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ORIGINAL ARTICLE

The transcription factor prolactin regulatory element-binding protein mediates prolactin transcription induced by thyrotropin-releasing hormone in GH3 cells

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Abstract The prolactin regulatory element-binding protein (PREB) is a transcription factor that regulates prolactin (PRL) promoter activity in the rat anterior pituitary. PRL gene expression and secretion are regulated by various hormones and growth factors, including dopamine, epidermal growth factor, and thyrotropin-releasing hormone (TRH). We examined the effect of TRH on PREB expression in pituitary cells. Western blots probed with a PREB-specific antiserum showed that the relative abundance of PREB in GH3 cells increased on treatment with TRH in a dose-dependent manner. The relative abundance of PREB mRNA also increased in a dose-dependent manner after treatment with TRH. TRH induced the expression of the luciferase reporter protein under the PREB promoter control. We used inhibitors of certain signal transduction pathways to show that TRH-induced PREB induction is sensitive to the protein kinase A (PKA) inhibitor. TRH stimulated the activity of the wild-type *PRL* promoter, whereas mutation of the PREB core-binding element on the *PRL* promoter reduced this ability. In summary, we have shown that TRH stimulated PREB expression in GH3 cells via the PKA pathway. PREB can function as a transcriptional regulator of *PRL* promoter activity and might be involved in TRH-induced PRL gene transcription.

Keywords Prolactin regulatory element-binding protein · Thyrotropin-releasing hormone · Prolactin · Pituitary · Promoter · Transcription

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Introduction

In pituitary lactotrophs, prolactin (PRL) is produced in a cell-type-specific fashion. This selective expression is due to a pituitary-specific transcription factor (Pit-1) that regulates the expression of the gene encoding PRL in these cells [1, 2]. The Pit-1 binding motif of *PRL* was used to screen a rat pituitary cell line library [3], and a cDNA encoding PRL regulatory element-binding protein (PREB) was isolated. PREB binds to and activates the *PRL* promoter, and it can also mediate the action of protein kinase A (PKA) [3]. The predicted amino acid sequence of PREB had three regions of high similarity with the tryptophan–aspartic acid (WD)-repeat consensus sequence and two

potential trans-regulatory PQ-rich regions. Thus, PREB belongs to the eukaryotic family of WD-repeat proteins. The highly conserved WD repeats in PREB exhibit sequence similarity to a subset of this family that consists of gene-regulatory proteins.

Human PRL (hPRL) gene expression and secretion are regulated by various hormones and growth factors, including dopamine, epidermal growth factor, and thyrotropin-releasing hormone (TRH) [4]. These factors modulate different signaling pathways (cyclic adenosine monophosphate [cAMP], Ca^{2+}, protein kinase C, and mitogen-activated protein kinases) to regulate *hPRL* gene transcription. These second messengers regulate *hPRL* gene activity via the proximal promoter region [4–6]. In the anterior pituitary, TRH binds to its receptor to activate several intracellular signal transduction pathways. The combined action of these intracellular signaling transduction pathways ultimately results in increased transcription of genes encoding TSH-β, α-subunit, and PRL [5, 7, 8]. Many pituitary proteins are phosphorylated in response to TRH, protein kinase C (PKC), and Ca^{2+}; however, most have not been identified [9].

In the present study, we examined the effect of TRH on PREB expression in pituitary cells. Our results suggest that PREB is involved in the mechanism underlying the induction of PRL gene transcription by TRH.

Materials and methods

Cell culture and animals

GH3 cells (Japan Health Sciences Foundation, Osaka, Japan) were cultured in Ham's F-10 medium (ICN Biomedicals Inc., Aurora, OH) supplemented with 15% fetal bovine serum and 2.5% horse serum in a humidified atmosphere containing 5% CO_2 as described in a previous study [10].

Antibodies

An antibody targeting a portion of the PREB protein sequence, between amino acid residues 330 and 410 [3], was generated. The corresponding cDNA fragment was amplified from rat pituitary cDNA by polymerase chain reaction (PCR). The amplified fragment was cloned into a pGEX-2T vector (GE Healthcare Bio-Sciences, Buckinghamshire, UK) and sequenced, and the protein was expressed in *Escherichia coli*. The fusion construct was isolated using glutathione-Sepharose 4B beads (GE Healthcare Bio-Sciences, Buckinghamshire, UK) and used to generate an antiserum in rabbits by a method described previously [11]. The IgG fraction obtained from the immunized animals was purified before use in western blotting and immunohistochemical analysis.

Western blot analysis

GH3 cells were washed and scraped in PBS and lysed according to a method described previously [12]. The proteins (15 μg) were separated on a 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel under reducing conditions and subsequently transferred onto polyvinylidene difluoride membranes for a western immunoblot assay. The membranes were incubated for 1 h at 4°C with 0.2% Tween 20 in PBS (PBS-T) containing anti-PREB antiserum (dilution, 1:250) according to a method described previously [13], or anti-β-tubulin antibody (Santa Cruz Biotechnology, CA; diluted to 1/1,000). Antibody binding was visualized using a chemiluminescence detection kit (ECL; Amersham Pharmacia Biotech, Buckinghamshire, UK).

Transfection of GH3 cells and luciferase reporter gene assay

The PRL luciferase reporter gene (pPRL-LUC) was constructed using 940 bp from the hPRL promoter linked to the luciferase reporter gene (PGBV2, ToyoInk, Tokyo, Japan) as described previously [10]. The mutant plasmid constructs (pPRL-mt-LUC) carrying mutations in the consensus binding sequence for PREB were generated as described previously [3]. A 1005-bp sequence from the PREB promoter (this region is defined as the start site of exon 1) was fused with the luciferase reporter gene (PGBV2; ToyoInk, Tokyo, Japan) to generate the pPREB-LUC construct, according to a procedure described in previous reports [3, 10, 12]. The reporter plasmid was transfected into GH3 cells (at 60% confluence) by using conventional cationic liposome transfection methods (Lipofectamine; Invitrogen Life Technologies, Gaithersburg, MD). Rous sarcoma virus-β-galactosidase (1 μg) was added to all the cells that were transfected to monitor their efficiency of DNA uptake. All assays were corrected for β-galactosidase activity, and the total amount of protein per reaction was identical.

Real-time polymerase chain reaction

The PREB cDNA was amplified by PCR performed on a LightCycler system (Roche Diagnostics). The following primer sequences for rat PREB were used as described previously [13]: sense primer, 5′-GTCATTTCCTGCCTC ACT-3′ and antisense primer, 5′-GTCACATCTGTCACC ACA-3′. A 146-bp fragment was synthesized by PCR using reverse transcribed RNA. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) served as

the control and was amplified and analyzed under conditions identical to those used for PREB cDNA by using primers described in previous reports. The expression level of PREB cDNA was determined as the relative ratio (RR) of the levels of PREB and GAPDH in the same sample.

Stable transfection

The PREB expression vector was transfected into the cultured INS-1 cells by the conventional cationic liposome transfection method as described previously [14]. The transfected cells were selected by adding G418 to the media, and clones showing high PREB production were identified using western blot analysis.

DNA analysis

The alignment procedure was performed with "CLUSTAL W" [15] (version 1.83) with its default setting. The reference genome sequences and their annotations were obtained from NCBI (National Center for Biotechnology Information) accessions NC_005116.2 for rat and NC_000 06.11 for human. We excised the corresponding upstream sequences according to the annotations of the reference genome sequences.

Statistical analysis

The data obtained were statistically compared by using one-way ANOVA and Student's *t* test; differences with *P* values < 0.05 were considered to be statistically significant.

Results

TRH-stimulated PREB expression in GH3 cells

Several reports have indicated that TRH is an important regulator of *PRL* gene expression in pituitary cells [9]. Moreover, Fliss et al. have reported that in pituitary cells, PREB mediates the transcriptional activation of *PRL* [3]. Therefore, we examined the effect of TRH on PREB expression in GH3 cells. Figure 1a showed the specificity of the antibody against PREB. Western blots probed with a PREB-specific antiserum indicated that the relative abundance of PREB in GH3 cells increased on treatment with TRH in a dose-dependent manner (Fig. 1a). In contrast, the basal level of the tubulin was not affected by TRH treatment. Furthermore, the relative abundance of PREB mRNA also increased after treatment with TRH (Fig. 1b). These results clearly suggest that TRH-stimulated PREB expression in GH3 cells.

Effect of TRH on *PREB* promoter activity in GH3 cells

We tested whether changes in the PREB expression mediated TRH induction of the gene by performing transient transfections of GH3 cells with a reporter gene extending from −1000 bp to +5 bp of the PREB promoter (Fig. 2). TRH-stimulated PREB promoter activity in GH3 cells.

TRH induces the PREB promoter via the PKA pathway

Interaction of TRH with its membrane receptor indicates the involvement of a signaling pathway. To test this hypothesis, we used known inhibitors of signaling pathways to disrupt the TRH induction of PREB promoter activity. We added compounds known to mitogen-activated extracellular signal-related kinase (ERK) (10 μM PD98059: PD), protein kinase C (1 μM bisindolylmaleimide I; Bis), p38 mitogen-activated protein kinase (MAPK) (1 μM SB203580; SB), or PKA (1 μM H-89) before exposing the transfected GH3 cells to TRH. The results (Fig. 3) showed that the inhibitors of ERK kinase, protein kinase C, or p38 MAPK pathways had no effect on the action of TRH; however, H-89, an inhibitor of PKA abrogated the induction of PREB by TRH. These observations suggest that the action of TRH is mediated via the PKA cascade.

PREB was involved in TRH-induced prolactin gene transcription

A previous report revealed that the *PRL* promoter sequence contains a 4-nucleotide motif (TGAT) corresponding to the deduced PREB core-binding element (PCBE) of *PRL* [3]. Figure 4a shows a schematic diagram of PREB-binding site on the human and mouse PRL gene. On the basis of this finding, we designed a plasmid construct, namely, pPRL-mt-LUC, which contained a mutated putative PCBE sequence (5′-GAT-3′ to 5′-AGC-3′). Transfection studies showed that PREB failed to induce any luciferase activity in cells that had been transfected with the pPRL-mt-LUC plasmid; however, it did stimulate luciferase activity in cells that were transfected with the wild-type pPRL-LUC plasmid (Fig. 4b). TRH stimulated the activity of the wild-type *PRL* promoter (Fig. 4c), whereas mutation in the PCBE reduced the ability of TRH to stimulate the *PRL* promoter activity. However, Pit-1 still stimulated the activity of mutant-type PRL promoter (Fig. 4d). These results suggest that the intact PCBE motif is required for TRH-induced *PRL* promoter activity.

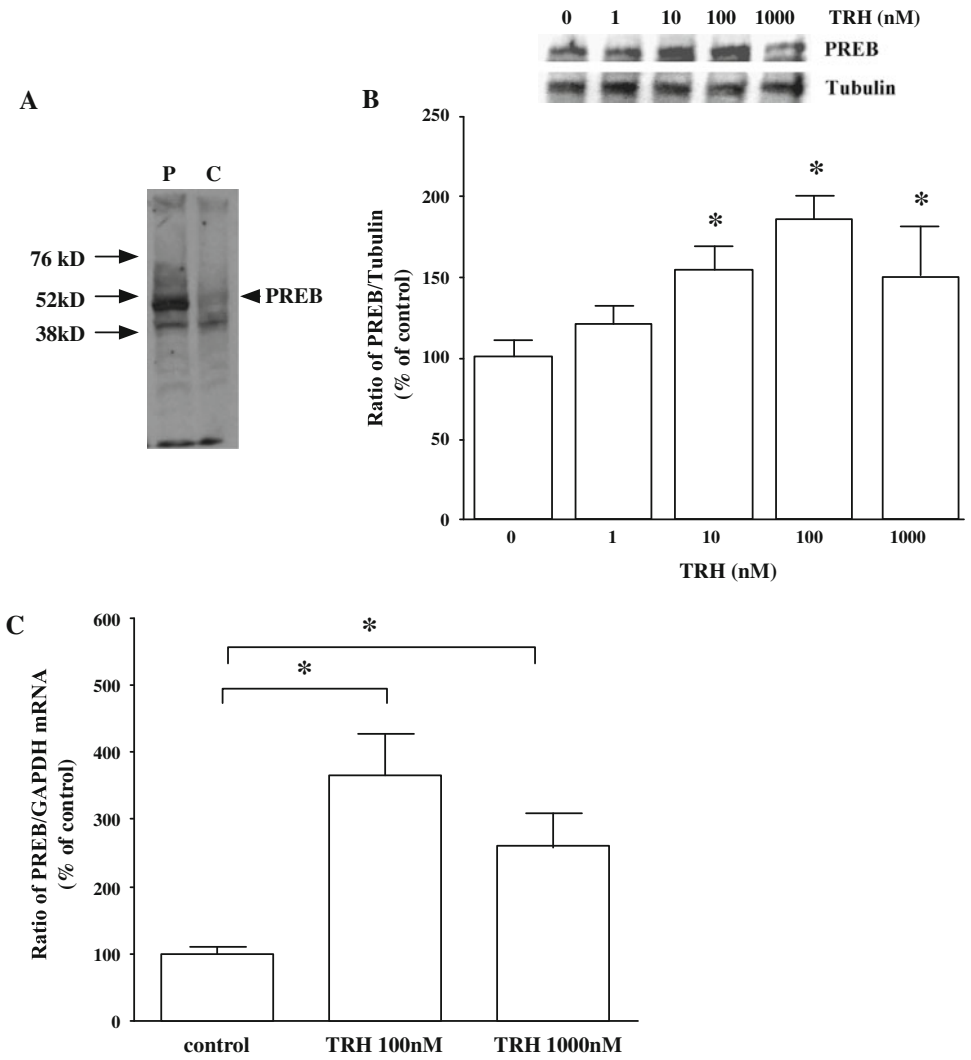

Fig. 1 Effects of TRH on PREB expression in GH3 cells. **a** The specificity of the antibody against PREB. Nuclear extract from the cells was extracted and subjected to western blot analysis with the antibody against PREB. *Lane C*: empty vector, pcDNA3 transfected cells. *Lane P*: PREB-transfected cells. **b** The nuclear extract obtained from GH3 cells treated with the indicated concentrations of TRH (nM). Western blot analysis was performed to examine PREB expression. β-Tubulin was used as the control; the results are shown in the *bottom lanes*. A plot showing the ratio of the PREB level to β-tubulin level is shown in the *bottom panel*. Results are presented as the mean (SE) of three independent experiments. The *asterisk* denotes a significant difference ($P \leq 0.05$). C) Total RNA was extracted from the GH3 cells treated with the indicated concentrations of TRH (nM) for 24 h. Real-time PCR was performed to analyze the PREB mRNA expression. Plot shows the ratio of PREB/GAPDH mRNA. Results shown are mean ± SEM of three experiments for each treatment group. The *asterisk* denotes a significant difference ($P \leq 0.01$)

Discussion

In a recent study, PREB cDNA was isolated from a rat pituitary cDNA library, and the protein product was shown to transactivate *PRL* promoter activity [3]. PREB mRNA transcripts were present not only in the pituitary, but also in the pancreas and the adrenal gland. In a recent study, we reported that PREB might participate in the regulation of insulin gene transcription in response to glucose stimulation and in steroidogenesis [12–14]. In this report, we examined the effect of TRH on PREB expression in GH3 cells. We observed that TRH-stimulated PREB protein and mRNA expression in GH3 cells. These results are consistent with the hypothesis that PREB is involved in TRH-induced PRL expression.

The pituitary-specific transcription factor, Pit-1, regulates the expression of growth hormone, thyroid-stimulating hormone, and PRL in the anterior pituitary [16]. Pit-1 can stimulate the activity of the *PRL* promoter by binding to the 1P motif [17]. PREB protein is encoded by a 1.9-kb transcript, which was shown to bind directly to the 1P site of the PRL promoter [3]. Analysis of the primary sequence of PREB revealed that it is a novel protein distinct from the Pit-1 sequence. The highly conserved WD repeats within

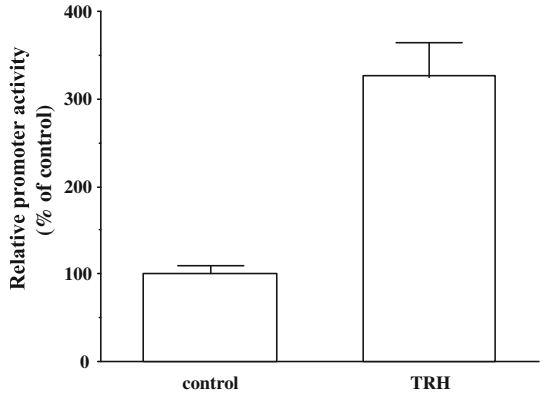

Fig. 2 Effect of TRH on PREB promoter activity: GH3 cells were co-transfected with 1 μg of pPREB-LUC, and then treated with 100 nM TRH. All assays were corrected for β-galactosidase activity and equal total amounts of protein per reaction were employed. The results are expressed as relative luciferase activities with that of control cells arbitrarily set at 100. Each data point shows the mean and SE ($n = 3$) of separate transfections. The *asterisk* denotes a significant difference ($P < 0.01$)

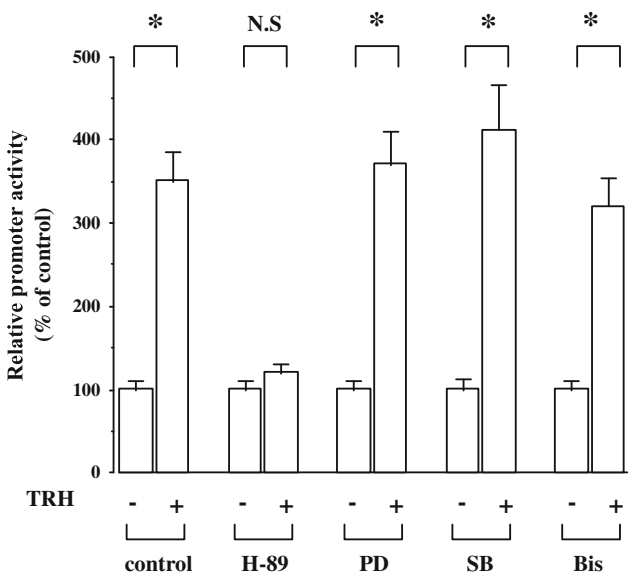

Fig. 3 Effect of PKA inhibitor on TRH-induced PREB transcription. GH3 cells were transfected with 1 μg pPREB-LUC and treated with 100 nM TRH for 24 h prior to cell harvesting. The effects of the PKC inhibitor (Bis), p38-MAPK inhibitor (SB), ERK inhibitor (PD), and PKA inhibitor (H-89) on PREB transcriptional activity in GH3 cells were examined with 100 nM TRH. All assays were corrected for β-galactosidase activity, and the total amount of protein in each reaction was identical. The results were expressed as relative luciferase activity compared with that in the control cells arbitrarily set at 100. Each data point shows the mean ± SE of four separate transfections that were performed on separate days. The *asterisk* denotes the significant difference ($P < 0.01$). *N.S.* no significant difference

PREB exhibit sequence similarity with a subset of this family consisting of proteins that act as gene regulators [18]. PREB affects transcriptional regulation in a manner quite different from that of other members of the WD-repeat protein family because PREB can stimulate gene expression by directly binding to DNA [3]. The treatment of GH3 rat pituitary cells with activators of either PKA or protein kinase C stimulated Pit-1 phosphorylation [19]; this suggests that Pit-1 phosphorylation mediates many hormonal responses that are localized to *PRL* promoter Pit-1 binding sites. However, some reports state that a number of these responses do not require Pit-1 phosphorylation [2, 20, 21]; this implies that factors other than Pit-1 are required for the regulation of PRL gene expression directed by binding sites for Pit-1. PREB represents a novel evolutionarily conserved transcription factor that may contribute to the regulation of *PRL* expression in the anterior pituitary.

Several of the hormones which regulate *PRL* transcription appear to act through changes in the activity of specific protein kinases. For instance, there is evidence that the effects of dopamine on *PRL* expression are mediated by intracellular changes in the concentration of cAMP [22], which leads to changes in the activity of the cAMP-dependent protein kinase. The ability of specific protein kinases to alter *PRL* transcription has been confirmed through the use of expression vectors encoding constitutively active protein kinases. Thus, the expression vector for the catalytic subunit of the cAMP-dependent protein kinase is sufficient for the activation of *PRL* transcription [23]. The importance of cAMP-dependent protein kinase for *PRL* expression has also been demonstrated using an expression vector for the heat-stable inhibitor of cAMP-dependent protein kinase [24].

Previous reports indicate that PREB can mediate PKA stimulation of the *PRL* promoter activity [3], and therefore suggest that this protein is involved in cAMP-mediated transcriptional responses. In this study, we showed that PKA inhibitor repressed TRH-induced PREB promoter activity. The activation of PREB via PKA-mediated phosphorylation could be a possible example for this function of PREB. Although it has not been determined whether PREB can serve as a PKA substrate either in vitro or in vivo, the predicted sequence of this protein contains a number of motifs that resemble PKA phosphorylation consensus sites [25]. Further studies are required to elucidate the specific regulatory mechanisms that underlie the regulation of PREB gene transcription by TRH.

In summary, we have shown that TRH stimulates PREB expression in GH3 cells via the PKA pathway. PREB can function as a transcriptional regulator of *PRL* promoter activity. PREB, like Pit-1, might be involved in TRH-induced *PRL* transcription. Further investigations are necessary to elucidate a possible physiological role of PREB in anterior pituitary cells.

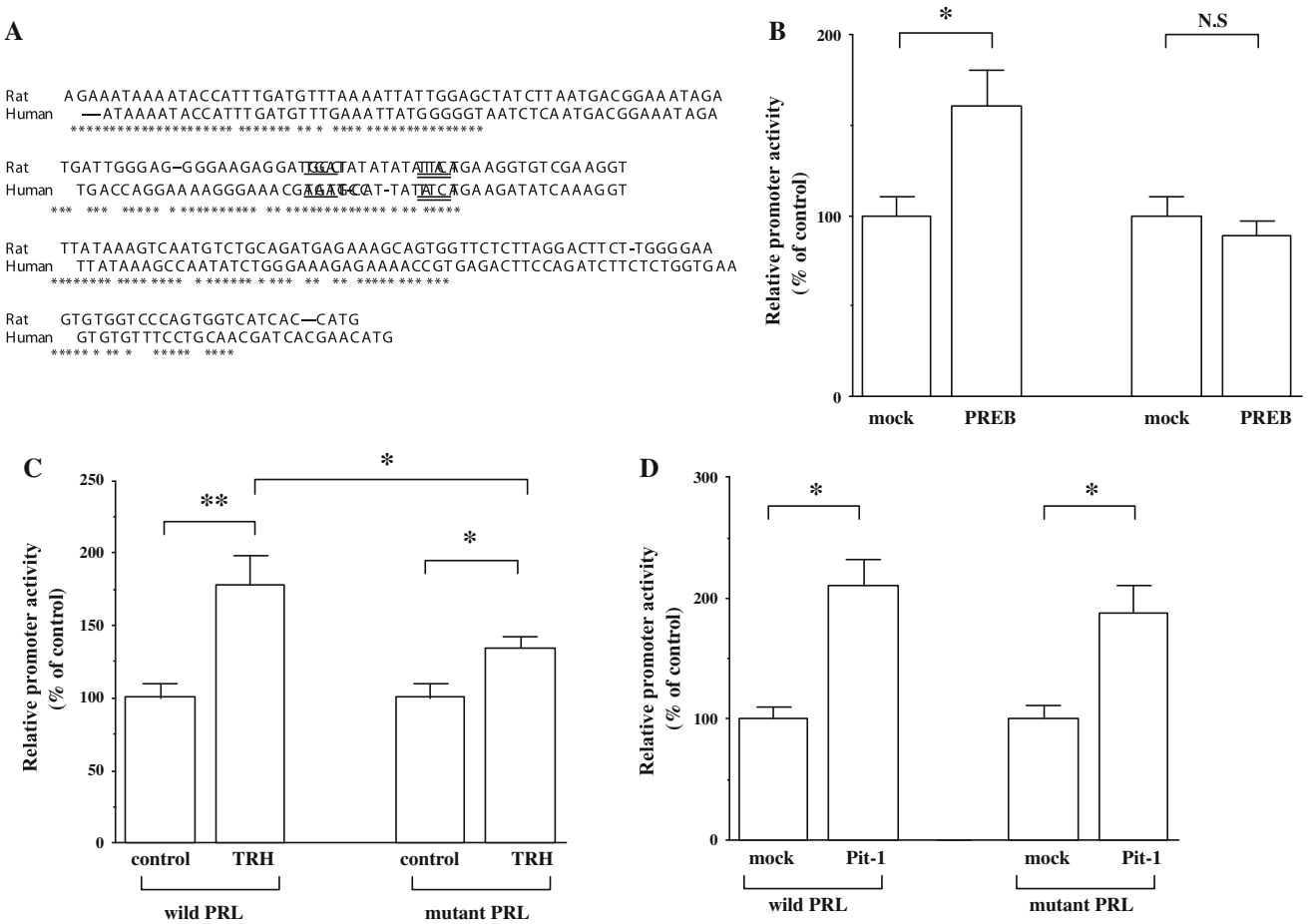

Fig. 4 Role of PREB on TRH-induced PRL promoter activity. **a** An alignment showing motifs' conservation between human and rat. An alignment is constructed using the human and rat reference genome sequences that each spans the first codon (ATG) and its 200-bp upstream stretch of the prolactin gene. An *asterisk* indicates a matched nucleotide site between human and rat. A conserved PREB-binding motif is marked with *underlined characters*, and a conserved Pit-1-biding motif with *double underlined characters*. The both motifs are shown to reside within a highly conserved region. **b**, **c** Site-directed mutagenesis of the PREB-binding site on PRL promoter region abrogates the response to PREB (**b**) or TRH (**c**). The binding site was disrupted by altering three base pairs in the PREB-binding site of the wild-type plasmid construct pPRL-LUC (wild PRL) to create the mutant plasmid construct pPRL-mt-LUC (mutant PRL) as described in "Materials and methods" section. All assays were corrected for β-galactosidase activity and equal total amounts of protein per reaction were employed. The results are expressed as relative luciferase activities with that of control cells arbitrarily set at 100. Each data point represents the mean ± SEM of three independent transfections. The *asterisk* denotes a significant difference (** $P < 0.01$, * $P < 0.05$). *N.S.* no significant difference. **d** Cells were transfected with the reporter genes containing, pPRL-LUC or pPRL-mt-LUC with Pit-1 expression vector (Pit-1) or empty vector (mock); the promoter activity was determined by luciferase assay. All assays were corrected for β-galactosidase activity and total amounts of protein per reaction were identical. The results are expressed as luciferase activities relative to those of control cells arbitrarily set at 100. Each data point shows mean ± SE ($n = 3$) of separate transfections ($P \leq 0.05$)

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