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Nongenomic Activation of Phosphatidylinositol 3-kinase Signaling by Thyroid Hormone Receptors

Fumihiko Furuya, Changxue Lu, Celine J. Guigon, and Sheue-yann Cheng

Laboratory of Molecular Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892-4264 USA

Abstract

Thyroid hormone (T3) is critical in growth, development, differentiation, and maintenance of metabolic homeostasis. Recent studies suggest that thyroid hormone receptors (TRs) not only mediate the biological activities of T3 via nucleus-initiated transcription, but also could act via nongenomic pathways. The striking phenotype of thyroid cancer exhibited by a knockin mutant mouse that harbors a dominant negative TR β mutant (TR $\beta^{PV/PV}$ mouse) allows the elucidation of novel oncogenic activity of a TR β mutant (PV) via extra-nuclear actions. PV physically interacts with the regulatory p85 α subunit of phosphatidylinositol 3-kinase (PI3K) to activate the downstream AKT-mammalian target of rapamycin (mTOR) and p70^{S6K} and PI3K-integrin-linked kinase-matrix metalloproteinase-2 signaling pathways. The PV-mediated PI3K activation results in increased cell proliferation, motility, migration, and metastasis. Remarkably, a nuclear receptor corepressor (NCoR) was found to regulate the PV-activated PI3K signaling by competing with PV for binding to the C-terminal SH2 domain of p85 α . Overexpression of NCoR in thyroid tumor cells of TR $\beta^{PV/PV}$ mice reduces AKT-mTOR- p70^{S6K} signaling. Conversely, lowering cellular NCoR by siRNA knockdown in tumor cells leads to over-activated PI3K-AKT signaling to increase cell proliferation and motility. Furthermore, NCoR protein levels are significantly lower in thyroid tumor cells than in wild type thyrocytes, allowing more effective binding of PV to p85 α to activate PI3K signaling, thereby contributing to tumor progression. Thus, PV, an apo-TR β , could act via direct protein-protein interaction to mediate critical oncogenic actions. These studies also uncovered a novel extra-nuclear role of NCoR in modulating the nongenomic actions of a mutated TR β in controlling thyroid carcinogenesis.

Keywords

thyroid hormone receptors; phosphatidylinositol 3-kinase; pituitary tumor transforming gene; steroid hormone receptor coactivator-3; nongenomic actions; thyroid hormone receptor mutants; mouse model; thyroid cancer; carcinogenesis

1. Introduction

Thyroid hormone (T3) has diverse effects on growth, development, differentiation, and maintenance of metabolic homeostasis. Thyroid hormone nuclear receptors (TRs) mediate these biological activities via transcriptional regulation. TRs are derived from two genes, α

Address correspondence to: Dr. Sheue-yann Cheng, Laboratory of Molecular Biology, National Cancer Institute, 37 Convent Dr, Room 5128, Bethesda, MD 20892-4264, Tel: (301) 496-4280; Fax: (301) 480-9676; chengs@mail.nih.gov.

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and β , that are located on two different chromosomes. Alternate splicing of primary transcripts gives rise to four T3-binding TR isoforms: $\alpha 1$, $\beta 1$, $\beta 2$, and $\beta 3$. The expression of these TR isoforms is developmentally regulated and tissue-dependent [1]. TRs regulate transcription by binding to the thyroid hormone response elements (TREs) in the promoter regions of T3-target genes [1]. In addition to the effects of T3 and the various types of TREs, transcription activity of TR is modulated by tissue- and development-dependent TR isoform expression [2,3] and by a host of corepressors and coactivators [3,4]. Studies using TR subtype knockout and TR total knockout mice have shown that TR isoforms have subtype-specific and redundant functions [5].

Recent studies, however, have indicated that TRs could also mediate T3 biological activities beyond TRE-mediated gene transcription. Simoncini et al. first reported T3-dependent TR-mediated activation of phosphatidylinositol 3-kinase (PI3K) activity in human endothelial cells [6]. This activation is through direct physical interaction of TR with the p85 α subunit of phosphatidylinositol 3-kinase (PI3K), leading to the phosphorylation and activation of AKT and endothelial nitric oxidase synthase [7]. This TR-mediated PI3K activation has also been demonstrated in other cell types including human fibroblasts, neonatal rat cardiomyocytes, and human and rat insulinoma cell lines [8–12]. These studies indicate that non-TRE-dependent effects of TR may contribute to important physiological effects of T3.

That unliganded TRs (apo-TRs) play critical roles is evident in that congenital hypothyroidism leads to cretinism with growth defects and mental retardation [13]. Studies of mice deficient in all TRs (TR $\alpha 1^{-/-}$ and TR $\beta^{-/-}$ mice) have shown that they exhibit a milder overall phenotype than the debilitating symptoms of severe hypothyroidism [5,14], highlighting the important role of apo-TRs in the pathogenesis of hypothyroidism. Current interest in nongenomic actions of TRs has focused more on the liganded state. Thus, whether apo-TRs could contribute to diseases via nongenomic actions is less well understood. The availability of a knockin mouse harboring a dominant negative TR β mutation (TR $\beta^{PV/PV}$ mouse) presents an opportunity to address this question. The PV mutation was identified in a patient with resistance to thyroid hormone (RTH). It is due to a C-insertion at codon 448 of the TR $\beta 1$ that leads to a mutant that has completely lost T3 binding and transcription activity [15,16]. The TR β^{PV} mouse faithfully reproduces human RTH with dysregulation of the pituitary-thyroid axis [17]. Remarkably, as TR $\beta^{PV/PV}$ mice age, they spontaneously develop follicular thyroid carcinoma similar to human thyroid cancer [18,19]. We therefore used this mouse model to ascertain whether apo-TR β could also signal via the PI3K pathway to mediate its oncogenic actions. This article will highlight recent advances in the understanding of the molecular mechanisms of the nongenomic actions of apo-TRs *in vivo* achieved with the use of a mouse model of thyroid cancer (TR $\beta^{PV/PV}$ mouse).

2. Activation of PI3K by PV via nongenomic pathways

2.1. Potent activation of PI3K by PV via protein-protein interaction

Amplification of the PIK3C gene and activation of PI3K are frequently observed in human follicular thyroid cancer [20]. TR $\beta^{PV/PV}$ mice that spontaneously develop follicular thyroid cancer provide a valuable tool to investigate whether an apo-TR β (PV) could activate PI3K signaling via nongenomic pathways. Analysis of PI3K activity in the thyroid extracts indicates that those of wild type mice show weak PI3K activity. The PI3K activity in the thyroid of TR $\beta^{PV/PV}$ mice is significantly higher than in wild type mice (40–50-fold) [21].

PI3K associates with TRs in human vascular endothelial cells and fibroblasts [7,8], but whether PV, an apo-TR β , is associated with PI3K in the thyroid was unknown. We therefore used monoclonal antibody J52 [22], which recognizes the N-terminal region of the A/B

domain of TR β and PV, to determine whether these two TRs are associated with PI3K. Fig. 1A shows that the antibody J52 precipitated from thyroid extracts of TR $\beta^{PV/PV}$ mice (Fig. 1A, bars 4 and 5) had 30-fold more PI3K activity than wild-type mice (bars 1 and 2). The increased PV-associated PI3K activity is not due to preferential binding of J52 with PV, because J52 interacts with TR β and PV with a similar affinity as J52 recognizes the epitope in the A/B domain shared by TR β and PV. To be certain that the increased PI3K activity is due to its association with PV, the same tumor extracts were first precipitated with a monoclonal antibody that specifically recognizes the C-terminal PV sequence (#302) [22] followed by kinase activity determination. As shown in bars 7 and 8 (Fig. 1A), a 26- to 85-fold increase in kinase activity was detected. The differences in fold of increases in PI3K activity between the J52 immunoprecipitates (the epitope is located in the AB domain of PV) and #302 immunoprecipitates (the epitope is located in the C-terminal 16 amino acids of PV) reflected the differences in the binding affinity of these two antibodies with PV. These findings indicate that a weak PI3K activity is associated with TR β , but a markedly high PI3K activity is associated with PV.

Whether the increased PI3K activity was due to physical interaction of PV with PI3K p85 α regulatory subunit was evaluated by co-immunoprecipitation analysis of thyroid extracts of TR $\beta^{PV/PV}$ mice. Indeed, the PI3K p85 β regulatory subunit was detected in a concentration-dependent manner in TR $\beta^{PV/PV}$ mice (lanes 5–7, Fig. 1B), but very weakly in wild type mice (lanes 2–4; Fig. 1B). These results indicate that more PV in the thyroid of TR $\beta^{PV/PV}$ mice is bound to p85 α than TR β in wild type mice. The increase in the binding of PV to PI3K shown in Fig. 1B is not due to increased PV protein abundance in the thyroid of TR $\beta^{PV/PV}$ mice because western blot analysis indicates a similar abundance of TR β protein in the thyroid of wild type mice and of PV protein in the thyroid of TR $\beta^{PV/PV}$ mice.

The sites of TR with which p85 α interacted were mapped by GST-pull down assays [21]. Using a series of sequentially truncated TRs, we mapped sites to the ligand-binding domain of TR [21]. The region of p85 α with which TR interacted is localized to the C-terminal SH2 domain of p85 α (Src homology 2) [24]. Taken together, these results indicate that physical interaction of PV with p85 α leads to marked increases in PI3K activity.

The functional consequences of interaction of PV with PI3K were evaluated by analyzing two downstream signaling pathways: the AKT-mTOR-p70^{S6K} and the integrin-linked kinase (ILK) pathways. The former is known to mediate cell growth and proliferation [25], and the latter is involved in cell migration, invasion, and an inhibition of apoptosis [26, 27]. Indeed, consistent with the activation of PI3K, the phosphorylated AKT, mTOR, and p70^{S6K} are increased in both the nuclear and cytoplasmic compartments without significant alteration of the respective total protein abundance, thereby indicating the activation of the signaling pathway via phosphorylation cascades [21]. The increased PI3K activity also activates the ILK-matrix metalloproteinase-2 (MMP2) pathway in the extracellular compartment to increase the degradation of the extracellular matrix that affects cancer cell invasion and metastasis [21, 26, 27]. These findings demonstrate that an apo-TR β (PV) could act via nongenomic signaling to exert its oncogenic effects.

2.3. Inhibition of PI3K signaling delays tumor progression and blocks metastasis of thyroid cancer

That this nongenomic PV-activated PI3K signaling is critical in the development and progression of thyroid cancer in TR $\beta^{PV/PV}$ mice was further demonstrated by treating TR $\beta^{PV/PV}$ mice with LY294002 (LY), a potent and specific PI3K inhibitor, and evaluating the effect of LY on the spontaneous development of thyroid cancer [24]. Analysis of Kaplan-Meier cumulative survival curves show that the 50% survival age for TR $\beta^{PV/PV}$ mice treated with LY or vehicle is 329 $\pm$ 64.5 days (n=24) or 244 $\pm$ 63.4 days (n=23),

respectively. The finding that LY-treated mice survive significantly longer suggests LY is effective in prolonging the survival of TR $\beta^{PV/PV}$ mice. LY treatment leads to a significant decrease (2-fold reduction) in thyroid weight of TR $\beta^{PV/PV}$ mice as compared with vehicle-treated controls.

Histopathological evaluation shows that whereas the untreated mice exhibit advanced hyperplasia, the treated mice show only early hyperplasia. While vascular invasion is frequent in untreated mice (~40%), it is rare in the treated mice (~5%) with frequent appearance of apoptotic bodies. In contrast to the untreated mice in which the occurrence of lung metastasis is frequent (25%), no metastasis occurs in the lung of treated mice. Thus, treatment with LY clearly delays the thyroid tumor progression and blocks metastatic spread to the lung [24].

To further confirm that the LY-mediated delay in tumor progression is acting through the PI3K-AKT signaling, we evaluated the AKT-mTOR-p70^{S6K} pathway known to be involved in cell growth and proliferation. As shown in lanes 3 and 4 in panels a, c, and e (Figure 2), p-AKT, p-mTOR, and p-p70^{S6K} in thyroid tumors of TR $\beta^{PV/PV}$ mice are consistently higher than those effectors in wild type mice. Remarkably, LY treatment of TR $\beta^{PV/PV}$ mice leads to a significant deactivation of the AKT-mTOR-p70^{S6K} pathway as evidenced by the reduced p-AKT, p-mTOR, and p-p70^{S6K} shown in lanes 5 and 6 in panels a, c, and e, respectively (Figure 2). The cellular levels of total AKT, total mTOR, and total p70^{S6K} shown in lanes 5 and 6 in panels b, d, and f, respectively (Figure 2), are not significantly altered, indicating that the deactivation of these effectors is mediated by the PI3K downstream phosphorylation cascade [24]. These results suggest that blocking this pathway by LY treatment leads to delay in tumor progression. Taken together, these findings further support the conclusion that the nongenomic action of PV in activating PI3K signaling is critical in thyroid carcinogenesis.

2.3. Nuclear receptor corepressor (NCoR) is a novel regulator of PV-activated PI3K signaling

Apo-TR β has been shown to associate with the nuclear receptor corepressor (NCoR), and this association modulates the dominant negative activity of the apo-TR β [28]. The finding that PV, an apo-TR β , activates the PI3K signaling via nongenomic action prompted us to ascertain whether NCoR could function to regulate the PV-mediated activation of PI3K signaling. Interestingly, we found that NCoR physically associates with p85 α . The amino terminal R1 and the C-terminal R4 and receptor interaction domain (RID) are the regions of NCoR to interact with p85 α . The C-terminal SH2 domain (CSH2) was identified as the region that interacts with the RID of NCoR [29]. Since both NCoR and TR interact with the same region of the CSH2 domain of p85 α , we further ascertained whether NCoR competes with TR β or PV for binding to p85 α . Using GST-pull down assays, we found that, indeed, in the presence of increasing concentrations of NCoR (RID), the binding of TR β to p85 α is decreased in a concentration-dependent manner (lanes 2–4, compared with lane 1; Figure 3A). NCoR also competes with PV for association with p85 α in a concentration-dependent manner (Figure 3B). In addition, we compared the binding of p85 α with TR β (Figure 3C), PV (Figure 3D) or NCoR (RID) in a concentration-dependent manner. As shown in the quantitative analysis, PV interacts with p85 α with the relative highest affinity in the rank order of PV > TR β > NCoR (Figure 3F).

The finding that PV and NCoR compete for binding to the same site of p85 α suggests that a decrease in the cellular NCoR would lead to an activation of PI3K activity and its downstream signaling. We therefore used siRNA approaches to ascertain the effect of knocking down the expression of NCoR on the PI3K activity of primary thyrocytes of wild type mice and tumor cells of TR $\beta^{PV/PV}$ mice. The western blot analysis (Figure 4A-a) shows

that NCoR expression is knocked down after the treatment of primary thyrocytes (compare lane 2 with lane 1) and tumor cells with specific siRNA (compare lane 4 with lane 3). Concomitant with the knocking down of NCoR, phosphorylation of its immediate downstream effector, AKT (pAKT), is increased in primary thyrocytes (compare lane 3 with lane 1; upper panel; Figure 4A-b) and tumor cells (compare lane 4 with lane 2; upper panel; Figure 4A-b). The increased p-AKT is not due to an increase in total AKT because total AKT is virtually unaltered by treatment of cells with siRNA (Figure 4A-b; middle panel). Taken together, these results indicate that the reduced NCoR protein levels would favor the interaction of TR β or PV with p85 α to activate PI3K-AKT signaling.

To obtain additional evidence in support of the conclusion that NCoR competes with TR β or PV for binding to p85 α , we over-expressed NCoR by transfection into primary thyrocytes of wild type mice and tumor cells of TR $\beta^{PV/PV}$ mice and determined the effect of its over-expression on PI3K signaling (Figure 4B). As shown by western blot analysis, NCoR is over-expressed in the transfected primary thyrocytes of wild type mice (compare lane 3 to lane 1; Figure 4B-a) and tumor cells of TR $\beta^{PV/PV}$ mice (compare lane 4 to lane 2; Figure 4B-a). In the absence of over-expressed NCoR, more p-AKT was observed in tumor cells of TR $\beta^{PV/PV}$ mice than in primary thyrocytes of wild type mice (compare lane 2 to lane 1, Figure 4B-b). When NCoR is over-expressed, a concurrent reduction in p-AKT was found in tumor cells as well as in thyrocytes of wild type mice (compare lane 4 to 2 and lane 3 to lane 1; Figure 4B-b). The observation that total AKT protein levels are not altered by NCoR over-expression (Figure 4B-c; the loading controls are shown in Figure 4B-d) further supports the conclusion that NCoR regulates the interaction of p85 α with PV or TR β , thus modulating the PI3K downstream signaling.

The results shown in Figure 4 suggest that the expression of NCoR in tumor cells of TR $\beta^{PV/PV}$ mice would be lower than that in thyroid of wild type mice. Indeed, analysis of the NCoR protein abundance shows that NCoR protein levels are 60% lower in thyroid tumor cells of TR $\beta^{PV/PV}$ mice than in normal thyrocytes [29]. The molecular model depicted in Figure 5 illustrates how PV and NCoR compete for binding to the CSH2 domain of p85 α shown by *in vitro* and *in vivo* findings (panel a). In tumor cells, the cellular levels of NCoR are reduced (panel b), thereby facilitating the binding of PV to p85 α to activate PI3K/AKT signaling to promote thyroid carcinogenesis by increasing cell proliferation and tumor metastasis. Thus, NCoR is a novel regulator of the PI3K signaling via nongenomic signaling.

Summary and Future Challenges

Using a mouse model of thyroid cancer, we uncovered a novel nongenomic action of PV, an apo-TR β mutant, that is critical for oncogenesis of the thyroid. This nongenomic action is mediated by direct protein-protein interaction in that binding of PV with the p85 α regulatory subunit increases the catalytic activity of p110. In the thyroid of wild type mice in which TR β is the major TR isoform [19], we found that TR β also interacts with p85 α . However, the PI3K activity is significantly lower in wild type mice than in TR $\beta^{PV/PV}$ mice [21]. We had mapped the interaction region of TR β with p85 α to the ligand-binding domain. Since PV has a frame-shift mutation at the carboxyl-terminus of TR β , it is reasonable to postulate that the conformational changes in this region favor PV to interact with p85 β more strongly than TR β . The interaction of another TR β mutant, G345R, with p85 α has been reported in human fibroblasts [8]. In that study, the over-expressed TR β and TR β G345R via adenoviral infection bind equally well with p85 α ; however, in contrast to PV, TR β G345R is unable to activate the PI3K activity [8]. The present findings suggest that the conformation of TR β at the carboxyl end of the ligand-binding domain is critical for the interaction with p85 α and the activation of the PI3K activity. Elucidation of the precise location and the binding motifs

of TR involved in the interaction with p85 α will advance our understanding of how TR activates PI3K signaling in the T3-dependent and T3-independent manner.

The present study uncovered NCoR as a novel regulator of PV-mediated activation of PI3K signaling. This modulation is via competition with PV for binding to the same CSH2 domain of p85 α (see Figure 5). Since PV acts as an oncogene via physical interaction with p85 α , NCoR could be considered as a newly identified tumor suppressor in the mouse model of thyroid cancer. However, it is important to explore further whether this tumor suppressor role of NCoR is unique in PV-mediated activation of PI3K signaling or also serves as a general modulator in other PI3K-mediated cellular functions. Activation of the PI3K activity via protein-protein interaction has precedents. The interaction of p85 α with other cellular proteins including insulin receptor, insulin receptor substrate, and several members of the Rho family is known to increase the PI3K activity to mediate a host of cellular functions [30–32]. Since NCoR is widely expressed in many tissues, it is likely that its regulatory role in PI3K signaling goes beyond the regulation of the oncogenic activity of PV in thyroid carcinogenesis to affect many other cellular functions.

Accumulated evidence in recent years has indicated that similar to many steroid hormone receptors, TRs can act via TRE-independent actions. However, a challenge is to demonstrate directly the membrane localization as it has been shown for estrogen [33–35], progesterone [35], androgen [35], mineralocorticoid [36] and vitamin D receptors [37]. TR has isoform-dependent actions as illustrated in TR subtype knockout and TR total knockout mice [5]. In addition, TR knockin mutant mice harboring the same PV mutation in the TR β (TR β PV mice) [17] or the TR α gene (TR α 1PV mice) [38] exhibit distinct abnormalities. It will further advance our understanding of the biology of TRs if the question of whether the nongenomic actions of TR could be isoform dependent is also elucidated in future studies.

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Figure 1.

Physical interaction of p85 α with TR β or PV in the thyroid extracts of wild type and TR $\beta^{PV/PV}$ mice, respectively. (A). The PI3K activity is associated with TR β or PV. One hundred micrograms of proteins derived from the total thyroid extracts of wild type (bars 1–3; three mice) or TR $\beta^{PV/PV}$ mice (bars 4–8) were immunoprecipitated with 5 μ g of anti-TR β 1 (J52, bars 1 & 2, wild type mice; bars 4 & 5, two TR $\beta^{PV/PV}$ mice), anti-PV (#302; bars 7 & 8, two mice) antibodies, or an irrelevant monoclonal antibody (MOPC) as control (bars 3 & 6, marked as C), and the PI3K activity was determined. (B). Co-immunoprecipitation of p85 α with TR β or PV. Increasing concentrations of lysates from pooled thyroid extracts of six wild type mice or three TR $\beta^{PV/PV}$ mice, respectively, were

immunoprecipitated with J52 antibody and subjected to immunoblot analysis probed with anti-p85 α antibody. Lane 1 is Jurkat cell lysate (#12-303, Up-State) as a positive control.

Figure 2.

LY deactivates the PV-induced activation of PI3K signaling in TR $\beta^{PV/PV}$ mice. Western blot analysis of thyroid extracts (50 μg) of TR $\beta^{PV/PV}$ mice at the age of 7.5 months was carried out for determination of p-AKT(S473) (a), total-AKT (b), p-mTOR (c), total-mTOR (d), p-p70^{S6K} (e) total-p70^{S6K} (f), and α -tubulin (g).

Figure 3.

NCoR (RID) competes with TR β or PV for binding to p85 α .

GST-p85 α fusion protein was incubated with 2 μ L of [35 S]-labeled TR β (A) or [35 S]-labeled PV (B) in the absence (lane 1) or presence of 1, 2, or 4 μ L of NCoR (RID) (lanes 2–4, respectively). (C – F) GST-p85 α fusion proteins were incubated with increasing quantities of [35 S]-labeled TR β (2.5, 5, 10, and 20 μ L; C), PV (3.2, 6.4, 12.8, and 25.6 μ L; D) or NCoR (RID) (1.7, 3.4, 6.8, and 13.6 μ L; E) (lanes 1 to 4). (F). PV binds to p85 α the strongest in the rank order of PV>TR β >NCoR. Band intensities were quantified and the relative binding affinity of TR β , PV, and NCoR (RID) to p85 α is shown.

Figure 4. Regulation of the PI3K downstream signaling by NCoR expression. (A-a) NCoR expression is knocked down in primary thyrocytes of wild type mice (lane 2) and tumor cells of TRP^{PV/PV} mice (lane 4) by siNCoR as compared with controls (lanes 1 and 3, respectively). Cellular extracts were analyzed by western blot analysis for NCoR (upper panel, A-a) and α -tubulin for loading controls (lower panel; A-a). (A-b) Western blot analysis was carried out for determination of p-AKT (upper panel; anti-phosphorylated S473 antibody), total AKT (middle panel), and α -tubulin (lower panel) as a control for protein loading. (B) Wild type primary thyroid cells or PV tumor cells were transfected with NCoR expression vector (pCMX-NCoR; 8 μ g). Western blot analysis was carried out to determine the effects of NCoR on the activity of p-AKT. Protein abundance of cellular NCoR (a), p-AKT (anti-phosphorylated S473 antibody) (b), total AKT (c), and α -tubulin as a control for protein loading (d).

Figure 5.

A proposed molecular model for the regulation of PI3K signaling by NCoR. (a). In cells, transfected PV and NCoR compete for binding to the CSH2 domain of the regulatory subunit, p85 α , of PI3K. (b). In thyroid tumors of TR $\beta^{PV/PV}$ mice, NCoR protein abundance is decreased (indicated by the arrow), thereby facilitating the association of PV with p85 α to activate PI3K-AKT signaling to increase cell proliferation and metastasis, thereby contributing to thyroid carcinogenesis of TR $\alpha^{PV/PV}$ mice.