Regulation of phosphatidylinositol-phosphate kinase IIγ gene transcription by thyroid-stimulating hormone in thyroid cells

S Park, W Lee, K H You, H Kim¹, J M Suh¹, H K Chung¹, M Shong¹ and O Y Kwon²

Department of Biology, College of Natural Sciences, Chungnam National University, Taejon 305–764, Korea
¹Department of Internal Medicine, College of Medicine, Chungnam National University, Taejon 301–040, Korea
²Department of Anatomy, College of Medicine, Chungnam National University, Taejon 301–131, Korea

(Requests for offprints should be addressed to O Y Kwon; Email: oykwon@cnu.ac.kr)

ABSTRACT

This study was performed to evaluate the effects of thyroid-stimulating hormone (TSH) on phosphatidylinositol-4-phosphate 5-kinase type IIγ (PIPKIIγ) gene expression in the thyrocytes of FRTL-5 cells. Although PIPKIIγ mRNA was expressed constantly in the absence of added TSH, its expression increased remarkably in the presence of 10⁻⁹ M TSH. This increase started within 6 h of the addition of TSH, and reached a maximum at 8 h. The mRNA expression properties of PIPKIIγ in the cells were identified using inhibitors. Actinomycin D blocked PIPKIIγ transcription strongly, while cycloheximide did not. In an experiment using 5,6-dichlo-1-β-d-ribofuranosyl-benzimidaxole, the half-life of PIPKIIγ mRNA was approximately 6 h in the presence or absence of TSH, and it was not affected by the stability of the PIPKIIγ mRNA. The effects of TSH on PIPKIIγ gene expression were specific, and other growth factors examined (transferrin, insulin and hydrocortisone) did not alter its expression. It is possible that the mechanism of PIPKIIγ gene expression is involved in the permissive effect of the TSH–cAMP cascade proper. Our results indicate, for the first time, that the expression of PIPKIIγ is regulated transcriptionally by TSH in thyrocytes.

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INTRODUCTION

Phosphoinositols (PtdIns) are found in all eukaryotes, and constitute 2–8% of the total cellular phospholipids that induce physiological responses, ranging from survival, growth, differentiation, cytoskeletal organization and vesicular secretion (Shibasaki et al. 1997, Toker 1998, Corvera et al. 1999). PtdIns kinases (PIPKs) are classified into two major subtypes (types I and II) on the basis of their chromatographic profiles and biochemical features. Each is comprised of three isoforms (Iα, Iβ, Iγ and IIα, IIβ and IIγ) (Ishihara et al. 1996, Loijens & Anderson 1996, Hinchliffe et al. 1998, Anderson et al. 1999), and it is believed that each has its own specific role.

The type I isozyme is activated by phosphatidic acid (Jenkins et al. 1994), which binds to the small GTPase, Rho and Rac (Tolias et al. 1995, Ren et al. 1996). This stimulation may be essential for the resynthesis of phosphatidylinositol 4,5-bisphosphate in response to phosphatidylinositol 4,5-bisphosphate hydrolysis by phosphatidic C, and the subsequent conversion of 1,2-diacylglycerol to phosphatidic acid (Nishikawa et al. 1998). Type I is also involved in Ca²⁺-dependent exocytosis and neurotransmitter release (Hay et al. 1995). In the fruit fly, Drosophila melanogaster, type I is required for cell viability, germline development, and the proper structural development of sensory bristles (Hassen et al. 1998). While type I is stimulated by both heparin and spermine, type II is inhibited by heparin, and either inhibited or not affected by spermine, which specifically binds to the receptor of p55 tumor necrosis factor (TNF) receptor, located in the cytoplasmic region, and which is regulated by...
TNF-α (Castellino et al. 1997). To date, the known biological function of the type II isozyme is much more limited than that of type I.

Phosphatidylinositol-4-phosphate 5-kinase type IIγ (PIPKIIγ) was recently isolated from rat fibroblasts as a novel PtdIns(4)P 5-kinase isozyme with 420 amino acids and 47,048 Da, and showed a higher homology to the type IIα and IIβ isoforms (61.1% and 63.7% amino acid identities respectively) of PIPKII than to the type I isoforms. Unlike the PIPKIIα and IIβ isoforms, PIPKIIγ is specifically localized in the endoplasmic reticulum (ER), where phosphorylation occurs through several stimuli such as serum, epidermal growth factor and platelet-derived growth factor (Itoh et al. 1998).

In previous studies, we have isolated thyroid-stimulating hormone (TSH)-dependently expressed genes, using a differential display-PCR method, from rat thyroid FRTL-5 cells. The results of the partial DNA sequencing demonstrated that one of these was a PIPKIIγ DNA fragment (Shong et al. 1999). At present, the effects of TSH on PIPKIIγ gene expression are still unclear. With this in mind, we have investigated the mRNA expression of PIPKIIγ in terms of the cellular response to TSH by Northern blot analysis, and have used actinomycin D (a transcription inhibitor), cycloheximide (another translation inhibitor), 5,6-dichlo-1-β-D-ribofuranosylbenzimidazole (DRB; a RNA polymerase II inhibitor) and growth factors (insulin, transferrin and hydrocortisone) to investigate the mRNA expression of PIPKIIγ in thyrocytes. The results of our study show that TSH dose- and time-dependently controls PIPKIIγ gene expression at the transcription level in thyrocytes, and that the expression of PIPKIIγ may be involved in the intracellular cAMP signal pathway.

MATERIALS AND METHODS

Materials

Highly purified bovine TSH was obtained either from the hormone distribution program of the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health (NIDDK-bTSH I-1; 30 U/mg; Bethesda, MD, USA) or from a previously described preparation (Kohn et al. 1995) (26 ± 3 U/mg) homogeneous in the ultracentrifuge, with a molecular weight of about 27,500 with the amino acid and carbohydrate composition of TSH. The [32P]dATP (3000 Ci/mmol) was from DuPont-New England Nuclear (Boston, MA, USA), and all other materials used in this study were obtained from Sigma Chemical Co. (St Louis, MO, USA) unless noted otherwise.

Cell culture

The rat thyroid cell line FRTL-5 (American Type Culture Collection, Rockville, MD, USA; CRL no. 8305) was cultured in Coon’s medium containing 5% calf serum and 10−9 M TSH, 5 µg/ml transferrin, 1 µg/ml insulin and 10 nM hydrocortisone at 37°C and under 5% CO2 conditions until confluent. Their doubling time with TSH was 36 ± 6 h, and they did not proliferate without TSH. The cells were diploid between the 5th and 20th passage. Fresh medium was added to the cells every 2 or 3 days and cells were passaged every 2 weeks. As a separate experiment, cells were washed three times with cold phosphate-buffered saline to remove...
the hormones completely and then incubated for 48 h without hormones in the growth medium.

RNA isolation and Northern blot analysis
After being incubated at various TSH concentrations, for different times and with other treatments, the collected cells were transferred into a sterile microtube and the total RNA of each sample was isolated as described in Materials and Methods and subjected to Northern blotting. An arrow indicates PIPKIγ (upper panel of A), and ethidium bromide staining of the 28S ribosomal RNA (lower panel of A) was used to indicate the equivalence of gel loading. (B) The results of quantification by phosphorimaging. Data represent means ± S.D. of three values per treatment.

RESULTS AND DISCUSSION
We determined whether the PIPKIγ gene was activated in the thyrocytes of FRTL-5 cells by stimulating the TSH. The cells were incubated in a control growth medium with only 5% calf serum and without TSH, transferrin, insulin or hydrocortisone (called 4H) for 2 days so as to completely remove the hormonal effect. Various concentrations of TSH were then added to the growth media and cultured for 24 h. The resulting total RNA were subjected to Northern blot analysis. As shown in Fig. 1, PIPKIγ mRNA was expressed constantly at all levels in the absence of TSH (control) and its expression increased in line with the TSH concentration. When 10^{-9} M TSH was added, a remarkable expression was induced in the FRTL-5 cells and this reached a maximum at 5 \times 10^{-9} M; increasing TSH concentrations further to 10^{-8} M decreased this expression. This is the first time that TSH has been shown to increase the mRNA level of PIPKIγ in thyrocytes. When one considers the fact that the PIPKIγ gene was expressed at all levels in the absence of TSH (control) and its expression enhanced TSH in response to the altered intracellular conditions.

Next, we examined the effects of TSH on PIPKIγ mRNA expression over time during a
24-h exposure. FRTL-5 cells were incubated for the number of hours indicated in the presence of $10^{-9}$ M TSH, after preincubation for 2 days in growth medium with 5% calf serum and without 4H. Although PIPKII$\gamma$ signal expression was detected when the cells were not chased, it increased gradually with chase periods for 24 h in $10^{-9}$ M TSH (Fig. 2). PIPKII$\gamma$ mRNA increased time dependently and peaked at 8 h, at approximately nine times its baseline value, and then gradually decreased.

To investigate the mode of TSH action on the regulation of PIPKII$\gamma$ gene expression, FRTL-5 cells were treated for 24 h with actinomycin D and cycloheximide in the presence or absence of $10^{-9}$ M TSH after preincubation for 2 days in growth medium with 5% calf serum and without 4H. The Northern blotting results are shown in Fig. 3. As has already been shown in Figs 1 and 2, TSH alone fully increased PIPKII$\gamma$ mRNA expression in FRTL-5 cells. When the cells were treated with $0.2 \mu g/ml$ actinomycin D in the presence or absence of TSH, the resulting signal of the PIPKII$\gamma$ mRNA almost vanished whether TSH was present or not. On the other hand, for the cells treated with $2 \mu g/ml$ cycloheximide in the presence or absence of TSH, the expression pattern of PIPKII$\gamma$ was similar to that of the control, although its expression was slightly weaker. This weakness was due to the activation of the pre-existing PIPKII$\gamma$ mRNAs, which had already become aggravated before the actinomycin D treatment. These results suggest that PIPKII$\gamma$ expression is completely regulated at the transcriptional level but not at the translational/post-transcriptional regulation levels in thyrocytes.

Although TSH alone fully enhanced PIPKII$\gamma$ expression, TSH promoted only one expression pathway for the transcriptional up- or down-regulation of PIPKII$\gamma$, as shown in Fig. 3. If TSH had taken one of the alternative pathways for PIPKII$\gamma$ expression, some strong signal should have been evident when cells were treated with TSH plus actinomycin D or plus cycloheximide.

We demonstrated the effects of TSH on the stability of PIPKII$\gamma$ mRNA using $25 \mu g/ml$ DRB, a specific inhibitor of RNA polymerase II, by determining its half-life in both the presence and absence of TSH. DRB treatment is known to prevent mRNA synthesis and permits the intracellular residue mRNAs to be monitored (Primorac et al. 1999). As is shown in Fig. 4, no signals were commonly detected in either the presence or absence of TSH after 8 h of treatment with DRB in FRTL-5 cells. This means that the previously aggravated PIPKII$\gamma$ mRNA had almost decayed, as the amount of PIPKII$\gamma$ mRNA became aggravated just by being treated with DRB. The half-life of PIPKII$\gamma$ mRNA in the FRTL-5 cells was approximately 6 h in the absence or presence of TSH.

![Figure 3](https://www.endocrinology.org)
By examining graphically the results of the DRB experiment, it was apparent that there was no serious difference between the control and TSH-treated groups, which implied that the stability of PIPKIIα mRNA was not largely affected by either the presence or absence of TSH in FRTL-5 cells. In other words, TSH is not involved in the post-transcriptional steps, and is effective only in the gene expression step. In addition, both signals seemed to be enhanced after 17 h of DRB treatment because of the inactivating effect of DRB, which had already been added by a single term of incubation at 37 °C, and because the signal appeared stronger when TSH was present than when TSH was absent.

We examined the effects of growth factors (e.g. transferrin, insulin and hydrocortisone, which are essential for growing FRTL-5 culture cells) on PIPKIIα gene regulation in the presence and absence of TSH. The cells were treated for 24 h with 5 µg/ml transferrin, 1 µg/ml insulin or 10 nM hydrocortisone, in the presence or absence of 10⁻⁹ M TSH after preincubation for 24 h in the growth media with 5% calf serum and without 4H.

As shown in Fig. 5, transferrin, insulin and hydrocortisone did not affect the level of PIPKIIα mRNA in FRTL-5 cells remarkably, in either the presence or the absence of TSH. When TSH was added together with the growth factors, PIPKIIα expression was not additive, and TSH alone was largely responsible for the increased PIPKIIα mRNA levels. This shows that TSH specifically affects PIPKIIα expression, at least in FRTL-5 cells, while the growth factors transferrin, insulin and hydrocortisone play an important role in the growth of FRTL-5 cells. If the growth factors had affected PIPKIIα expression, some strong signals should have been evident when the cells were treated with a combination of TSH, transferrin, insulin or hydrocortisone. However, as shown in Fig. 5, no strong signals were found when the cells were treated with additional growth factors, compared with the signal produced by treatment with TSH.
It is not yet fully understood why a TSH stimulatory signal is involved in the intracellular cascade pathway for PIPKIIγ expression. TSH actions were mimicked by forskolin (enhancer of intracellular cAMP), which confirmed that the cAMP cascade mediated its expression in FRTL-5 cells, and stimulated RNA synthesis and proliferation (Van Sande et al. 1989, Dremier et al. 1997). To confirm PIPKIIγ expression when the thyrocytes were treated with forskolin, approximately the same PIPKIIγ expression was detected with both the TSH and the forskolin treatments (Fig. 6). The results shown in Fig. 6 suggest that forskolin is one of the transcription factors in the expression of the PIPKIIγ gene in thyrocytes. We also hypothesize that TSH stimulates the concentration of intracellular cAMP which, in turn, triggers other intracellular factors associated with PIPKIIγ gene transcription.

Considering that the PIPKIIγ mRNA was constantly expressed in the cells that were not treated with TSH, and bearing in mind the results of previous reports, PIPKIIγ would seem to act as a kinase to produce both phosphatidylinositol 4-bisphosphate and phosphatidylinositol 4,5-bisphosphate (Anderson et al. 1999). In addition, the results of our study demonstrated that TSH strictly controls PIPKIIγ gene expression at the transcriptional level in thyrocytes. It was suggested by Itoh et al. (1998) that each subfamily of PIPK has a distinct localization and function. It is interesting that PIPKIIγ is specifically localized in the ER (Helms et al. 1991, Wong et al. 1997), although most PIPK activity is detected in the plasma membrane and the cytosol, where the phosphorylated form of PIPKIIγ was detected in response to extracellular stimuli (e.g. growth factors or hormones) and binds to one of the ER resident molecular chaperones of immunoglobulin-binding protein (Bip) (Itoh et al. 1998). Binding with Bip makes it possible for phosphorylated PIPKIIγ, induced by stimulation, to be involved in the post-translational step of protein folding and the assembly of newly synthesized proteins in the ER as distinct functions in vivo. Further studies are necessary to clarify the roles of PIPKIIγ as an ER chaperone in thyrocytes, where it might participate in the folding and assembly of thyroglobulin, the major thyroid secretory glycoprotein.

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