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Overexpression of RGPR-p117 Enhances Regucalcin Gene Promoter Activity in Cloned Normal Rat Kidney Proximal Tubular Epithelial Cells: Involvement of TTGGC Motif

Natsumi Sawada and Masayoshi Yamaguchi*

Laboratory of Endocrinology and Molecular Metabolism, Graduate School of Nutritional Sciences, University of Shizuoka, 52-1 Yada, Suruga Ward, Shizuoka 422-8526, Japan

Abstract A novel protein RGPR-p117 was discovered as regucalcin gene promoter region-related protein that binds to the TTGGC motif using a yeast one-hybrid system. RGPR-p117 is localized in the nucleus of kidney cells, and overexpression of RGPR-p117 can modulate regucalcin protein and its mRNA expression in the cloned normal rat kidney proximal tubular epithelial NRK52E cells. This study was undertaken to determine whether overexpression of RGPR-p117 enhances the regucalcin promoter activity using the −710/+18 LUC construct (wild-type) or −710/+18 LUC construct (mutant) with deletion of −523/−435 including TTGGC motif. NRK52E cells (wild-type) or stable HA-RGPR-p117/phCMV2-transfected cells (transfectant) were cultured in Dulbecco's minimum essential medium (DMEM) containing 5% bovine serum (BS). Wild-type cells or transfectants were transfected with the −710/+18 LUC construct vector or the −710/+18 LUC construct with deletion of −523/−435. Wild-type cells or transfectants with subconfluency were cultured for 48 h in a DMEM medium containing either vehicle, BS (5%), or parathyroid hormone (1–34) (PTH; 10^{-7} M). Luciferase activity in wild-type cells was significantly increased with culture of BS or PTH. This increase was significantly blocked in the presence of various protein kinase inhibitors (staurosporine and PD 98059). Luciferase activity in transfectants was significantly increased as compared with that of wild-type cells in the absence of BS or PTH. The increase in luciferase activity in transfectants was completely decreased in mutant with deletion of −523/−435 sequence of regucalcin promoter. This was also seen using the −710/+18 LUC construct with deletion of −523/−503 sequence containing TTGGC motif. The increase in luciferase activity in transfectants was not significantly enhanced with culture of BS (5%), PTH (10^{-7} M), Bay K 8644 (10^{-6} M), phorbol 12-myristate 13-acetate (PMA; 10^{-6} M), or N^6, 2′-dibutyryl cyclic adenosine 3′, 5′-monophosphate (DcAMP; 10^{-4} M). The increase in luciferase activity in transfectants was completely inhibited with culture of dibucaine (10^{-6} M), staurosporine (10^{-9} M), PD 98059 (10^{-8} M), wortmannin (10^{-8} M), genistein (10^{-6} M), vanadate (10^{-6} M), or okadaic acid (10^{-6} M) which are inhibitors of various kinases and protein phosphatases. This study demonstrates that RGPR-p117 can enhance the regucalcin promoter activity which is related to the NF-1 consensus sequences including TTGGC motif, and that its enhancing effect is partly mediated through phosphorylation and dephosphorylation in NRK52E cells. J. Cell. Biochem. 99: 589–597, 2006.

Key words: RGPR-p117; regucalcin gene promoter region-related protein; regucalcin; transcription factor; normal rat kidney proximal tubular epithelial cells (NRK52E)

RGPR-p117 was discovered as a regucalcin gene promoter region-related protein [Misawa and Yamaguchi, 2001]. Regucalcin has been demonstrated to play a multifunctional role as regulatory protein in the intracellular signaling system of many cell types [Yamaguchi, 2000a,b, 2005; in review]. Regucalcin gene expression has been regulated by various signaling factors [Murata and Yamaguchi, 1998, 1999; Misawa and Yamaguchi, 2000]. The nuclear factor binding site, which contains a nuclear factor I

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*Correspondence to: Masayoshi Yamaguchi, PhD, Laboratory of Endocrinology and Molecular Metabolism, Graduate School of Nutritional Sciences, University of Shizuoka, 52-1 Yada, Suruga Ward, Shizuoka 422-8526, Japan.
E-mail: yamaguch@u-shizuoka-ken.ac.jp

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(NF-1)-like motif, TTGGC(N)$_6$CC, is present in the promoter region of the rat regucalcin gene [Misawa and Yamaguchi, 2000, 2002a]. An unknown protein that binds to the TTGGC sequence in the promoter region of the rat regucalcin gene was identified using a yeast one-hybrid system [Misawa and Yamaguchi, 2001]. The protein was termed as a regucalcin promoter region-related protein (RGPR-p117) [Misawa and Yamaguchi, 2001].

RGPR-p117 is conserved in various vertebrate species, including human, rat, mouse, bovine, rabbit, and chicken [Misawa and Yamaguchi, 2001; Sawada and Yamaguchi, 2005a], indicating a great conservation of RGPR-p117 genes throughout evolution. The entire human RGPR-p117 cDNA consists of 3,989 bp, which contains an open reading frame (ORF) of 3,180 bp, encoding a protein of 1,060 amino-acid residues [Misawa and Yamaguchi, 2001]. A comparison of human RGPR-p117 sequence with the genomic sequence database indicates that the gene consists of at least 26 exons, spanning ~41 kb, and localized on human chromosome 1q25.2 [Misawa and Yamaguchi, 2001]. RGPR-p117 mRNA is ubiquitously expressed in many tissues including liver, kidney, heart, and brain of rats [Misawa and Yamaguchi, 2002b], and stably expressed for the physiologic change in rat liver [Yamaguchi et al., 2003].

RGPR-p117 is suggested to play a role in nuclear function. RGPR-p117 has been found to localize in the cytoplasm and nucleus with immunocytochemical and Western blot analysis using HA-RGPR-p117/phCMV2-transfected normal rat kidney proximal tubular epithelial NRK52E cells [Sawada et al., 2005]. Overexpression of RGPR-p117 enhances the expression of regucalcin mRNA and its protein level in NRK52E cells [Sawada and Yamaguchi, 2005b]. RGPR-p117 may play a role as a transcriptional factor that is related to TTGGC motif.

This study was undertaken to determine whether overexpression of RGPR-p117 enhances the regucalcin promoter activity using the −710/+18 LUC construct (including TTGGC sequences) in NRK52E cells and HA-RGPR-p117/phCMV2-transfected NRK52E cells. We found that RGPR-p117 can enhance the regucalcin promoter activity that is involved in the NF-1 consensus sequences including TTGGC motif in NRK52E cells.

MATERIALS AND METHODS

Chemicals

Non-essential amino acid solution, bovine serum (BS), and penicillin-streptomycin solution (5,000 U/ml penicillin; 5,000 μg/ml streptomycin) were obtained from Gibco Laboratories (Grand Island, NY). Dulbeco's modified Eagle's medium (DMEM), staurosporine, PD98059, dibucaine, Bay K 8644, PTH [synthetic human PTH (1–34)], aldosterone, DcAMP (N^6, 2′-dibutyryl cyclic adenosine 3′, 5′ monophosphate), phorbol 12-myristate 13-acetate (PMA), wortmannin, genistein, and okadaic acid were obtained from Sigma Chemical (St. Louis, MO). Vanadate and other chemicals were purchased from Wako Pure Chemical Co. (Osaka, Japan). pGL3-basic vector and pRL-TK vector were purchased from Promega (Madison, WI). Reagents used were dissolved in distilled water or ethanol, and some reagents were passed through ion exchange resin to remove metal ions.

Cell Culture

The cloned normal rat kidney proximal tubular epithelial cells (NRK52E; 1×10^5 cells) [Rice et al., 2003] were maintained in DMEM supplemented with 5% BS, non-essential amino acids solution, 50 U/ml penicillin, and 50 μg/ml streptomycin in humidified 5% CO_2/95% air at 37°C to obtain subconfluent monolayers.

Selection of Stable Transfectant

Expression plasmid for HA-tag fused rat RGPR-p117, HA-RGPR-p117/phCMV2 was constructed as described previously [Sawada et al., 2005]. NRK52E cells were grown on 35-mm dishes to approximately 70% confluence and transfected with HA-RGPR-p117/phCMV2 vector using the synthetic cationic lipid components, a Lipofectamine reagent (Invitrogen), according to the manufacturer's instructions. At 48 h after transfection, neomycin (0.8 mg/ml Geneticin G418, Sigma) was added to culture medium for selection and transfected cells were plated at limiting dilution. Multiple surviving clones were isolated, transferred to 35-mm dishes, and grown in the medium without neomycin. HA-RGPR-p117 was stably expressed in the transfectants. In experiments, transfectants were cultured for 72 h in DMEM containing 5% BS.

RT-PCR Analysis

Total RNAs were prepared using Trizol regent (Invitrogen), according to the manufacturer's instruction [Chomczynski and Sacci, 1987]. RT-PCR was performed with a Titan™ One-Tube RT-PCR kit (Roche Molecular Biochemicals) as recommended by the supplier. Primers for amplification of rat regucalcin cDNA [Shimokawa and Yamaguchi, 1993] were: 5′-GGAGGCTATGTTGCCACCATTGGA-3′ (sense strand, position 292–314 of cDNA sequence) and 5′-CCCTCCAAAGCAGCATGA AGTTG-3′ (antisense strand, position 849–827). For semiquantative PCR, glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as an internal control to evaluate total RNA input. Primers for amplification of G3PDH were: 5′-GATTTGGCCGTATCGGACGC-3′ (sense strand) and 5′-CTCCTTGGAGGCCAT-GTAGG-3′ (antisense strand). The pair oligonucleotide primers were designed to amplify a 977 bp sequence from the mRNA of G3PDH. RT-PCR was performed using reaction mixture (20 μl) containing 2 μg of total RNAs, supplied RT-PCR buffer, Titan™ enzyme mixture (AMV and Expand High Fidelity), 0.2 mM dNTP, 5 mM DTT, 5U RNase inhibitor, and 0.3 μM primers. Samples were incubated at 50°C for 30 min, and then amplified for 35 cycles under the following conditions; denaturation for 30 sec at 94°C, annealing for 30 sec at 60, and extension for 60 sec for 68°C. The amplified products were separated by electrophoresis on 1.5% agarose gel and visualized by ethidium bromide staining.

Construction of the Reporter Gene Plasmids

The reporter gene plasmids were generated with subcloning of restriction fragment from −710/+18 TA vector [Misawa and Yamaguchi, 2000]. The DNA fragment −710/+18 of the rat regucalcin gene was prepared from a vector by *Kpn*I/*Xho*I restriction digestion, and then cloned into the pGL3-basic promoterless plasmid containing the firefly luciferase gene [Murata and Yamaguchi, 1998]. This wild-type promoter construct was named the −710/+18 LUC (WT). The DNA fragment (−710/−523) was amplified by two primers 5′-AGGTACC-GAATTCCTGACTGATCTTT-3′ and 5′AATGA GCTCCAGGCAAAGAACATTCTAT-3′, and digested by *Eco*RI and *Sac* I, and the fragment was ligated into *Eco*RI and *Sac* I site of −710/+18 LUC (WT), and its construct was named Mut 1. The DNA fragment (−710/−523) amplified by 5′-AGGTACCGAATTCCTGACT-GATCTTT-3′ and 5′-ATGGATCCCACAGG-CAAAGAACA-3′ was digested by *Eco*RI and *Bam*HI. The DNA fragment (−505/+18) amplified by 5′-AGGATCCTCATGCAAGGAAGCA-AG-3′ and 5′-ACACTCGAGGGTTGTAATG-ACTCCTGGC-3′ was digested by *Bam*HI and *Xho*I. These two DNA fragments were ligated into *Eco*RI and *Xho*I site of −710/+18 LUC (WT), and the plasmid was named Mut 2. The DNA fragment (−710/−505) amplified by 5′-AGGTACCGAATTCCTGACTGATCTTT-3′ and 5′-ATGAGCTCGTTTGGCAGGGAGCCTTG-3′ was digested by *Eco*RI and *Sac*I. The fragment was ligated into *Eco*RI and *Sac*I site of −710/+18 LUC (WT), and the plasmid was named Mut 3.

Cell Culture and Transfection

The cloned normal rat kidney proximal tubular epithelial NRK52E cells were cultured as previously described [Nakagawa and Yamaguchi, 2005]. For the transfection experiments, the cells were grown on 24-well plate to approximately 70% confluence and washed once with serum-free DMEM. Either 300 ng of pGL3-basic plasmid or an equivalent molar amount of test plasmid was co-transfected into NRK52E cells along with 300 ng of pRL-TK plasmid using the synthetic cationic lipid component, a lipofectamin reagent, according to the manufacturer's instructions (Invitorogen). The pRL-TK vector containing the Renilla luciferase gene under control of the herpes simplex virus thymidine kinase promoter was used as an internal control for differences in transfection efficiency and cell number. For analysis of regulation of the regucalcin promoter by signaling factors, the transfected cells were maintained for 48 h in serum-free DMEM containing either vehicle, 5% BS, hormones, or various inhibitors. After culture, the transfectants were lysed, and the luciferase activity in the cell lysates was measured using dual-luciferase reporter assay system (Promega).

Statistical Analysis

The significance of difference between values was estimated by Student's t test. A P-value of less than 0.05 was considered significant. Also, we used a multiway ANOVA multiple comparison to compare the treatment groups.

RESULTS

Effects of Hormones on Regucalcin mRNA Expression in NRK52E Cells

NRK52E cells (wild-type) or stably HA-RGPR-p117/phCMV2-transfected cells (transfectants) were cultured for 72 h in medium with BS (5%), and the cells with subconfluency were cultured for 24 h in the presence of BS (5%). The expression of regucalcin mRNA was significantly increased in transfectants as compared with that of wild-type cells (Fig. 1A), indicating that overexpression of RGPR-p117 enhances regucalcin mRNA, and its protein expressions in NRK52E cells as shown previously [Sawada and Yamaguchi, 2005b]. When wild-type cells or transfectants with subconfluency were cultured for 24 or 48 h in a medium containing either vehicle, PTH (10^{-7} M), aldosterone (10^{-5} M), or PMA (10^{-6} M) in the absence of BS, regucalcin mRNA expression in wild-type cells (Fig. 1B), or transfectants (Fig. 1C) was significantly increased in the presence of PTH or PMA. G3PDH mRNA levels were not changed in transfectants (data not shown).

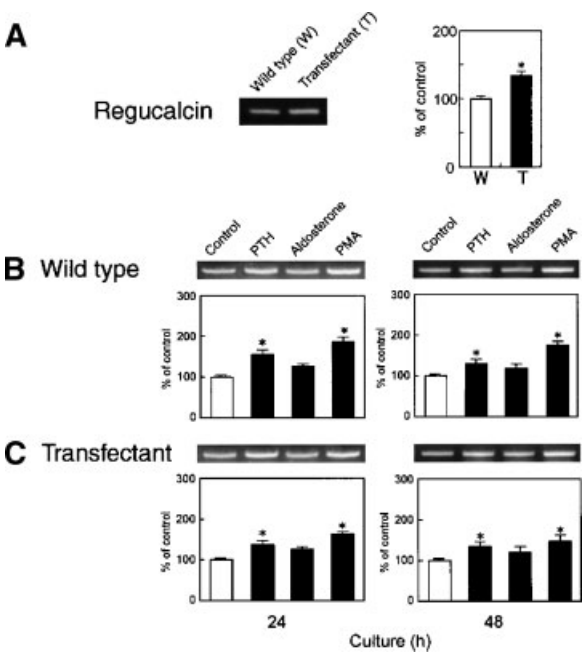

Fig. 1. Effects of PTH, aldosterone, or PMA in regucalcin mRNA expression in the cloned normal rat kidney proximal tubular NRK52E cells (wild-type; W) or stable HA-RGPR-p117/phCMV2-transfected cells (transfectant; T). After culture for 72 h with 5% BS, wild-type cells and transfectants with subconfluency were cultured for 48 h in the absence of BS (**A**). Wild-type cells (**B**) or transfectants (**C**) with subconfluency were cultured for 24 or 48 h in DMEM medium containing either vehicle, PTH (10^{-7} M), aldosterone (10^{-5} M), or PMA (10^{-6} M) in the absence of BS. After culture, total RNAs (2 μg) extracted from cells were analyzed by RT-PCR using specific primers. The figure shows one of four experiments with separates samples. The densitometric data for each mRNA level with the control (mean ± SEM for four experiments). *$P < 0.01$, compared with the control (none) value.

Involvement of the NF-1 Consensus Sequences Including TTGGC Motif in Regucalcin Promoter in NRK52E Cells Overexpressing RGPR-p117

To clarify whether the NF-1 consensus sequences including TTGGC motif in the regucalcin promoter region is involved in the enhancement of regucalcin mRNA expression in NRK52E cells overexpressing RGPR-p117, NRK52E cells wild-type, or stable transfectants with subconfluency were transfected with either −710/+18 LUC construct (WT) or chimeric constructs containing serial deletions [−523/−435 (mutant 1), −523/−503 (mutant 2), or −504/−435 (mutant 3)] of the regucalcin promoter region (Fig. 2A). The cells were cultured for 48 h in medium without BS. Luciferase activity in NRK52E cells in stable NRK52E cells overexpressing RGPR-p117 (transfectants) was significantly increased as compared with that of wild-type cells with the −710/+18 LUC construct (Fig. 2B). Luciferase activity in the mutant 1, 2, or 3 was not significantly changed as compared with that of wild-type cells with the −710/+18 construct. In transfectants overexpressing RGPR-p117, however, mutant 1 or 2 was unresponsive. The enhancement of luciferase activity in the transfectants overexpressing RGPR-p117 was seen in mutant 3. These results indicate that the nucleotide −523/−503 containing TTGGC motif in the regucalcin promoter region are essential in the enhancement of regucalcn promoter activity in NRK52E cells overexpressing RGPR-p117.

Effects of Signaling Factors on Regucalcin Promoter Activity in NRK52E Cells Overexpressing RGPR-p117

The effects of signaling factors on the regucalcin promoter activity in NRK52E cells (wild-type) or stable RGPR-p117-overexpressing transfectants transfected with the −710/+18 LUC construct (WT) or the −523/−435 deletion (Mut 1) was examined. Wild-type cells or transfectants with subconfluency were cultured for 48 h in a medium containing either vehicle, BS (5%), PTH (10^{-7} M), Bay K 8644

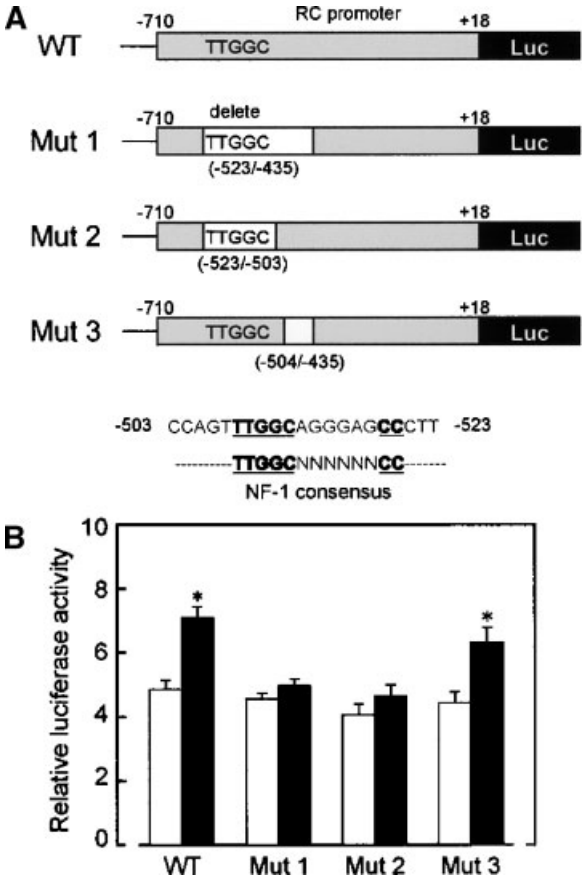

Fig. 2. Basal expression of wild-type and mutant derivative of the rat regucalcin promoter in the cloned normal rat kidney proximal tubular NRK52E cells (wild-type) and stable HA-RGPR-p117 overexpressing NRK52E cells (transfectant). A DNA fragment was ligated into the pGL3-basic promoterless plasmid (Basic LUC) and −710/+18 (WT). The mutant reporter plasmids (Mut 1, Mut 2, and Mut 3) were generated as described in Materials and Methods. Their constructions are described in subpart **A**. NRK52E cells were transfected with WT, Mut 1, Mut 2, and Mut 3, and each cell were co-transected with pRL-TK (internal control plasmid). Wild-type cells and RGPR-p117 overexpressing cells (transfectant) with transfection of the reporter gene plasmids were cultured for 48 h in medium without 5% BS (**B**). Luciferase activity was measured using the dual-luciferase reporter assay system. The firefly luciferase activity of the test plasmids was corrected for Renilla luciferase activity of the pRL-TK plasmid. The results are expressed as a fold-stimulation in comparison with the luciferase activity measured after transfection with Basic LUC, which was set as 1.0. Each value is the mean ± SEM of six separate experiments with different cell cultures. $*P < 0.01$ compared with the control value obtained from wild-type cells. White bars, wild-type cells; black bars, transfectants overexpressing RGPR-p117.

(10^{-6} M), PMA (10^{-6} M), or DcAMP (10^{-4} M). In wild-type cells, culture with BS or PTH caused a significant increase in luciferase activity in the cells transfected with the −710/+18 LUC construct (WT) (Fig. 3). This increase was not

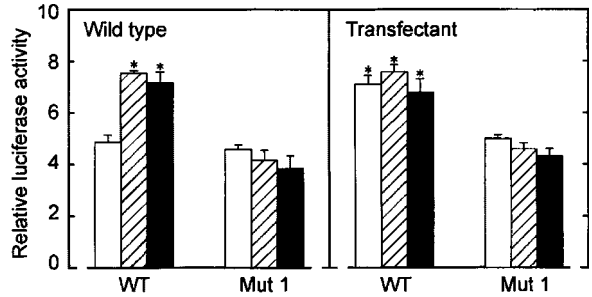

Fig. 3. Effects of BS or PTH on promoter activities from the regucalcin promoter (−710/+18) reporter gene plasmid WT or Mut 1 (with −523/−450 deletion) in the cloned normal rat kidney proximal tubular NRK52E cells (wild-type) or stable NRK52E cells transfected with HA-RGPR-p117/phCMV2 (transfectants). The constructions of wild type (WT) and Mut 1 in the reporter gene plasmids for regucalcin promoter are shown in Figure 1A. NRK52E cells (wild-type) or stable HA-RGPR-p117-overexpressing transfectants with subconfluency were cultured for 48 h in medium containing vehicle, BS (5%), or PTH (10^{-7} M). Luciferase activity in the cell lysate was measured as described in the legends of Figure 2. Each value is the mean ± SEM of six separate experiments with different cell cultures. $*P < 0.01$ compared with the control value obtained from wild-type cells. White bars, control (none); hatched bars, with BS; black bars, with PTH.

seen in Mut 1. The presence of PMA caused a significant increase in luciferase activity in wild-type cells transfected with the −710/+18 LUC construct (Fig. 4). Such an increase was

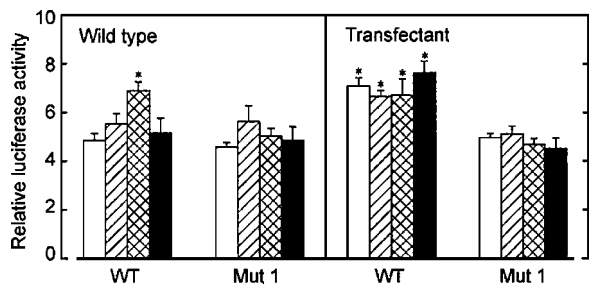

Fig. 4. Effects of Bay K 8644, PMA, or DcAMP on promoter activities from the regucalcin promoter (−710/+18) reporter gene plasmid WT or Mut 1 (with −523/−435 deletion) NRK52E cells (wild-type) or stable NRK52E cells transfected with HA-RGPR-p117/phCMV2 (transfectants). The constructions of wild-type (WT) and Mut 1 in the reporter gene plasmids for regucalcin promoter are shown in Figure 1A. NRK52E cells (wild-type) or stable HA-RGPR-p117-overexpressing transfectants with subconfluency were cultured for 48 h in medium containing either vehicle, Bay K 8644 (10^{-6} M), PMA (10^{-6} M), or DcAMP (10^{-4} M). Luciferase activity in the cell lysate was measured as described in the legends of Figure 2. Each value is the mean ± SEM of six separate experiments with different cell cultures. $*P < 0.01$ compared with the control value obtained from wild-type cells. White bars, control (none); hatched bars, Bay K 8644; double hatched bar, PMA; black bars, DcAMP.

not seen in the presence of Bay K 8644 or DcAMP. Luciferase activity in transfectants overexpressing RGPR-p117 was not significantly enhanced with culture of BS, PTH, Bay K 8644, PMA, or DcAMP (Figs. 3,4). The enzyme activity in transfectants in the presence or absence of various signaling factors was not enhanced in the Mut 1 (Figs. 3,4).

Effects of Signaling Inhibitors on Regucalcin Promoter Activity in NRK52E Cells Overexpressing RGPR-p117

The effects of signaling inhibitors on the regucalcin promoter activity in NRK52E cells (wild-type) or stable RGPR-p117-overexpressing transfectants transfected with the −710/+18 LUC construct (WT) or the −710/+18 LUC construct with −523/−435 deletion (Mut 1) was examined. Wild-type cells or transfectants with subconfluency were cultured for 48 h in a medium containing either vehicle, dibucaine (10^{-6} M), staurosporine (10^{-9} M), PD 98059 (10^{-8} M), wortmannin (10^{-8} M), genistein (10^{-6} M), vanadate (10^{-6} M), or okadaic acid (10^{-6} M) in the presence or absence of BS (5%), or PTH (10^{-7} M). In wild-type cells, the effects of BS or PTH increasing luciferase activity in the cells (wild-type) transfected with the −710/+18 LUC construct was completely blocked with the culture of staurosporine or PD 98059 (Fig. 5).

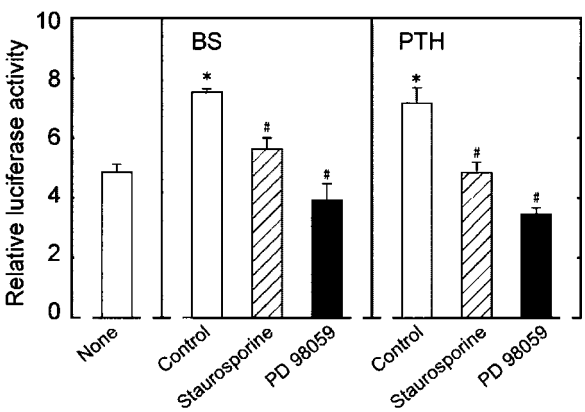

Fig. 5. Effects of intracellular signaling inhibitors on the BS or PTH-enhanced promoter activities in the cloned normal rat kidney proximal tubular NRK52E cells (wild-type). NRK52E cells with subconfluency were cultured for 48 h in a medium containing wither vehicle, staurosporine (10^{-9} M), or PD98059 (10^{-8} M) in the presence or absence of BS (5%), or PTH (10^{-7} M). Luciferase activity in the cell lysate was measured as described in the legends of Figure 2. Each value is the mean ± SEM of six separate experiments with different cell cultures. *$P < 0.01$ compared with the control (none) value. White bars, control; hatched bars, staurosporine; black bars, PD 98059.

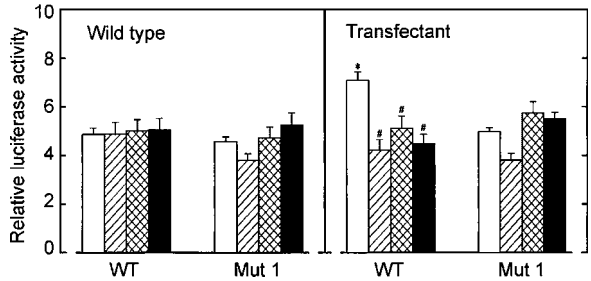

Fig. 6. Effects of dibucaine, staurosporine, or PD 98059 on promoter activities from the regucalcin promoter reporter gene plasmid WT or Mut 1 (with −523/−435 deletion) in NRK52E cells (wild-type) or stable NRK52E cells transfected with HA-RGPR-p117/phCMV2 (transfectants). The constructions of wild type (WT) and Mut 1 in the reporter gene plasmids for regucalcin promoter are shown in Figure 2A. NRK52E cells (wild-type) or HA-RGPR-p117-overexpressing transfectants with subconfluency were cultured for 48 h in medium containing either vehicle, dibucaine (10^{-6} M), staurosporine (10^{-9} M), or PD 98059 (10^{-8} M). Luciferase activity in cell lysate was measured as described in the legends of Figure 2. Each value is the mean ± SEM of six separate experiments with different cell cultures. *$P < 0.01$ compared with the control value obtained from wild-type cells. White bars, control (none); hatched bars, dibucaine; double hatched bars, staurosporine; black bars, PD 98059.

In wild-type cells with culture in the absence of BS or PTH, luciferase activity in the wild-type or mutant 1 was not significantly changed in the presence of dibucaine, staurosporine, PD 98059, wortmannin, genistein, or okadaic acid

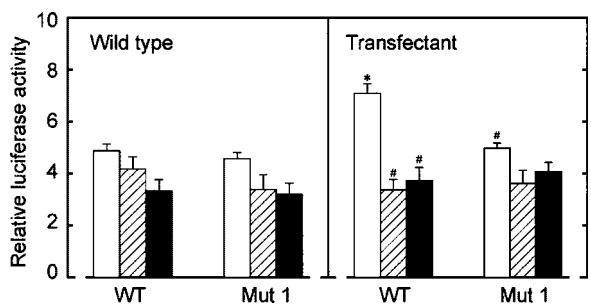

Fig. 7. Effects of wortmannin or genistein on promoter activities from the regucalcin promoter reporter gene plasmid WT or Mut 1 (with −523/−435 deletion) in NRK52E cells (wild-type), or stable NRK52E cells transfected with HA-RGPR-p117/phCMV2 (transfectants). The constructions of wild type (WT) and Mut 1 in the reporter gene plasmids for regucalcin promoter are shown in Figure 2A. NRK52E cells (wild-type) or stable HA-RGPR-p117-overexpressing transfectants with subconfluency were cultured for 48 h in medium containing either vehicle, wortmannin (10^{-8} M), or genistein (10^{-6} M). Luciferase activity in the cell lysate was measured as described in the legends of Figure 2. Each value is the mean ± SEM of six separate experiments with different cell cultures. *$P < 0.01$ compared with the control value obtained from wild-type cells. White bars, control (none); hatched bars, wortmannin; black bars, genistein.

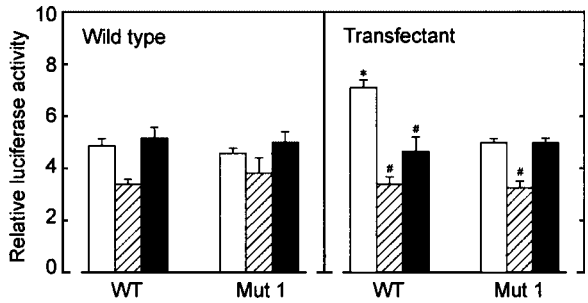

Fig. 8. Effects of vanadate or okadaic acid on promoter activities from the regucalcin promoter gene plasmid WT or Mut 1 in NRK52E cells (wild-type) or stable NRK52E cells transfected with HA-RGPR-p117/phCMV2 (transfectant). The constructions of wild type (WT) and Mut 1 in the reporter gene plasmids for regucalcin promoter are shown in Figure 2A. NRK52E cells (wild-type) or stable HA-RGPR-p117-overexpressing transfectants with subconfluency were cultured for 48 h in medium containing either vehicle, vanadate (10^{-6} M), or okadaic acid (10^{-6} M). Luciferase activity in the cell lysate was measured as described in the legends of Figure 2. Each value is the mean ± SEM of six separate experiments with different cell cultures. $^{*}P < 0.01$ compared with the control value obtained from wild-type cells. White bars, control (none); hatched bars, vanadate; black bars, okadaic acid.

(Figs. 6–8). Culture with vanadate caused a significant decrease in luciferase activity in wild-type cells with the −710/+18 LUC construct (Fig. 8). Such a decrease was not seen in mutant 1. In RGPR-p117-overexpressing NRK52E cells (transfectants), the enhancement of luciferase activity in the transfectants with the −710/+18 LUC construct was significantly inhibited in the presence of dibucaine, staurosporine, PD 98059, wortmannin, genistein, vanadate, or okadaic acid (Figs. 6–8). These inhibitory effects were not seen in Mut 1, whereas the effect of vanadate was observed.

DISCUSSION

RGPR-p117 has been demonstrated to localize in the nucleus of cloned normal rat kidney proximal tubular epithelial NRK52E cells in vitro [Sawada et al., 2005]. Overexpression of RGPR-p117 enhances the expression of regucalcin mRNA and its protein level in NRK52E cells [Sawada and Yamaguchi, 2005b]. RGPR-p117 has a leucine zipper motif, and its location is highly conserved in RGPR-p117 of the mammalian species [Misawa and Yamaguchi, 2001; Sawada and Yamaguchi, 2005a]. RGPR-p117 may play a role as a transcription factor. This study demonstrates that overexpression of RGPR-p117 in stable transfectants enhances the regucalcin promoter activity in the cloned normal rat kidney proximal tubular epithelial NRK52E cells in vitro, supporting the view that the protein has a role as a transcription factor. The enhancement of the regucalcin promoter activity was also observed in transient transfection assays (data not shown).

The expression of regucalcin mRNA was significantly increased with culture of PTH or PMA, activator of protein kinase C, in NRK52E cells. These effects were also observed in stable NRK52E cells overexpressing RGPR-p117 (transfectants). The effect of RGPR-p117 in increasing regucalcin mRNA expression may partly be mediated through signaling factors in NRK52E cells.

RGPR-p117 was discovered as a regucalcin promoter region-related protein that binds to the TTGGC$(N)_6$CC sequences (NF-1 consensus sequences), which is present in the promoter region of the rat regucalcin gene [Misawa and Yamaguchi, 2000, 2002], using a yeast one-hybrid system [Misawa and Yamaguchi, 2001]. The regucalcin promoter activity in the −710/+18 LUC constructs, which contains TTGGC sequence, was significantly enhanced in NRK52E cells overexpressing regucalcin. This enhancement was found to disappear in NRK52E cells transfected with the regucalcin promoter −710/+18 LUC construct with deletion of the −523/−435 or −523/−503 sequences. The effect of RGPR-p117 in enhancing the promoter activity was seen in NRK52E cells transfected with the −710/+18 LUC construct with deletion of −504/−435 sequence. These results demonstrate that NF-1 consensus sequences including TTGGC motif is essential in the enhancing of the regucalcin promoter activity in NRK52E cells overexpressing RGPR-p117.

The regucalcin promoter activity in NRK52E cells (wild-type) was significantly increased with culture of BS, PTH, or PMA. Such an increase was not observed in NRK52E cells transfected with the regucalcin promoter −710/+18 LUC construct that the −523/−435 sequence was deleted. The effect of BS or PTH on the promoter activity in NRK52E cells (wild-type) was inhibited with the culture of protein kinase inhibitors. These results suggest that the regucalcin promoter activity with −523/−503 LUC construct is stimulated through

signaling factors. PMA is an activator of protein kinase C. The hormonal effect of PTH is partly mediated through protein kinase C [Verheijen and Defize, 1997]. Presumably, the transcription factor that is related to TTGGC motif in the regucalcin promoter is partly regulated through phosphorylation with protein kinase C. The effect of BS, PTH, or PMA in increasing the regucalcin promoter activity was not enhanced in NRK52E cells overexpressing RGPR-p117. It appears that phosphorylation of RGPR-p117 may be saturated.

The regucalcin promoter activity in NRK52E cells (wild-type) transfected with −710/+18 LUC construct was not significantly inhibited with culture of dibucaine, an inhibitor of Ca^{2+}/calmodulin-dependent protein kinase [Vincenzi, 1982]; staurosporine, an inhibitor of protein kinase [Tamaoki et al., 1986]; PD 98059, an inhibitor of mitogen activating protein (MAP) kinase [Peleck et al., 1993]; wortmannin, an inhibitor of PI_3 kinase [Yamaguchi and Daimon, 2005]; or genistein, an inhibitor of protein tyrosine kinase [Spinozzi et al., 1994], with effective concentration. Meanwhile, the regucalcin promoter activity in NRK52E cells overexpressing RGPR-p117 transfected with the −710/+18 LUC construct was found to decrease with culture of each inhibitor. The effect of RGPR-p117 in enhancing the regucalcin promoter activity may partly be mediated through phosphorylation with various protein kinases.

Vanadate and okadaic acid are inhibitors of protein phosphatases [Hunter, 1995]. The regucalcin promoter activity in NRK52E cells (wild-type) transfected with −710/+18 LUC construct was significantly inhibited with culture of vanadate, an inhibitor of protein tyrosine phosphatase, with effective concentration. Such an effect was not observed in the case of okadaic acid, an inhibitor of protein serine/threonine phosphatase. It is speculated that the regucalcin promoter activity with the −710/+18 LUC construct is partly mediated through dephosphorylation that is involved in protein tyrosine phosphatase. Meanwhile, the enhancement of the regucalcin promoter activity in NRK52E cells overexpressing RGPR-p117 was significantly inhibited with culture of vanadate or okadaic acid. The inhibitory effect with vanadate was also observed in RGPR-p117 overexpressing NRK52E cells transfected with the −710/+18 LUC construct with deletion of −523/−435 sequence. The effect of RGPR-p117 in enhancing the regucalcin promoter activity may partly be involved in dephosphorylation that is mediated through protein phosphatases.

It is speculated that the effect of RGPR-p117 in enhancing the regucalcin promoter activity, which is related to NF-1 consensus sequences, may be mediated through the recruitment of phosphorylation and dephosphorylation, which is regulated by intracellular signaling factor in NRK52E cells.

In conclusion, it has been demonstrated that overexpression of RGPR-p117 can enhance the regucalcin promoter activity which is related to the NF-1 consensus sequences including TTGGC motif in the cloned normal rat kidney proximal tubular epithelial NRK52E cells.

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