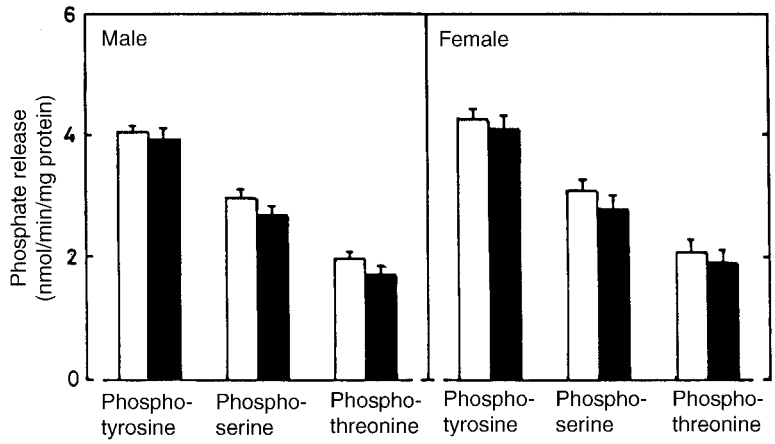

Fig. 2. Change in protein phosphatase activity in the liver cytosol of regucalcin TG rats. The enzyme activity toward phosphotyrosine, phosphoserine, or phosphothreonine was measured in a reaction mixture containing the cytosolic protein of livers prepared from normal (wt) or regucalcin TG rats. Each value is the mean ± SEM of five experiments with different rats. White bars, normal (wt) rats; black bars, TG rats.

(Fig. 1B). This result may be related to regucalcin expression in the kidney cortex cytosol of TG male and female rats.

The effect of anti-regucalcin monoclonal antibody on protein phosphatase activity toward phosphotyrosine in the kidney cortex cytosol of TG female rats is shown in Figure 4. Endogenous regucalcin in rats kidney cortex cytosol has been shown to have a suppressive effect on protein phosphatase [Morooka and Yamaguchi, 2001]. The presence of anti-regucalcin monoclonal antibody (100 ng/ml) in the enzyme reaction mixture caused a significant increase in protein tyrosine phosphatase activity in the kidney cortex cytosol of normal (wt) and TG rats. This increase was completely abolished by the addition of regucalcin (1 μM) in the reaction mixture. Similar results were obtained in protein phosphatase activity toward phosphoserine and phosphothreonine. These findings indicate that the decrease in protein phosphatase activity, which was seen in the kidney cortex cytosol of TG rats is resulted from a potent expression of endogenous regucalcin.

Change in Regucalcin Expression and Ca^{2+}-ATPase Activity in the Microsomes of Heart Muscle of TG Rats

The expression of regucalcin in the cytosol and microsomes of heart muscle of TG rats is

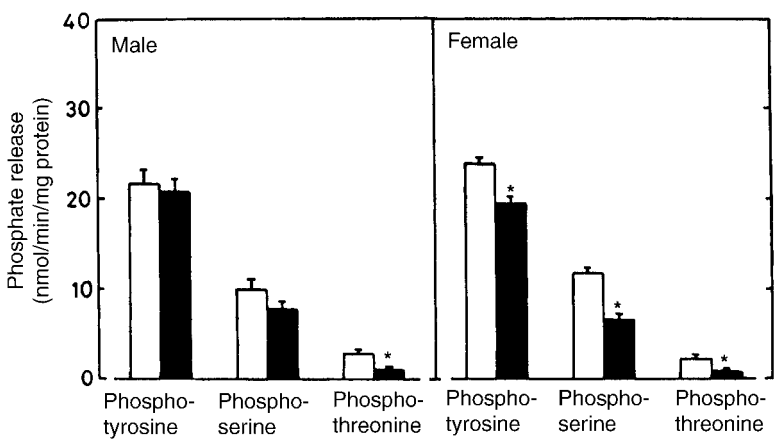

Fig. 3. Change in protein phosphatase activity in the kidney cortex cytosol of regucalcin TG rats. The enzyme activity toward phosphotyrosine, phosphoserine, or phosphothreonine was measured in a reaction mixture containing the cytosolic protein of kidney cortex prepared from normal (wt) or regucalcin TG rats. Each value is the mean ± SEM of five experiments with different rats. $^*P < 0.01$, compared with the control value from normal (wt) rats. White bars, normal (wt) rats; black bars, TG rats.

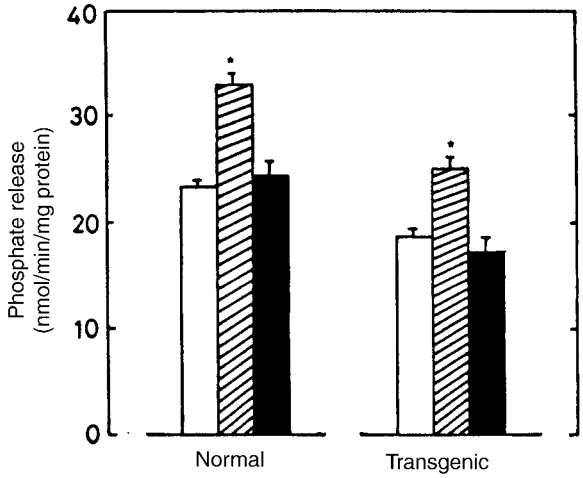

Fig. 4. Effect of anti-regucalcin monoclonal antibody on protein phosphatase activity in the kidney cortex cytosol of regucalcin TG female rats. The enzyme activity toward phosphotyrosine was measured in a reaction mixture containing either vehicle, anti-regucalcin monoclonal antibody (100 ng/ml), or the antibody (100 ng/ml) plus regucalcin (1 μM) in the presence of the cytosolic protein of kidney cortex prepared from normal (wt) or regucalcin TG rats. Each value is the mean ± SEM of five experiments with different rats. $^*P < 0.01$, compared with the control (none) value. White bars, control (none); hatched bars, the antibody addition; black bars, the antibody plus regucalcin addition.

shown in Figure 5. A remarkable expression of regucalcin was seen in the cytosol (Fig. 5A) and microsomes (Fig. 5B) of heart muscle of TG male and female rats as compared with that of normal (wt). A potent expression of regucalcin was observed in the heart muscle microsomes (sarcoplasmic reticulum) of TG female rats as compared with that of TG male rats. Four TG male and female rats used gave the same results.

Regucalcin has been demonstrated to activate Ca^{2+}-ATPase, which functions as Ca^{2+} pump in the sarcoplasmic reticulum of heart muscle microsomes [Yamaguchi and Nakajima, 2002]. The change in Ca^{2+}-ATPase and Mg^{2+}-ATPase activities in the microsomes of heart muscle of TG rats is shown in Figure 6. Ca^{2+}-ATPase activity was significantly enhanced in the heart muscle microsomes of TG male and female rats as compared with that of wt rats (Fig. 6A). Mg^{2+}-ATPase activity was significantly increased in the heart muscle microsomes of female TG rats (Fig. 6B). The increase in Ca^{2+}-ATPase and Mg^{2+}-ATPase activities in the heart muscle microsomes obtained from TG female rats was completely abolished in the presence of anti-regucalcin monoclonal antibody (100 ng/ml) in the enzyme reaction mixture (Fig. 7).

Change in Body Weight and Serum Findings in Regucalcin TG Rats

The change in body weight and serum biochemical findings in regucalcin TG rats is shown in Table I. TG male and female rats were killed by bleeding at 38 days after birth. The body weight of TG female rats was significantly lowered as compared with that of normal (wt) rats. Such a decrease was not seen in TG male rats. Serum inorganic phosphorus concentration was significantly raised in TG male and female rats as compared with that of normal rats. Serum calcium, glucose, triglyceride, free cholesterol, albumin, and urea nitrogen concentrations were not significantly changed in TG male and female rats.

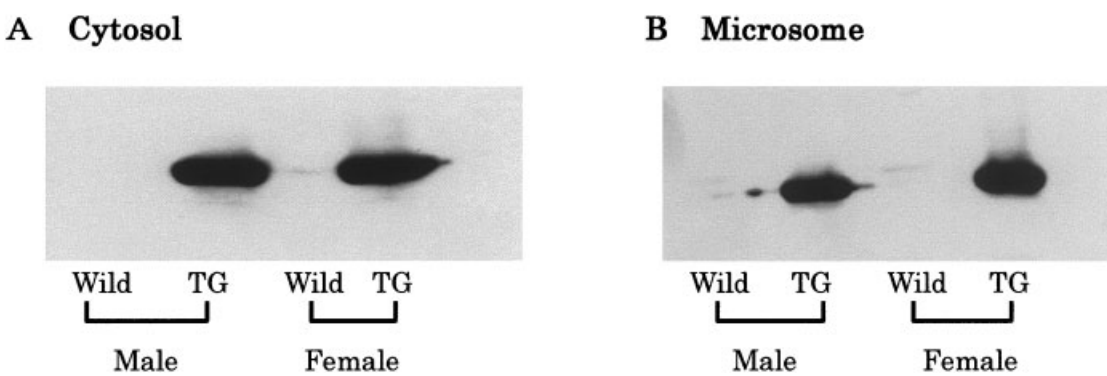

Fig. 5. Analysis of regucalcin protein in the cytosol (**A**) of microsomes (**B**) of heart muscle of regucalcin TG rats. Western blot analysis was carried out on the extracts (10 or 25 μg of proteins from cytosol or microsomes, respectively) obtained from normal (wt) or TG rats. The figure shows one of four experiments with separate rats.

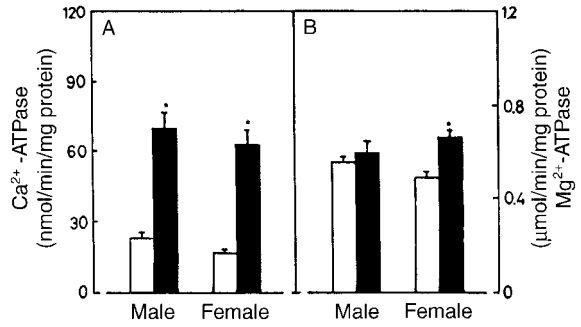

Fig. 6. Change in Ca^{2+}-ATPase (**A**) or Mg^{2+}-ATPase (**B**) activity in the microsomes of heart muscle of regucalcin TG rats. The enzyme activity was measured in a reaction mixture containing the microsomes of heart prepared from normal (wt) or regucalcin TG rats. Each value is the mean ± SEM of five experiments with different rats. $^*P < 0.01$, compared with the control value from normal (wt) rats. White bars, normal (wt) rats; black bars, TG rats.

DISCUSSION

We generated regucalcin TG rats in this study. Regucalcin homozygote male and female rats expressed a prominent regucalcin protein in the tissues. The expression of regucalcin was remarkable in many tissues of TG female rats; its expression of male rats was seen in the liver, kidney cortex, heart, lung, and stomach. The tissue expression of regucalcin in TG rats were sexual differences.

The enhancement of regucalcin expression in the liver of TG male and female rats was only slightly as compared with that of wt rats.

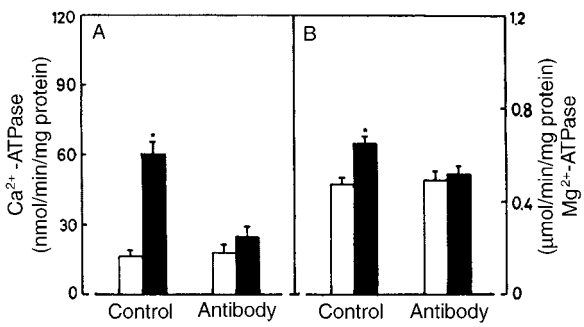

Fig. 7. Effect of anti-regucalcin monoclonal antibody on Ca^{2+}-ATPase (**A**) or Mg^{2+}-ATPase (**B**) activity in the microsomes of heart muscle of female regucalcin TG rats. The enzyme activity was measured in a reaction mixture containing either vehicle, anti-regucalcin monoclonal antibody (100 ng/ml) in the presence of the microsomes of heart muscle prepared from normal (wt) or regucalcin TG rats. Each value is the mean ± SEM of five experiments with different rats. $^*P < 0.01$, compared with the control value from normal (wt) rats. White bars, normal (wt); rats; black bars, TG rats.

Regucalcin is predominantly expressed in the liver of wt rats [Yamaguchi and Isogai, 1993]. Regucalcin in rat kidney is mainly present in the cortex, but not the medulla [Yamaguchi and Kurota, 1995]. Kidney cortex regucalcin expression was slightly enhanced in male TG rats, while its expression was great in the female rats. In addition, regucalcin expression was markedly increased in the heart muscle of male and female rats. Then, the regulatory effect of endogenous regucalcin in vivo was estimated in the liver, kidney cortex, and heart muscle of TG male and female rats.

Regucalcin has been shown to have a suppressive effect on protein phosphatase activity in the cytosol of liver and kidney cortex of wt rats [Omura and Yamaguchi, 1998; Morooka and Yamaguchi, 2001]. Dephosphorylation of many phosphorylated proteins is regulated by protein phosphatase in cells [Hunter, 1995]. Protein phosphatase activity toward phosphotyrosine, phosphoserine, and phosphothreonine was not significantly changed in the liver cytosol of TG male and female rats as compared with that of wt rats. This result may be related to the observation that regucalcin expression in liver cytosol was not appreciably enhanced by transgene. However, protein phosphatase activity toward phosphothreonine in the kidney cortex cytosol of TG male rats was significantly suppressed as compared with that of wt rats. Moreover, a significant decrease in protein phosphatase activity toward three phosphoamino acids was observed in the kidney cortex cytosol of TG female rats. Such a decrease was associated with the enhancement of regucalcin expression in TG rats. The present finding clearly demonstrates that endogenous regucalcin has a suppressive effect on protein phosphatase activity in vivo.

Regucalcin has been demonstrated to increase Ca^{2+}-ATPase activity in the microsomes (sarcoplasmic reticulum) of rat heart muscle [Yamaguchi and Nakajima, 2002], The Ca^{2+} current is one of the most important components in cardiac excitation–contraction coupling. This coupling mechanism is based on the regulation of intracellular Ca^{2+} concentration by Ca^{2+} pump in the sarcoplasmic reticulum of heart muscle [Langer, 1992]. TG male and female rats caused a remarkable increase in regucalcin expression in the cytosol and microsomes of heart muscle. The microsomal Ca^{2+}-ATPase activity was significantly raised in the heart muscle of TG male

TABLE I. Change in Body Weight and Serum Findings in Regucalcin TG Rats

	Male		Female	
	Normal	TG	Normal	TG
Body weight (g)	161.6 ± 6.8	160.5 ± 7.0	136.8 ± 5.1	116.7 ± 5.7**
Serum (mg/dl)				
Calcium	10.68 ± 0.20	10.52 ± 0.14	10.02 ± 0.23	10.20 ± 0.24
Inorganic phosphorus	10.06 ± 0.22	10.65 ± 0.23*	9.03 ± 0.25	9.83 ± 0.26*
Glucose	144.1 ± 3.8	138.5 ± 2.6	139.9 ± 4.1	144.2 ± 3.9
Triglyceride	58.1 ± 14.3	59.7 ± 5.6	40.2 ± 6.5	42.8 ± 3.7
Free cholesterol	20.2 ± 0.88	24.2 ± 1.28	26.3 ± 2.19	23.9 ± 1.13
Albumin	3,502 ± 109	3,528 ± 36	3,816 ± 85	3,834 ± 100
Urea nitrogen	14.2 ± 1.16	16.7 ± 2.08	13.8 ± 0.91	19.3 ± 2.56

Rats were killed by bleeding at 38-day-old. Each value is mean ± SEM of eight rats.
*$P < 0.01$ and **$P < 0.025$, compared with the control (wt) value.

and female rats. This finding may support the view that endogenous regucalcin has an activatory effect on Ca^{2+}-ATPase activity in the microsomes of rat heart muscle in vivo.

Kidney cortex cytosolic protein phosphatase activity, which was suppressed in TG female rats, was completely abolished by the addition of anti-regucalcin monoclonal antibody in the enzyme reaction mixture in vitro. Likewise, the increase in heart muscle microsomal Ca^{2+}-ATPase activity seen in TG female rats was completely abolished by the addition of anti-regucalcin monoclonal antibody. These results indicate that the alteration in their enzyme's activity in the tissues of regucalcin TG rats is related to an increase in endogenous regucalcin. Thus, regucalcin TG rats should be a useful model to define a regulatory role of endogenous regucalcin in various tissues in vivo.

The body weight of regucalcin TG female rats was significantly lowered as compared with that of wt rats. Serum inorganic phosphorus concentration was significantly increased in regucalcin TG male and female rats, although serum calcium, glucose, albumin, triglyceride, free cholesterol, and nitrogen urea concentrations were not significantly changed in regucalcin TG rats. These observations suggest that a specific metabolic disorder, which is involved in the regulation of serum inorganic phosphorus concentration, is induced in regucalcin TG rats. This remains to be elucidated.

In conclusion, it has been demonstrated that endogenous regucalcin has a regulatory effect using a regucalcin TG rats model in vivo. This TG rats should be useful to define a regulatory role of endogenous regucalcin on various tissues in vivo.

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