1α(OH)ase gene promoter (Murayama *et al*, 1998). However, to our surprise, neither homologous nor related to the previously reported nVDREs in the PTH and PTHrP gene promoters were present in the 1α(OH)ase gene promoter (Demay *et al*, 1992; Falzon, 1996). To our knowledge, the present study was the first to identify the core sequence of 1αnVDRE and to explore the molecular basis of 1α,25(OH)$_2$D$_3$-induced transrepression.

Although the reported nVDREs resemble positive VDREs in that they contain directly repeated AGGTCA motifs spaced by 3 bp (DR3) (Demay *et al*, 1992; Falzon, 1996), the identified 1αnVDRE sequence was composed of two E-box-like motifs and conferred a negative responsiveness to 1α,25(OH)$_2$D$_3$ in a kidney cell line that expressed endogenous 1α(OH)ase gene. Unlike the reported nVDREs, direct DNA binding of VDR/RXR to 1αnVDRE was not detected. The cDNA cloning of a binding factor for 1αnVDRE by yeast expression screening allowed us to identify a bHLH-type transcription factor designated as VDR interacting repressor (VDIR). VDIR acted as an activator on 1αnVDRE by recruiting p300 HAT co-activator complexes in response to activated-PKA signaling. However, 1α,25(OH)$_2$D$_3$-dependent interaction between VDR and VDIR induced p300 dissociation and association of HDAC and Sin3A co-repressors, which resulted in ligand-induced transrepression. Thus, our present findings decipher a novel molecular mechanism of ligand-induced transrepression by a NR.

Results

Mapped core element in 1αnVDRE conferred a positive response to PKA signaling

To identify the core element of the nVDRE in the human 1α-hydroxylase (1α(OH)ase) gene promoter, functional analysis was performed using a series of promoter deletion mutants in a transient expression assay using MCT cells. The MCT cell line is derived from a mouse proximal tubulal cell line that expresses endogenous 1α(OH)ase gene with a negative responsiveness to 1α,25(OH)$_2$D$_3$ (Murayama *et al*, 1998). Using reporter plasmids to supply a thymidine kinase TATA box to potentiate basal transcriptional activity, the core nVDRE region was mapped from −537 to −514 bp upstream of the transcription start site (Figure 1A). 1α,25(OH)$_2$D$_3$-induced repression via the identified 1αnVDRE was confirmed using a synthetic element (data not shown). The mapped sequence, designated as 1αnVDRE, was distinct from the reported DR3-like nVDREs, being composed of two E-box-like motifs (Figure 1B, box). We found that

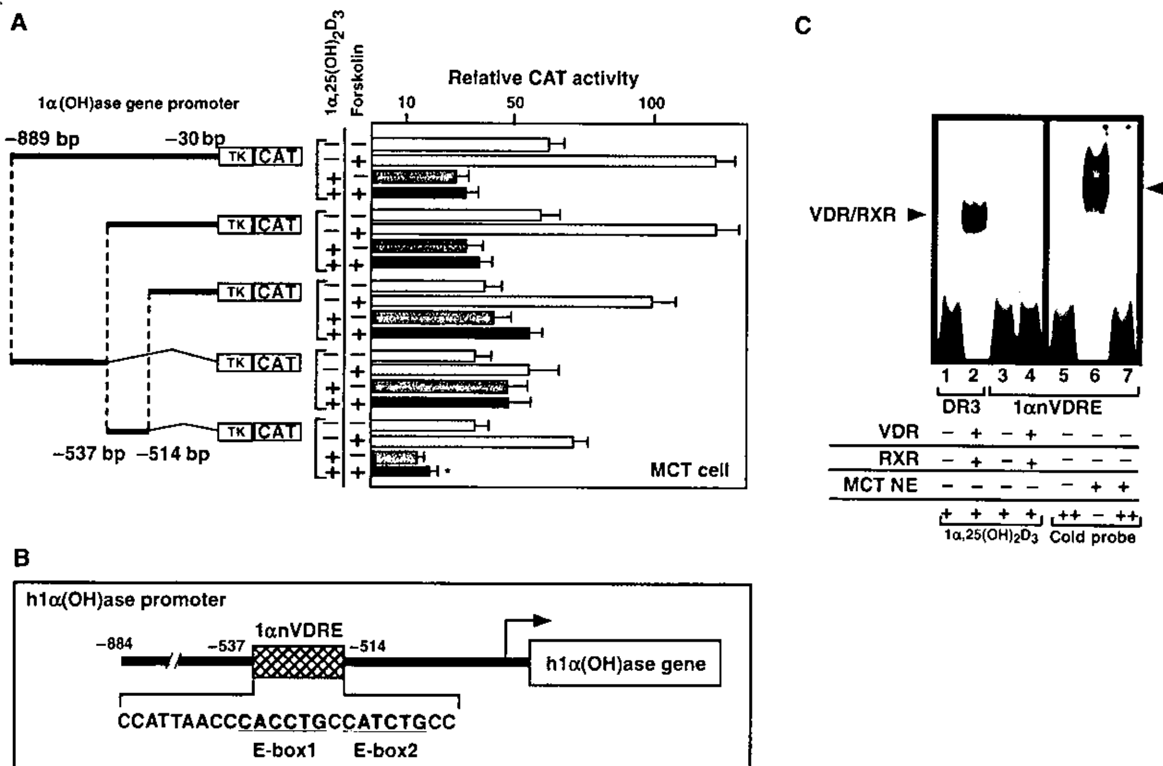

Figure 1 Identification of 1αnVDRE. (A) CAT assay using a series of human 1α(OH)ase gene promoter deletion mutants in MCT cells. After 3 h, forskolin (1×10^{-8} M), which activates PKA signaling, and 1α,25(OH)$_2$D$_3$ (1×10^{-8} M) were added, respectively. 1α(OH)ase gene promoter deletion constructs (−889/−30, −537/−30, −514/−30, −889/−537 and −537/−514) as indicated were transfected in MCT cells. Results shown are representative of five independent experiments. (B) Sequence of the 1αnVDRE core element. The 1αnVDRE was composed of two E-box-like motifs in the 1α(OH)ase gene promoter −537 to −514 bp. (C) Absence of direct binding between VDR/RXR and 1αnVDRE. A gel mobility shift assay was performed using bacterially expressed recombinant VDR and RXR proteins or MCT cell nuclear extracts together with a radiolabeled probe (10 ng) comprising 1αnVDRE sequence (lanes 3-7). Unlabeled 1αnVDRE oligonucleotides (100 ng) were used as cold competition (lanes 5-7). Radiolabeled probe DR3 (consensus positive VDRE) (10 ng) was used as positive control for DNA binding of liganded VDR/RXR (lanes 1and 2).

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this mapped element also conferred responsiveness to forskolin, an agent used to activate PKA signaling. Interestingly, negative regulation due to 1α,25(OH)$_2$D$_3$ was more pronounced when forskolin was used to potentiate transcription (Figure 1A). As 1α(OH)ase gene expression is induced by PKA signaling downstream of PTH/PTHrP activity (Henry, 1985; Brenza *et al*, 1998), it was possible that the putative core element served as a dual regulatory element for the two oppositely acting hormones. We also found a 1αnVDRE sequence with the identical core motif (−537 to −514 bp) in the mouse 1α(OH)ase promoter, which also exhibited a negative response to 1α,25(OH)$_2$D$_3$ (M Kim, unpublished results).

Previous reports have shown that 1α,25(OH)$_2$D$_3$-induced transrepression through DR3-like nVDREs in the PTH and PTHrP gene promoters requires direct DNA binding of VDR/RXR heterodimers to the nVDREs (Demay *et al*, 1992; Falzon, 1996). Therefore, we examined the DNA binding of VDR/RXR to 1αnVDRE core elements by electrophoresis mobility shift assay (EMSA). Recombinant VDR/RXR heterodimers expressed in *Escherichia coli* effectively bound to a consensus positive VDRE (DR3) containing two AGGTCA core motifs (Ebihara *et al*, 1996; Takeyama *et al*, 1999), while no DNA binding was detected using 1αnVDRE (Figure 1C, left panel). This result confirmed the difference between 1αnVDRE and the reported nVDREs. However, a clear band was observed on 1αnVDRE using MCT nuclear extracts (Figure 1C, right panel), which suggested the presence of an unknown factor that directly bound to 1αnVDRE.

Molecular cloning of a bHLH-type transcription factor, VDIR, as a direct binding factor for 1αnVDRE

To isolate and identify the 1αnVDRE-binding factor, a yeast one-hybrid assay using 1αnVDRE was employed to screen a yeast expression cDNA library derived from MCT cells. Out of 8×10^9 colonies, seven candidates were identified, of which five represented overlapping sequences that encoded a protein designated as VDIR (Figure 2A). VDIR was found to be a bHLH-type factor and appeared to be a mouse homolog of the human E47 (Figure 2B). VDIR also exhibited strong homology, in terms of both motif sequences and genetic organization, to the rat Pan-1 and Pan-2 transcription factors (Vierra and Nelson, 1995) (Figure 2B). The VDIR gene was ubiquitously expressed in many tissues, including the kidney (Figure 2C). To test if VDR controls expressions of VDIR, we examined VDIR transcript levels in VDR-null mouse (Yoshizawa *et al*, 1997). In the mouse kidney, VDIR transcript levels were not altered at all, which suggested that unlike the 1α(OH)ase gene, the VDIR gene was not under the transcriptional control of VDR (Figure 2D).

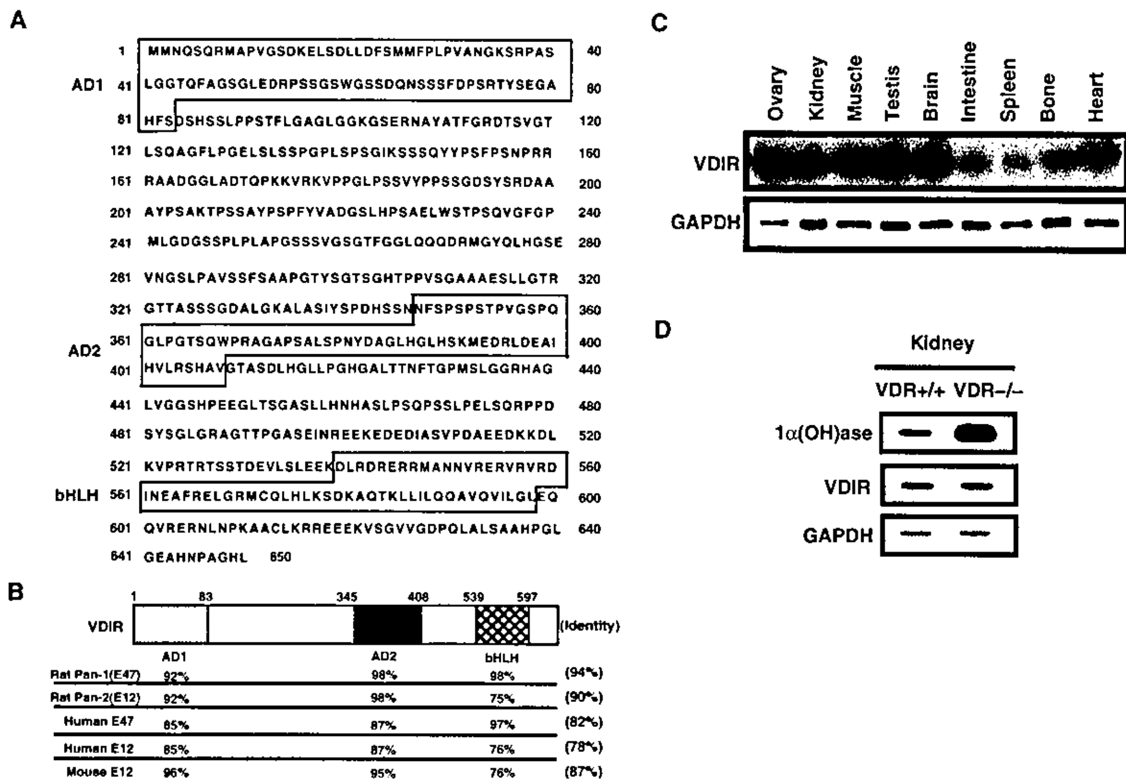

Figure 2 Cloning of the 1αnVDRE-binding factor, VDIR. (**A**) Sequence of VDIR. VDIR has two transactivation domains (AD1 and AD2), and a bHLH motif. (**B**) Functional domain sequence homology between VDIR and members of the bHLH-type activator family (rat Pan-1, E47; rat Pan-2, E12; human E47; human E12; mouse E12). VDIR exhibits a high homology with rat Pan-1 (E47). (**C**) Analysis of VDIR mRNA expression in various tissues. Northern blotting analysis was performed using VDIR open reading frame as a probe. GAPDH was used as an internal control. (**D**) 1α(OH)ase and VDIR gene expression in the kidneys of normal and VDR-deficient mice by Northern blotting. VDR$^{+/+}$: wild-type mice; VDR$^{-/-}$; VDR-deficient mice.

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VDIR is an activator for 1αnVDRE

As VDIR appeared to be a bHLH-type factor and 1αnVDRE was composed of two E-box-like motifs, we tested whether VDIR acted as a DNA sequence-specific regulator on 1αnVDRE using a transient expression assay with MCT cells (Figure 3A). To our surprise, VDIR effectively activated transcription through 1αnVDRE in a plasmid-dose-dependent manner (Figure 3A, left panel). To verify this activator function of VDIR on 1αnVDRE, we also examined other bHLH-type transcription factors, mTFE3 and hE47 (Figure 3A, left panel). hE47 belongs to a family of E2A-type bHLH transcription factors, and is thought to function as an activator, as a homodimer or a heterodimer (Murre *et al*, 1989a, b). mTFE3 is another bHLH-type family factor that binds E-box in functional association with E2A-type bHLH transcription factors (Beckmann *et al*, 1990; Ohkido *et al*, 2003). As expected, hE47 homodimer potently activated transcription of a luciferase reporter gene with 1αnVDRE, while mTFE3 exhibited no activity on 1αnVDRE. Thus, it is likely that VDIR binds, presumably as a homodimer, to 1αnVDRE and activates transcription. Supporting these findings, recombinant VDIR protein effectively bound 1αnVDRE in the absence and presence of VDR/RXR heterodimer. Moreover, while the presence of VDR/RXR heterodimer induced a further band-shift of VDIR, it appeared not to modify VDIR DNA binding (Figure 3B, lanes 6 and 7).

Ligand-induced transrepression of VDIR activation function is mediated by the N-terminal region of VDR

We then tested whether VDR suppressed the VDIR activator function on 1αnVDRE in a ligand-dependent manner (Figure 3C). VDR clearly and potently suppressed VDIR-mediated transcription only in the presence of 1α,25(OH)$_2$D$_3$, while marked ligand-induced transrepression was observed when transcription was activated by VDIR (Figure 3C, lane 6). These findings suggested that liganded VDR-mediated transrepression did not occur in response to basal transcription of the 1α(OH)ase gene, but rather significantly operated only when promoter function was potentiated by active regulators, such as PTH/PTHrP.

The VDR region responsible for ligand-induced VDIR transrepression was mapped using several VDR deletion mutants in a transient expression assay (Figure 3C, middle

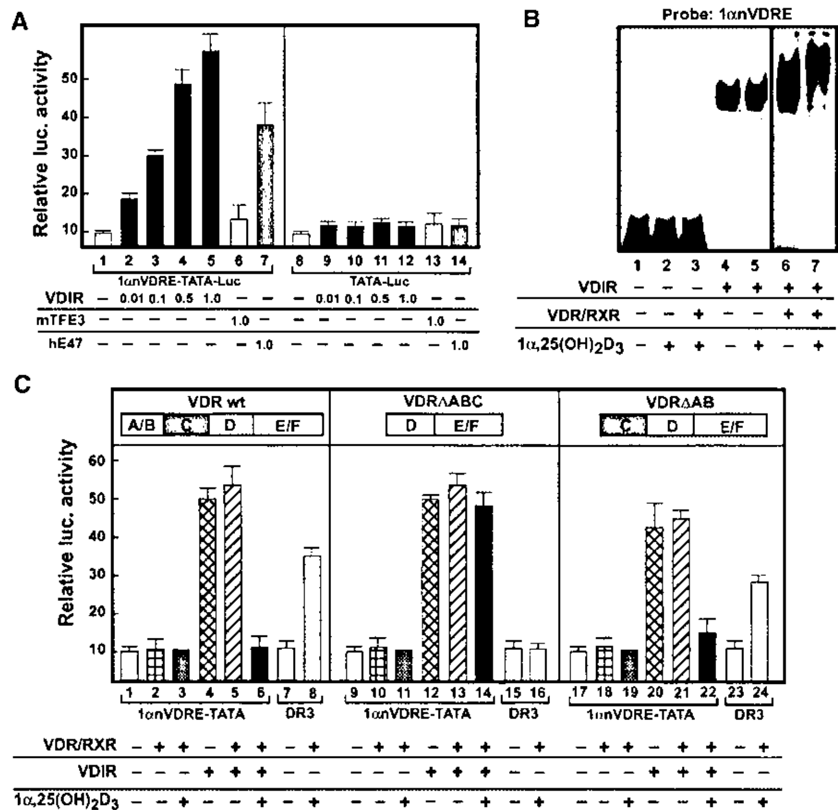

Figure 3 VDIR as an activator for 1αnVDRE. (**A**) Plasmid dose dependency of VDIR activation of nVDRE. Luciferase activity under the control of 1αnVDRE after the transfection of VDIR, mTFE3 or hE47 into MCT cells. MCT cells were cotransfected with LUC reporter plasmid (0.3 μg of nVDRE pGL3 TATA-LUC vector), rat VDR, rat RXR expression vector (0.1 μg of pSG5-rat VDR, pSG5-rat RXR), mTFE3(1.0 μg of pcDNA3-mTFE3), hE47 (1.0 μg of pcDNA3-hE47) and increasing amounts of pcDNA3-VDIR (0.01–1.0 μg). Empty vector (pcDNA3) was used to keep the total DNA concentration the same. LUC activity is represented as fold induction. Values are mean±s.d. (**B**) Gel mobility shift assay using bacterially expressed recombinant VDIR, VDR and RXR proteins together with a radiolabeled probe containing 1αnVDRE. The closed arrow indicates VDIR, and the open arrow indicates supershift of the VDR/RXR-VDIR complex. (**C**) Luciferase activity under the control of 1αnVDRE in MCT cells. Wild-type and mutated VDR, RXR, VDIR and 1α,25(OH)$_2$D$_3$ (1×10^{-8} M) were added as indicated. DR3-Luc was used as a positive control for VDR/RXR and 1α,25(OH)$_2$D$_3$. VDR wt: wild-type VDR; VDR ΔABC and VDRΔAB: VDR mutants with deleted N-terminal A–C and AB domains, respectively.

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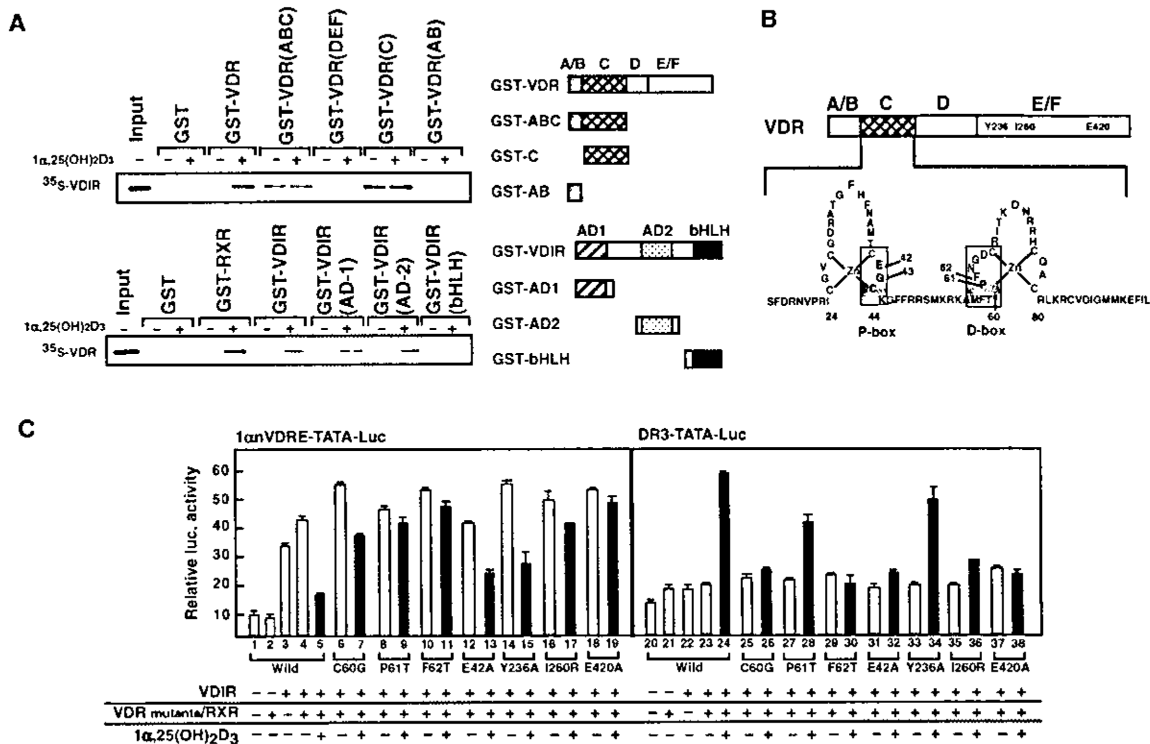

Figure 4 The DNA-binding domain (C-domain) of VDR leeds to the binding of VDIR. (**A**) GST pull-down assay using either GST alone, GST wild-type VDR or GST-fused VDRs deletion mutants together with [^{35}S]-labeled VDIR in the presence or absence of 1α,25(OH)$_2$D$_3$ (1×10^{-6} M) (upper panel). GST pull-down assay was observed using either GST alone, GST wild-type VDIR or GST-variant VDIRs together with [^{35}S]-labeled VDR in the presence or absence of 1α,25(OH)$_2$D$_3$ (1×10^{-6} M) (lower panel). Right panel: Schematic diagrams of wild-type and variant VDR or VDIR proteins. The specific residues present in each VDR or VDIR variant are indicated. (**B**) Schematic diagram of wild-type VDR and the structure of VDR DNA-binding domain. The P-box is located in the bottom of the first Zn finger, and the D-box is located in the second Zn finger. Amino-acid residues indicating shadow replaced into alanine or threonine residues, which inhibit DNA binding (E42A, P61T and F62T). Y236A and E420A mutants lack co-activator-binding activity. I260R (isoleucine→arginine) mutant lacks heterodimerization of VDR and RXR. (**C**) Transrepression of VDIR via VDR mutants in luc assay. Luciferase activities were tested in either 1αnVDRE or DR3 after co-transfection of either wild-type VDR or point mutant VDRs into MCT cells in the presence or absence of 1α,25(OH)$_2$D$_3$ (1×10^{-8} M). This experiment is representative of five independent experiments performed.

and right panels). As expected from the ligand dependency results, ligand-induced transrepression was abolished in mutants that lacked ligand-binding activity (data not shown). A VDR mutant with deleted N-terminal A–C domain was found to be inactive (Figure 3C), although that with a deleted N-terminal A/B domain mutant was active. These data indicate that the C domain of VDR is critically important for ligand-induced VDIR transrepression.

To verify the ligand-induced association between VDR and VDIR, GST pull-down assay with VDR deletion mutants fused to GST protein was performed to detect interactions with full-length VDIR (Figure 4A). The interaction of VDIR with wild-type VDR was dependent on 1α,25(OH)$_2$D$_3$ binding, and only the VDR C domain exhibited clear but ligand-independent interaction with VDIR (Figure 4A, upper panel). Although the VDR DEF domain appeared not to serve as a direct interface for VDR on its own, the DEF domain may contribute to ligand-induced interactions with VDIR through intramolecular associations with the VDR C domain, perhaps altering its structure to make it more accessible for VDIR. In the VDIR molecule, both transactivation domains (AD1 and AD2), which were mapped by generating fusion mutants with GAL4 DNA-binding domain (data not shown), appeared to associate with liganded VDR, while the bHLH domain C-terminal DNA-binding domain showed no interaction with VDR (Figure 4A, lower panel).

To map more precisely the contact site of VDR with VDIR, a series of point mutations were introduced into VDR (Figure 4B). As expected from the ligand-induced interaction between VDIR and VDR, the C-terminal AF-2 core domain appeared to be essential, and its functional state faithfully reflected the level of ligand-induced transactivation or transrepression exhibited by the point mutants (Figure 4C). The E420A mutant, which is lost in co-regulator recruitment but retains its heterodimerization activity for RXR (Kraichely *et al*, 1999), exhibited neither positive nor negative response to 1α,25(OH)$_2$D$_3$ in transcription (Figure 4C, lanes 18, 19, 37 and 38). Another mutant (Y236A), which lacks co-activator-binding activity (Jurutka *et al*, 1997), retained the activity of ligand-induced transrepression, but not transactivation (Figure 4C, lanes 14, 15, 33 and 34). However, the 1α,25(OH)$_2$D$_3$-induced transrepression was undetectable in a mutant (I260R) lacking heterodimerization (Figure 4C, lanes 17 and 36). Thus, these results suggested that heterodimerization with RXR is critical for ligand-induced transrepression.

The replacement of a glutamic acid residue with alanine at amino-acid position 42 (E42A) in the P-box at the base of the

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first Zn finger in the DNA domain abolished ligand-induced transactivation of VDR (Figure 4C, compare lane 31 with 32). This result was in agreement with previous findings that the P-box is critical for the recognition and direct binding of specific DNA elements by cognate nuclear receptors (Schena *et al*, 1989). Interestingly, ligand-induced transrepression was still retained in this mutant (Figure 4C, lane 13), which suggested that no specific VDRE binding of VDR was required for ligand-induced transrepression. However, both ligand-induced transactivation and transrepression were abolished when an alanine replaced phenylalanine at position 62 residue, part of the D-box of the DNA-binding domain (Jakacka *et al*, 2001) (Figure 4C, lanes 11 and 30). Thus, together with the observation that VDR does not bind directly to 1αnVDRE (Figure 1C), it is likely that the structure of the VDR DNA-binding domain, particularly the second Zn-finger motif, is critical for ligand-induced interaction and presumably the transrepression of VDIR.

Phosphorylation of VDIR by PKA induced p300 co-activator recruitment

As VDIR acted as an activator on 1αnVDRE, we presumed that VDIR mediated the positive effects of PTH/PTHrP on 1α(OH)ase gene expression through downstream PKA signaling (Henry, 1985; Brenza *et al*, 1998). Indeed, expression of the PKA catalytic subunit α (PKAα) potentiated VDIR transactivation function (Figure 5A). This potentiation by PKAα was likely to have involved association with the p300 co-activator, initially identified as a PKAα-regulated co-activator (Chrivia *et al*, 1993), as synergistic potentiation of combined p300 and PKAα was observed (Figure 5A).

Then, to test whether PKAα phosphorylation was linked to p300 recruitment to VDIR, we characterized potential PKAα phosphorylation sites in the VDIR. A series of alanine point mutations that prevented PKAα phosphorylation were introduced into the putative phosphorylation sites (only three representative mutations are displayed). A significant reduction in the

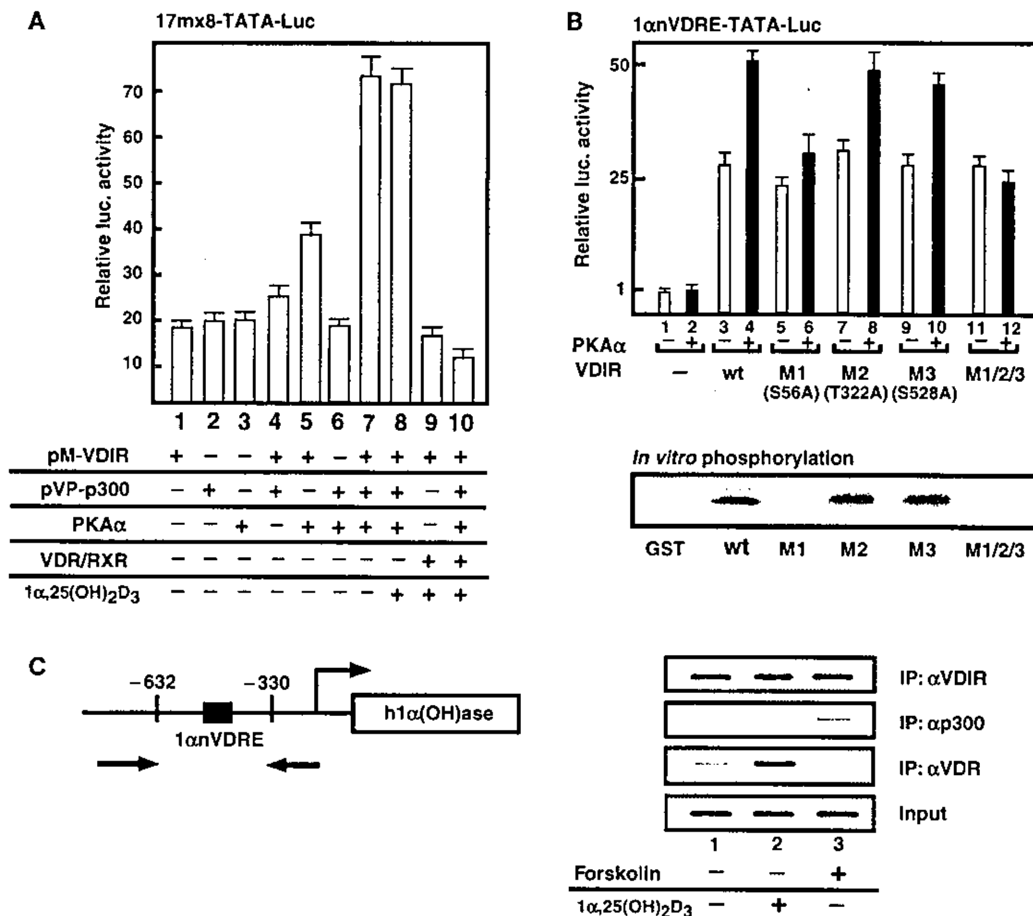

Figure 5 Phosphorylation of VDIR by PKA induced a p300 co-activator recruitment. (**A**) Association of VDIR and p300 in the mammalian two-hybrid assay. The expression plasmids of fusion proteins with GAL4-DBD (pM) and VP16-AD (pVP) were transiently transfected into MCT cells with a GAL4-DBD-regulated 17mer × 8 TATA luciferase reporter. PKAα or VDR/RXR was co-transfected in the absence or presence of 1α,25(OH)$_2$D$_3$ (1×10^{-8} M) as indicated. (**B**) Phosphorylation of VDIR by PKAα. Luciferase activity of either wild-type VDIR or its point mutants of potential PKAα phosphorylation residue to alanine was tested on 1αnVDRE with or without PKAα in MCT cells. S56A (M1), T322A (M2) and S528A (M3) were replaced alanine residue, respectively. M1/M2/M3 mutant was indicated to replace alanine residues to all of S56, T322 and S528 amino residues. In the lower panel, the *in vitro* phosphorylation of the VDIR mutants fused with GST by PKAα is shown by *in vitro* phosphorylation assay. (**C**) ChIP assays demonstrate co-localization of VDIR and p300 in MCF7 cells. In the left schematic diagram, the 1αnVDRE-contained region amplified by PCR in ChIP assays is illustrated. Antibodies used in each assay are indicated on the right panel.

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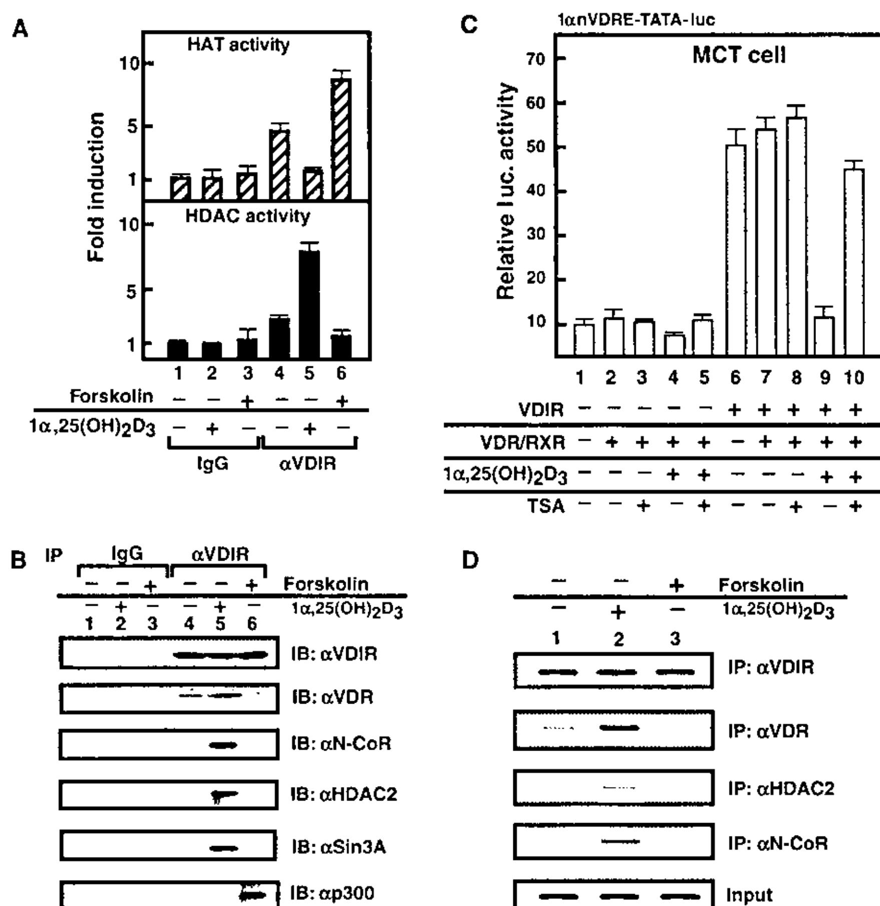

Figure 6 Co-regulator switching upon VDIR for the ligand-induced transrepression by VDR. (A) HAT and HDAC activities of the immunoprecipitated VDIR complexes in the MCT cells. Assays were determined in MCT cells after treatment, in the absence or presence of $1\alpha,25(OH)_2D_3$ and forskolin. Representative graphs corresponding to means±s.d. for triplicate independent experiments are shown. (B) Forskolin-dependent interaction between p300 and VDIR, and $1\alpha,25(OH)_2D_3$-dependent interaction between HDAC complex and VDIR. Western blotting of the immunoprecipitates with α-VDIR, α-VDR, α-NCoR, α-HDAC2 and α-Sin3A antibodies. (C) Effects of HDAC inhibitor TSA on repression by $1\alpha,25(OH)_2D_3$. Transfections were performed in the presence of TSA (3 mM) in MCT cells. TSA reduced $1\alpha,25(OH)_2D_3$-dependent transrepression. (D) Co-localization of VDIR complex components on 1αnVDRE in ChIP assay. Soluble chromatin was prepared from MCT cells treated with $1\alpha,25(OH)_2D_3$ (1×10^{-8} M) for 45 min and immunoprecipitated with the indicated antibodies.

potentiation of VDIR function by PKAα was found for a mutation at the Ser^{56} residue (Figure 5B, lane 6 in the upper panel), which supported the hypothesis that phosphorylation of serine residues by PKAα enhanced the association of VDIR with p300/CBP, which then potentiated transcription. Reflecting this PKAα-mediated potentiation, PKAα phosphorylation of the VDIR mutant (S56A) *in vitro* was significantly impaired (Figure 5B, lower panel). Furthermore, to test whether PKAα induced p300 recruitment to the VDIR activation region in endogenous gene promoters, ChIP analysis was performed using the human 1α(OH)ase gene promoter region containing 1αnVDRE in MCT cells (Figure 5C). VDIR appeared to be present at 1αnVDRE, while p300 was clearly recruited after forskolin treatment (Figure 5C). The p300 recruitment to VDIR upon the forskolin treatment was also detected in the VDIR immunoprecipitant (Figure 6B).

Ligand-induced transrepression of VDIR by VDR coupled with p300 HAT dissociation and HDAC association

To gain an insight into the ligand-induced VDR transrepression of VDIR function, we examined whether co-repressor complexes associated with VDIR via ligand-induced interaction with VDR (Takeyama *et al*, 1999), thereby suppressing transcription, and whether p300 co-activators disassociated from VDIR upon interaction with liganded VDR. Measurement of HAT and HDAC activities in VDIR immunoprecipitates showed that the highest HAT activity was detected when PKA signaling was induced by forskolin treatment (Figure 6A, upper panel, lane 6). $1\alpha,25(OH)_2D_3$ treatment markedly reduced HAT activity, which was reflected by the dissociation of p300 and the acquisition of HDAC activity (Figure 6A). Treatment with TSA, an HDAC inhibitor (Yoshida *et al*, 1990), abrogated $1\alpha,25(OH)_2D_3$-induced transrepression by VDIR/VDR (Figure 6C), which confirmed the HDAC recruitment. The putative p300/HDAC switching mechanism was further supported by results obtained using VDIR immunoprecipitants (Figure 6B). Moreover, several major HDAC co-repressor components, including N-CoR, HDAC2 and Sin3A, were co-immunoprecipitated with VDIR in a $1\alpha,25(OH)_2D_3$-dependent manner (Figure 6B), and were recruited to the 1α(OH)ase promoter

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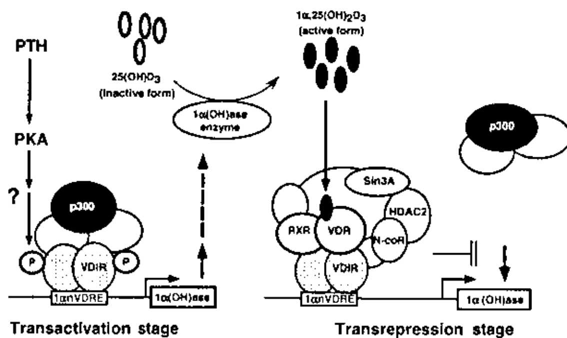

Figure 7 Schematic illustration of the proposed molecular mechanism of $1\alpha,25(OH)_2D_3$-induced transrepression in the 1α-hydroxylase gene promoter. Upon activated-PKA signaling due to PTH, the 1α-hydroxylase gene is transactivated through recruitment of a HAT co-activator complex to VDIR bound to 1αnVDRE, leading to increased serum concentrations of $1\alpha,25(OH)_2D_3$. $1\alpha,25(OH)_2D_3$ binding to VDR induces association with VDIR, and leads to the dissociation of the HAT co-activator complex, and the recruitment of an HDAC co-repressor complex. This results in ligand-induced transrepression of the 1α(OH)ase gene due to co-regulator switching on VDIR.

as shown by ChIP analysis (Figure 6D). Thus, our findings showed the $1\alpha,25(OH)_2D_3$-dependent switching of co-regulators via VDIR, such that the HDAC co-repressor complex recruited by liganded VDR led to the dissociation of p300 from VDR–VDIR complexes (Figure 7).

Discussion

Identification of a novel nVDRE in the human 1α(OH)ase gene promoter

The 1α(OH)ase gene is one of the best-characterized VDR target genes (Haussler *et al*, 1998). While the VDR target genes are distinguished by being negatively regulated by liganded VDR, regulation of 1α(OH)ase gene expression is more complicated as it is also regulated by PKA signaling activated by liganded PTH/PTHrP receptor (Henry, 1985; Brenza *et al*, 1998; Panda *et al*, 2001). We previously showed that 1α(OH)ase gene expression was highly upregulated in VDR KO mice (Takeyama *et al*, 1997; Murayama *et al*, 1998), similar to hereditary type II rickets patients who suffer from VDR malfunction (Kitanaka *et al*, 1999). Hence, in the present study, we mapped and characterized an nVDRE (1αnVDRE) in the human 1α(OH)ase gene promoter. Our results showed that the identified nVDRE conferred a positive responsiveness to activated-PKA signaling, and that this element appeared to act downstream of PTH/PTHrP. Distinct from the previously reported nVDREs (Demay *et al*, 1992; Falzon 1996), 1αnVDRE contained no AGGTCA-like core motif, present in the binding core elements of many NRs including VDR (Mangelsdorf *et al*, 1995; Ebihara *et al*, 1996; Haussler *et al*, 1998). Instead, 1αnVDRE was composed of two E-box-like motifs. Moreover, no DNA sequences similar to the reported DR3-like nVDREs were present in the entire promoter region, up to 5 kb upstream, in both the human and mouse 1α(OH)ase genes (M Kim, unpublished results). Reflecting the sequence attributes of 1αnVDRE, no direct binding of VDR/RXR heterodimers to the mapped sequence was detected, in contrast to the previously reported nVDREs that readily bind VDR/RXR heterodimers (Demay *et al*, 1992; Falzon 1996). However, EMSA analysis showed that an unknown nuclear factor appeared to bind effectively to 1αnVDRE.

Cloning and characterization of a novel bHLH-type activator as a 1αnVDRE-binding factor

To identify the 1αnVDRE-binding factor, a yeast one-hybrid assay was performed using an MCT cell line cDNA library. This led to the identification of a factor designated VDIR that exhibits motif organization typical of E2A-type activators, including N-terminal transactivation domains (AD) and a C-terminal bHLH-type DNA-binding domain. VDIR appeared to be the mouse homolog of hE47 as the two molecules shared 97% amino-acid sequence identity. Like hE47 (Murre *et al*, 1989a, b; Beckmann *et al*, 1990), VDIR appeared to bind as a homodimer to 1αnVDRE, as determined by EMSA assay using recombinant VDIR. It has been reported that hE47-type transcriptional factors, which are widely expressed, can both homodimerize and heterodimerize with tissue specific-type bHLH proteins, and be responsible for the biological activity of these proteins *in vivo* (Davis *et al*, 1990; Lassar *et al*, 1991). Therefore, we cannot exclude the possibility that an unidentified factor may form a heterodimer with VDIR for more stable DNA binding.

As expected from the VDIR amino-acid sequence and the two E-box-like motifs in 1αnVDRE, VDIR effectively activated transcription via 1αnVDRE binding. 1αnVDRE served as an enhancer, and its function was potentiated through PKA signaling, that is activated by the PTH/PTHrP cell membrane receptors (Henry, 1985). We further found that VDIR was phosphorylated *in vitro* by PKA at several phosphorylation sites in the transactivation domains. A series of point mutations identified the Ser^{58} residue as a significant PKA phosphorylation site, such that phosphorylation of Ser^{58} appeared to be a prerequisite for the PKA-induced transactivation function of VDIR. Thus, VDIR appeared to act as an activator downstream of PKA, and may be responsible, at least in part, for the role of PTH/PTHrP in 1α(OH)ase gene induction.

Ligand-induced transrepression by VDR is mediated via direct binding of VDIR to 1αnVDRE

While ligand-induced transrepression by VDR via 1αnVDRE was detected in the absence of exogenous VDIR expression, it was relatively of low level. However, ligand-induced transrepression by VDR was more evident when transcription was augmented by activated-PKA signaling. Likewise, when higher basal promoter activity was achieved by replacing the intact basal 1α(OH)ase promoter with the much stronger tk promoter, ligand-induced VDR transrepression was much more evident. Supporting these findings, ligand-induced association between VDR and VDIR was detected at the human 1α(OH)ase gene promoter by ChIP analysis (Kitagawa *et al*, 2003). This association was further supported by findings *in vivo* and *in vitro* by nuclear co-immunoprecipitation and GST pull-down assays, respectively.

Modulation of the transactivation function of one activator class by another activator class through their direct association has already been described (McNamara *et al*, 2001; Xu *et al*, 2001). As observed in this study, the ligand-induced association of some nuclear receptors with bHLH-type activators has been shown to either potentiate or suppress the transactivation function of the bHLH activators. Recently, McNamara *et al* reported that nuclear retinoid receptors

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(RARα and RXRγ) suppressed the transactivation function of CLOCK and MOP4, bHLH-type activators, in a ligand-dependent manner, blocking CLOCK/MOP4-mediated gene expression. Further detailed analysis revealed that ligand-induced association of RAR/RXR prevented CLOCK and MOP4 from binding their DNA targets, resulting in suppressed retinoid activity in the CLOCK/MOP4-mediated gene cascade. Like the interaction between VDR and VDIR, the C-terminal AF-2 core motif of RAR/RXR is required for ligand-induced association. However, unlike the VDIR AD domain, the DNA-binding bHLH domains in MOP4 appear to be involved in direct interaction. This discrepancy in the functional domains in terms of interaction with nuclear receptors is hardly surprising due to the completely distinct motif organization between MOP4/CLOCK and VDIR irrespective of the fact that they belong to the same class of bHLH-type activators. This difference may also explain the different modes of nuclear receptor suppressive function on gene expression, as liganded VDR had no inhibitory effect on VDIR DNA binding.

Co-regulator switching in ligand-induced transrepression by VDR

Thus, the present study revealed a novel mechanism of ligand-induced transrepression by nuclear receptors based on co-regulator switching rather than preventing DNA binding of another activator class. The transactivation function of VDIR appeared to require p300 co-activator, presumably as part of a HAT complex (Glass and Rosenfeld, 2000). The functional and physical association of p300 with VDIR was potentiated via the PKA-mediated phosphorylation of several serine residues in the VDIR AD1 domain. This may explain, at least in part, the induction of the 1α(OH)ase gene by the PKA-mediated PTH/PTHrP upregulation, although it is likely from previous reports that there may be other positive regulatory element(s) in the gene promoter (Brenza *et al*, 1998). Interestingly, the association between p300 and VDIR was abrogated by the ligand-induced association of VDR along with major co-repressor complex components. Thus, VDR appeared to be highly effective in switching HAT co-activator complexes to HDAC co-repressor complexes in a ligand-dependent manner upon binding of VDIR to 1αnVDRE, as illustrated in Figure 7. This hypothesis was verified by the finding of both HAT and HDAC activities in immunoprecipitated VDIR complexes. Together, these findings clearly show that co-regulator switching underlies ligand-induced transrepression by VDR.

The molecular mechanism of ligand-induced co-regulator switching involving VDIR remains to be investigated. However, it is evident from its ligand dependency that the VDR LBD plays a crucial role, although this switching is in effect opposite to that of ligand-induced transactivation accompanied by co-activator recruitment. It is presumed from our present findings that ligand-induced association with VDIR allows liganded VDR to retain co-repressor complexes without the recruitment of co-activator complexes. Such ligand-induced switching of co-repressors on VDIR is likely to be accomplished by unique ligand-induced structural alterations in VDR present, thus a unique VDR–VDIR co-repressor complex may be formed. To test this idea, purification and identification of VDR–VDIR complex components is clearly needed to uncover the molecular basis of ligand-induced transrepression by VDR.

Materials and methods

Plasmids

Transfection studies included constructs of a chimeric gene in which the human 1α(OH)ase promoter (−889/−30) and deletion mutants (−537/−30, −514/−30, −889/−537, −537/−514) were inserted into the pGL thymidine kinase (tk)–chloramphenicol acetyltransferase (CAT), and nVDRE (−537/−514) were inserted into the pGL3-Luciferase vector (Promega) driven by TATA promoter. Full-length rat VDR and rat RXR plasmid were described previously (Takeyama *et al*, 1999). Rat VDR point mutants, by PCR mutagenesis, were inserted into pcDNA3 (Invitrogen). Full-length mouse VDIR plasmids were inserted into pcDNA3. Chimeric GST proteins fused with rat VDR and mouse VDIR deletion mutant series were expressed in pGEX-4T (Pharmacia Biotech). pcDNA3-mTFE3 plasmid was kindly provided by Dr K Miyamoto (Tokshima University).

Cell culture and transient transfection assay

MCT cells were maintained in DMEM supplemented with 5% FBS (GIBCO BRL) at 37°C in 5% CO_2. For transfection, cells were plated in DMEM supplemented with 5% charcoal-stripped FBS in 12-well plates 1 day before transfection. Transfections were performed using Lipofectamin Plus (GIBCO BRL) according to the manufacturer's instructions. After 3 h, 1α,25(OH)$_2$D$_3$ (1×10^{-8} M) and/or forskolin (1×10^{-8} M) were added to the culture medium, and the cells were incubated continuously at 37°C for 24 h. CAT and Luciferase assays were performed as described previously (Murayama *et al*, 1998).

Yeast one-hybrid system

The yeast strain YM4271 (CLONETECH), transformed with the yeast expression plasmids pHISi and pLacZi (CLONETECH) containing 3 × 1αnVDRE motifs (CCCACCTGCCATCTGCC), was used to screen a yeast GAL4 activation domain fusion MCT cDNA library (a detailed procedure for the library construction is available upon request). Positive clones were selected on SD medium that lacked Leu and His, but contained 25 mM 3-amino-triazol (3AT). Surviving colonies were assayed for β-galactosidase (X-gal) activity using a colony filter lift assay and incubation in the presence of 5-bromo-4-chloro-3-indolyl β-D-galactosidase according to the manufacturer's instructions (CLONETECH). cDNA from LacZ-positive clones were sequenced across the Gal4/library cDNA and analyzed using the NCBI BLAST search tool.

Gel electrophoresis mobility shift assay

Nuclear extracts were prepared from MCT cells. Recombinant rat VDR, rat RXR proteins fused to GST, were expressed in *E. coli* and bound to glutathione–sepharose 4B beads. GST fusion proteins bound to glutathione–sepharose were cleaved by thrombin protease treatment (25 U/24 h). Double-stranded oligonucleotide DR3 (consensus VDRE, 5'-AGCTTCAGTTCAGGAAGTTCAGT-3') and human 1αnVDRE (h1αnVDRE 5'-CCATTAACCCACCTGCCATCTGCC-3') were end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (Takeyama *et al*, 1999). Reactions were performed using 0.5 μg nuclear extracts in binding buffer (10 mM Tris (pH 7.5), 75 mM KCl, 5 mM EDTA, 1 mM $MgCl_2$, 4% glycerol, 1 mM DTT, 1 μg poly dI–dC) in a final volume of 20 μl and labeled probes of 10 ng. Samples were incubated for 30 min at room temperature and resolved on 5% polyacrylamide gels run in 0.5 × TAE buffer. Gels were then dried and subjected to autoradiography (Ebihara *et al*, 1996).

Northern blotting

Northern blot analysis was performed as previously described (Takeyama *et al*, 1997). cDNA fragments of N-terminal mouse 1α hydroxylase and VDIR full-length were used as probes.

GST pull-down assay

VDIR and VDR deletion mutant proteins fused to GST were expressed in *E. coli* and bound to glutathione–sepharose 4B beads (Pharmacia Biotech). [^{35}S]methionine labeling of proteins was carried out by *in vitro* translation using a TNT-coupled transcription-translation system (Promega). GST-VDR (or GST-VDIR) was preincubated with 1α,25(OH)$_2$D$_3$ (10^{-6} M) for 15 min at room temperature. GST fusion proteins and [^{35}S]methionine-labeled proteins were then incubated in Net-N+ buffer for 2 h. After

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successive washes in Net-N+ buffer, proteins were resolved by SDS-PAGE and visualized by autoradiography (Kitagawa *et al*, 2003).

HAT/HDAC assay

Whole MCT cell lysates were immunoprecipitated with a-VDIR antibody and then incubated with or without 10 μg calf thymus histones (Sigma) and [^{3}H]-labeled acetyl CoA (4.7 Ci/mmol, Amersham) for 30 min at 30°C, spotted onto Whatman P-81 filters, and washed extensively with sodium carbonate buffer (pH 9.1). Radioactivity remaining on the filter was then quantitated by liquid scintillation counting (Yanagisawa *et al*, 2002). HDAC assays were carried out using the HDAC fluorescent activity assay kit according to the manufacturer's instructions (BIOMOL, Inc.).

Mammalian two-hybrid assay

MCT cells were co-transfected with 17mer × 8-Luc reporter plasmid, pM-VDIR and pVP-p300 with pSG5-rat VDR and pSG5-rat RXR in the presence of PKAα. After 3 h, 1α,25(OH)$_2$D$_3$ was added to the culture medium, and the cells were incubated for 24 h at 37°C. Luciferase assays were performed as described above.

In vitro kinase assay

MCT cells transfected with pcDNA3-Flag-PKAα were lysed in lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1.5 mM MgCl$_2$, 2 mM EDTA, 12.5 mM β-glycerophosphate, 10 mM NaF, 1 mM sodium vanadate, 1 mM PMSF, 1% Triton-X) with protease inhibitors (Kato *et al*, 1995). Whole cell lysate supernatants were immunoprecipitated with Anti-FLAG M2-Agarose Affinity Gel (Sigma), and washed three times in TBS buffer (20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 1 mM PMSF, 2 mM DTT, 1 mM sodium vanadate) with protease inhibitors and twice in Tris-HCl (pH 7.5) buffer. Reactions consisted of 4 μl 5 × kinase buffer (100 mM Tris-HCl (pH 7.5), 50 mM MgCl$_2$, 0.5 mM ATP), 2 μl immunoprecipitate, [γ-^{32}P]ATP and GST-VDIR in a final volume of 20 μl and were incubated for 20 min at 30°C. Reaction products were resolved by SDS-PAGE and visualized by autoradiography (Watanabe *et al*, 2001).

Immunoprecipitation

Whole cell lysate supernatants in TNE buffer (10 mM Tris-HCl (pH 7.8), 1 mM EDTA, 0.15 M NaCl, 0.1% NP-40) containing protease inhibitors were immunoprecipitated with α-VDIR antibody and then added to G-sepharose beads. After successive washes in TNE buffer, proteins were resolved by SDS-PAGE and Western blotted using α-VDR antibody (Neo Markers), α-HDAC2 antibody (ABR), α-p300 antibody (Santa Cruz Biotechnology) or α-Sin3A antibody (Santa Cruz Biotechnology) (Yanagisawa *et al*, 1999).

ChIP assay

ChIP analyses were performed using the ChIP assay kit (Upstate Biotechnology), as described previously (Kitagawa *et al*, 2003). Whole cell lysates of MCF7 cells were immunoprecipitated with antibodies against the indicated proteins. Specific primer pairs were designed (h1αp5'(632) 5'-ATTCCCATGTCTGGAAGGAG-3' and h1αp3'(−330) 5'-CAGTGAGCCCAGCCCCTTTA-3') and PCR conditions optimized to allow semiquantitative measurement. Conditions used were 25 cycles of 30 s at 90°C, 15 s at 58°C and 1 min at 72°C. PCR products were visualized on 2% agarose/TAE gels.

Acknowledgements

We thank S Kitanaka, K Unno, H Kitagawa and I Takada for helpful discussion and Chugai Pharmaceutical Co., Ltd for 1α,25(OH)$_2$D$_3$ relative compound. We are also grateful to I Ohkido and K Miyamoto (Tokushima University) for mTFE3 expression vector, and Y Nagasawa for preparation of the manuscript. A part of this research was supported by a grant-in-aid for Basic Research Activities for Innovative Biosciences (BRAIN) and priority areas from the Ministry of Educations, Science, Sports and Culture of Japan (to SK).

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0888-8809/05/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 19(1):102–112
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doi: 10.1210/me.2004-0093

Thyroid Hormone Induces Rapid Activation of Akt/Protein Kinase B-Mammalian Target of Rapamycin-$p70^{S6K}$ Cascade through Phosphatidylinositol 3-Kinase in Human Fibroblasts

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We have demonstrated that T_3 increases the expression of ZAKI-4α, an endogenous calcineurin inhibitor. In this study we characterized a T_3-dependent signaling cascade leading to ZAKI-4α expression in human skin fibroblasts. We found that T_3-dependent increase in ZAKI-4α was greatly attenuated by rapamycin, a specific inhibitor of a protein kinase, mammalian target of rapamycin (mTOR), suggesting the requirement of mTOR activation by T_3. Indeed, T_3 activated mTOR rapidly through S2448 phosphorylation, leading to the phosphorylation of $p70^{S6K}$, a substrate of mTOR. This mTOR activation is mediated through phosphatidylinositol 3-kinase (PI3K)-Akt/protein kinase B (PKB) signaling cascade because T_3 induced Akt/PKB phosphorylation more rapidly than that of mTOR, and these T_3-dependent phosphorylations were blocked by both PI3K inhibitors and by expression of a dominant negative PI3K (Δp85α). Furthermore, the association between thyroid hormone receptor β1 (TRβ1) and PI3K-regulatory subunit p85α, and the inhibition of T_3-induced PI3K activation and mTOR phosphorylation by a dominant negative TR (G345R) demonstrated the involvement of TR in this T_3 action. The liganded TR induces the activation of PI3K and Akt/PKB, leading to the nuclear translocation of the latter, which subsequently phosphorylates nuclear mTOR. The rapid activation of PI3K-Akt/PKB-mTOR-$p70^{S6K}$ cascade by T_3 provides a new molecular mechanism for thyroid hormone action. (*Molecular Endocrinology* 19: 102–112, 2005)

CALCINEURIN (Cn) WAS first purified as a calmodulin-binding protein from bovine brain (1). It is the only known serine/threonine protein phosphatase under the control of Ca^{2+}/calmodulin. The discovery that immunosuppressants, cyclosporin A and FK506, inhibit Cn activity through binding with their cognate immunophilins [cyclophilin A and FK506-binding protein (FKBP)] established Cn as a mediator involved in the regulation of multiple biological processes such as T cell activation (2), muscle hypertrophy (3), memory development (4), glucan synthesis (5), ion homeostasis (6), and cell cycle control (7).

Recently, several physiological inhibitors of Cn have been identified, including CBP1/calcipressin (8) and regulator of calcineurin 1 in yeast (9), DSCR1 (Down syndrome candidate region 1) (10), ZAKI-4 (also termed DSCR1L1 by gene nomenclature committee) (11), and DSCR1L2 (12) in human. DSCR1 homolog in hamster was identified as Adapt78 (13) and in mouse as modulatory calcineurin-interacting protein 1 (14). These proteins were shown to bind with Cn and to inhibit Cn activity *in vitro* and *in vivo*.

The endogenous Cn inhibitor ZAKI-4 has two isoforms, α and β. Both isoforms inhibit Cn activity by binding through the common C-terminal region, whereas only the expression of ZAKI-4α, but not β, responds to thyroid hormone in human skin fibroblasts (15). Regulation of gene expression by T_3 is mediated through the thyroid hormone receptor (TR), usually acting as a ligand-dependent nuclear transcription factor (16). Liganded TR binds with its cognate *cis*-element (thyroid hormone-responsive element), present in the regulatory region of target genes, and promotes their transcription. This T_3 action is often referred to as genomic action. However, we found that T_3-dependent expression of ZAKI-4α is not mediated by such genomic action, because there is no canonical thyroid hormone-responsive element in the promoter of ZAKI-4α gene, and because a protein synthesis inhibitor cycloheximide

First Published Online September 23, 2004

Abbreviations: CHX, Cycloheximide; Cn, Calcineurin; DSCR1, Down syndrome candidate region 1; 4E-BP1, eukaryotic initiation factor 4E-binding protein-1; FBS, fetal bovine serum; FKBP, FK506-binding protein; IRS, insulin receptor substrate; M.O.I., multiplicity of infection; mTOR, mammalian target of rapamycin; NP-40, Nonidet P-40; PKB, protein kinase B; PI3K, phosphatidylinositol 3-kinase; $PtdIns(3,4,5)P_3$, phosphatidylinositol-3,4,5-triphosphate; $PtdIns(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; SH2, Src homology 2; TR, thyroid hormone receptor.

***Molecular Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

(CHX) abrogated T_3-dependent ZAKI-4α expression, suggesting that *de novo* protein synthesis is required (11).

On the other hand, it was reported that expression of regulator of calcineurin 1, yeast homolog of ZAKI-4, was increased by intracellular Ca^{2+} mobilization, and that this increase was sensitive to FK506 (9). More recently, it was reported that forced expression of Cn in the heart of transgenic mice increased expression of modulatory calcineurin-interacting protein 1 (17). These findings suggest a common mechanism regulating the expression of endogenous Cn inhibitors through activation of Cn.

We therefore investigated a possible involvement of Cn in T_3-dependent expression of ZAKI-4α by utilizing a Cn inhibitor FK506 and its structural analog rapamycin, which specifically prevents the activation of a protein kinase, mammalian target of rapamycin (mTOR). Unexpectedly, rapamycin, but not FK506, greatly attenuated T_3-induced ZAKI-4α expression. This finding implicated mTOR in the control of ZAKI-4α expression by T_3.

mTOR is a member of the phosphatidylinositol kinase-related protein kinase family. Its carboxyl-terminal region is highly homologous to lipid kinases. However, evidence supports a role for mTOR as a serine/threonine protein kinase (18). It was discovered biochemically, based on its binding properties to FKBP-rapamycin complex (19). In mammals the complex inhibits mTOR through interaction with the FKBP-rapamycin binding domain in mTOR (20). The minimal FKBP-rapamycin binding domain spans residues 2025–2114 and lies N-terminal to the catalytic kinase domain. mTOR mediates signaling in response to nutrients and growth factors, such as insulin and IGF-I, and controls the mammalian translation initiation machinery through activation of the $p70^{S6K}$ protein kinase (p70 ribosomal S6 kinase) and through inhibition of the eukaryotic initiation factor 4E inhibitor, 4E-BP1 (eukaryotic initiation factor 4E-binding protein-1) (21, 22). For mTOR activation, phosphorylation of two serine residues, S2448 and S2481, located in the C-terminal repressor domain, seems to be important. Deletion of this domain renders the mutant constitutively active (23). Because S2481 phosphorylation does not occur in a kinase-inactive mTOR, this site is considered to be autophosphorylated (24). Rapamycin was shown to have no effect on S2481 phosphorylation. In contrast, three stimuli, insulin, amino acids, and muscle loading, have been demonstrated to induce S2448 phosphorylation and to activate mTOR kinase (25, 26). However, significance of this phosphorylation remains to be established because substitution of S2448 with alanine was reported not to affect mTOR kinase activity (23).

In the present study we demonstrate, for the first time, a novel nongenomic action of T_3 that phosphorylates S2448 of mTOR and activates the kinase rapidly. This T_3 action is initiated by activation of phosphatidylinositol 3-kinase (PI3K)-Akt/protein kinase B (PKB) signaling by liganded TR, leading to an increased expression of ZAKI-4α.

RESULTS AND DISCUSSION

T_3-Dependent ZAKI-4α Expression Is Sensitive to Rapamycin

Human skin fibroblasts cultured in a medium containing T_3-depleted serum were incubated with T_3 for 12 h in the presence or absence of a Cn inhibitor, FK506, or its analog, rapamycin (Fig. 1A). As we previously reported, ZAKI-4α mRNA was increased by physiological dose of T_3. Unexpectedly, pretreatment with FK506 had no effect on the T_3-dependent induction, whereas rapamycin attenuated T_3-dependent ZAKI-4α expression. Because both FK506 and rapamycin require FKBP for their function, the effect of rapamycin warrants the presence of FKBP in human fibroblasts, and lack of FK506 effect could not be due to the absence of FKBP. These results therefore demonstrate that T_3-dependent ZAKI-4α expression is mediated by activation of mTOR but not by Cn signaling. The unexpected effect of rapamycin prompted us to investigate the T_3-dependent signaling cascade leading to mTOR activation because rapamycin-sensitive signaling pathway has not been described in the T_3 action.

T_3 Induces mTOR Activation and Its S2448 Phosphorylation

We investigated whether T_3 induces mTOR activation by phosphorylating S2448. Activation of mTOR was

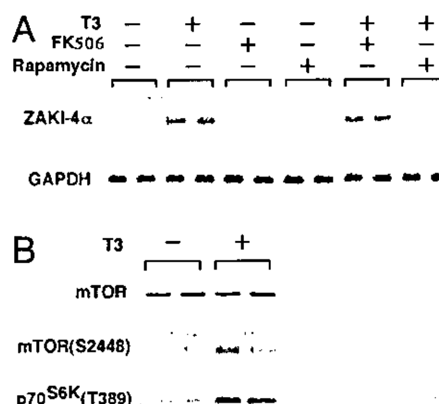

Fig. 1. T_3 Induces ZAKI-4α Expression through Activating mTOR

Human skin fibroblasts were cultured in DMEM with 5% T_3-depleted FBS for 2 d and were treated with T_3 (10^{-8} M) for 12 h. FK506 (1 μM) and rapamycin (1 μM) were added 30 min before T_3 addition. Total RNAs were subjected to Northern blot analysis and whole-cell lysates to Western blot analysis. Panel A shows the image of the Northern blot hybridized with ZAKI-4α-specific probe. Glyceraldehyde-3 phosphate-dehydrogenase (GAPDH) was used as an internal control. Panel B shows T_3-dependent activation of mTOR and $p70^{S6K}$.

ascertained by phosphorylation of $p70^{S6K}$ at T389, because it was established that mTOR is an upstream kinase for $p70^{S6K}$, phosphorylating the residue in a rapamycin-sensitive manner (27).

We first studied the effect of 12 h exposure to T_3 on S2448 phosphorylation of mTOR to match the exposure time for Northern blot analysis (Fig. 1A). As shown in Fig. 1B, an increase in S2448 phosphorylation was detected after 12 h exposure to T_3. Note that total mTOR protein levels were not affected by T_3. The phosphorylation is associated with a marked increase in T389 phosphorylation of $p70^{S6K}$. These results demonstrate T_3-dependent phosphorylation of S2448 activates mTOR kinase.

T_3 Induces Rapid and Persistent Activation of mTOR in a CHX-Insensitive Manner

We next examined the earlier time course of T_3 effect on mTOR activation. As shown in Fig. 2, A and B, S2448 phosphorylation of mTOR was detected as early as 10 min after T_3 addition, followed by a peak level at 25 min and preservation of the phosphorylated form up to 12 h, whereas total protein levels were not altered. This phosphorylation is correlated with T389 phosphorylation of $p70^{S6K}$, which started 10 min after T_3 treatment and gradually increased up to 25 min. These results confirmed the rapid and sustained activation of mTOR by T_3. This T_3 action did not require *de novo* protein synthesis, because treatment with a protein synthesis inhibitor CHX did not affect S2448 phosphorylation of mTOR even after 30 min exposure of T_3 (Fig. 2C). Rapid and CHX-insensitive activation of mTOR strongly suggests that this T_3 action is nongenomic, implying that transcriptional activation by TR is not required.

T_3-Dependent Activation of mTOR Is Mediated by PI3K-Akt/PKB Signaling Pathway

We next examined the possible involvement of PI3K-Akt/PKB signaling pathway in T_3-dependent activation of mTOR, because it is known that insulin activates mTOR through this pathway (23). Binding of insulin to the membrane receptor results in the phosphorylation of its receptor and insulin receptor substrate (IRS) proteins. These proteins then interact with Src homology 2 (SH2) domain-containing proteins such as p85α, the regulatory subunit of PI3K (28). PI3K is subsequently recruited to the membrane where it converts lipid phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2], to phosphatidylinositol-3,4,5-triphosphate [PtdIns(3,4,5)P_3]. The synthesis of PtdIns(3,4,5)P_3 recruits the proteins possessing pleckstrin homology domain, such as Akt/PKB and phosphoinositide-dependent protein kinase 1, from the cytoplasm to the plasma membrane. Then, phosphorylation and activation of Akt/PKB occur near the plasma membrane. Phosphorylations of T308 and S473 in Akt/PKB are required for its full activation

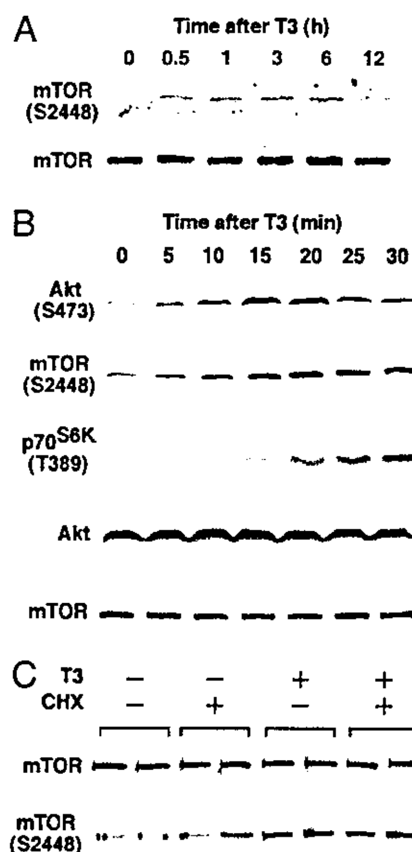

Fig. 2. T_3 Induces Phosphorylation of mTOR and $p70^{S6K}$ Rapidly in a CHX-Insensitive Manner

Human skin fibroblasts were cultured in DMEM with 5% T_3-depleted FBS for 2 d. They were then treated with T_3 (10^{-8} M) and harvested at intervals (A and B) for Western blot analysis. In panel C, CHX (10 μg/ml) was added 30 min before T_3 treatment, and cells were harvested 30 min after T_3 addition.

(29). Phosphorylation of the former site is directly catalyzed by phosphoinositide-dependent protein kinase 1. However, a kinase responsible for the latter phosphorylation has not yet been identified.

The involvement of Akt/PKB in T_3-dependent S2448 phosphorylation of mTOR was determined by Western blot analysis using a specific antibody against Akt/PKB phosphorylated at S473. As shown in Fig. 2B, S473 phosphorylation of Akt/PKB was induced by T_3 as early as 5 min, whereas total amount of Akt/PKB was not altered by T_3. Note that this S473 phosphorylation precedes S2448 phosphorylation of mTOR, suggesting that T_3-dependent mTOR activation is mediated by Akt/PKB.

The involvement of PI3K was examined using PI3K-specific inhibitors, wortmannin and LY294002. Human skin fibroblasts cultured in the medium containing T_3-depleted serum were exposed to T_3 for 30 min in the presence or absence of wortmannin and LY294002. As shown in Fig. 3A, T_3-dependent S473 phosphorylation of

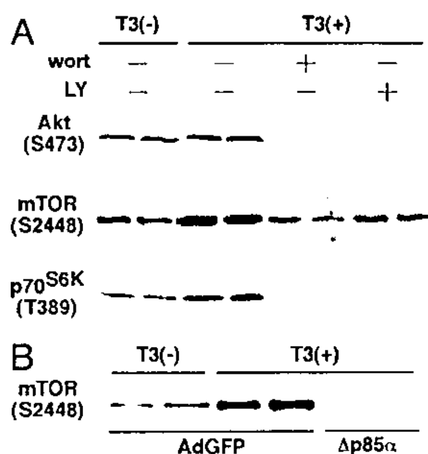

Fig. 3. Inhibition of PI3K Abrogates T_3-Dependent Activation of mTOR

A, Cells incubated in DMEM with 5% T_3-depleted FBS for 2 d were treated with T_3 (10^{-8} M) for 30 min. PI3K inhibitors, wortmannin (wort, 2 μM) and LY294002 (LY, 50 μM), were added 30 min before T_3 addition. Whole-cell extracts were subjected to Western blot analysis. B, Adenoviruses expressing either green fluorescent protein (AdGFP, Δp85α minus) or Δp85α were infected at a M.O.I of 200 for 1 h. They were then incubated in DMEM with 5% T_3-depleted FBS for 2 d and treated with T_3 (10^{-8} M) for 30 min.

Akt/PKB, S2448 phosphorylation of mTOR, and T389 phosphorylation of $p70^{S6K}$ were all inhibited by wortmannin and by LY294002. These results suggest that PI3K mediates T_3-dependent activation of Akt/PKB, mTOR, and $p70^{S6K}$. More importantly it indicates that T_3-dependent PI3K activation is an initial event leading to the activation of Akt/PKB, mTOR, and $p70^{S6K}$.

However, Brunn *et al.* (30) reported that both wortmannin and LY294002 are able to inhibit mTOR directly. To confirm the contribution of PI3K to T_3-dependent activation of mTOR, a mutant p85 subunit of PI3K (Δp85α) lacking an interacting domain with p110 was overexpressed. Δp85α has been shown to inhibit insulin-dependent PI3K activation (31). Its inhibitory action is explained by the disruption of interaction between IRS and p85/p110 PI3K due to the predominant association of Δp85α with IRS.

Human skin fibroblasts infected with a recombinant adenovirus expressing Δp85α were exposed to T_3 for 30 min. As shown in Fig. 3B, overexpression of Δp85α abolished T_3-dependent S2448 phosphorylation of mTOR. These results clearly demonstrate the role of PI3K in T_3-mediated mTOR activation and indicate the presence of a certain protein(s) associating with Δp85α is involved in T_3 action. The most likely candidate to mediate the action is TR.

TRβ1 Complexes with p85α Subunit of PI3K in a Ligand-Independent Manner

TR belongs to a steroid hormone receptor superfamily also including estrogen receptor, glucocorticoid receptor, progesterone receptor, retinoic acid receptor and so on. The genomic actions of these receptors have been well demonstrated. Recently, their nongenomic actions, outside the nucleus, were also identified. For example, estrogen and retinoic acid activate PI3K rapidly through the nontranscriptional action of their receptors (32–35). Furthermore, estrogen receptor-α was demonstrated to activate PI3K through binding with p85α either in a ligand-dependent manner in endothelial cells (32) or a ligand-independent manner in epithelial cells (33).

We therefore investigated whether TR interacts with p85α using recombinant adenoviruses expressing wild-type TRβ1 or its dominant negative mutant TR G345R. This mutant has been shown to lack T_3-binding property, but to preserve dimerization property with wild-type TR and thereby inhibit its transactivation function in a dominant negative manner (36). The cells with overexpression of either wild-type TRβ1 or TR G345R were treated with T_3 for 30 min. The cell lysate was then immunoprecipitated with anti-p85α or anti-TRβ1 antibody and probed with the antibodies to each other. As shown in Fig. 4A, immunoprecipitation with anti-p85α antibody resulted in pulling down of

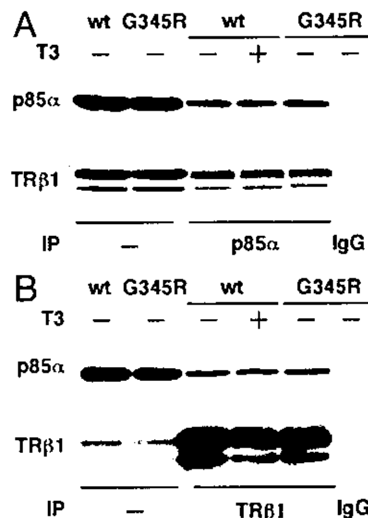

Fig. 4. TRβ1 Complexes with p85α in a Ligand-Independent Manner

Human skin fibroblasts were incubated in serum-free DMEM containing recombinant adenoviruses expressing wild-type TRβ1 (wt) or TR G345R (G345R) at a M.O.I. of 200 for 1 h. They were then cultured in the medium with 5% T_3-depleted FBS for 2 d and treated with T_3 (10^{-8} M) for 30 min. Whole-cell extracts were immunoprecipitated with anti-p85α antibody (panel A) or anti-TRβ1 antibody (panel B) and then subjected to immunoblot analysis probed with antibodies to each other. A goat antimouse IgG immobilized on CNBr-activated Sepharose 4 Fast Flow was used to show the nonspecific binding, and the samples without immunoprecipitation were used to confirm the expression of each protein. Our recombinant adenovirus of wild-type TRβ1 (wt) or TR G345R (G345R) produces two bands at molecular mass of 52 and 49 kDa because of the alternative initiation.

TRβ1 in the presence or absence of T_3. In addition, TR G345R without T_3-binding ability also associates with p85α. As shown in Fig. 4B, immunoprecipitation with anti-TRβ1 antibody confirmed that TRβ1 forms complex with p85α in a ligand-independent manner. Two bands were detected for TRβ1 and TR G345R because of alternative initiation.

p85α has been shown to bind with several proteins through its functional domains including SH2, Rho-GAP, and SH3 domain, resulting in subsequent activation of PI3K. Binding of insulin receptor and IRS to SH2 domain (37, 38) or binding of proline-rich protein to SH3 domain (39, 40) or binding of Rho family protein such as Rac and Cdc42 to Rho-GAP domain (41, 42) leads to a stimulation of PI3K activity. Insulin receptor and IRS bind to SH2 domain due to their tyrosine phosphorylation (37, 38). Although TR was shown to be a phosphoprotein, tyrosine phosphorylation was not reported (43, 44). It is thus unlikely that p85α binds with TRβ1 through SH2 domain. It is noteworthy that Rho-GAP domain contains three repeats of LXXLL motif (Swiss-Prot accession no. Q63787), which is considered as a sequence interacting with nuclear receptor directly and found in many nuclear receptor-associating proteins, such as steroid receptor coactivator-1/p160, transcriptional intermediary factor-2/glucocorticoid receptor interacting protein-1, and CBP/p300 (45). It is thus possible that Rho-GAP domain of p85α might comprise a surface for a direct interaction with TRβ1, although we could not exclude a possible involvement of SH3 domain. The interaction between TRβ1 and p85α might take place in the cytosol or near plasma membrane, because the presence of TR in the cytoplasm has been shown even though its amount is less than that in the nucleus (44, 46). Further studies are required to substantiate this speculation.

Activation of PI3K Requires T_3 Binding with TRβ1-p85α

Demonstration of T_3-independent complex formation between TRβ1 and p85α and T_3-dependent phosphorylation of Akt/PKB and mTOR suggested that T_3 binding with TRβ1 is required for the activation of PI3K. To confirm this hypothesis, PI3K activity in the cells with or without overexpression of wild-type TRβ1 or TR G345R was determined by competitive ELISA. As shown in Fig. 5A, overexpression of wild-type TRβ1 alone did not increase PI3K activity. Further treatment of these cells with T_3 resulted in a similar degree of T_3-dependent induction of PI3K activity as in the cells infected with control adenovirus (AdGFP), suggesting that a sufficient amount of TR is expressed in human skin fibroblasts. When the cells were infected with the virus expressing TR G345R, T_3-dependent activation of PI3K could not be detected, demonstrating the involvement of liganded TRβ1 in PI3K activation. These results are compatible with a report by Simoncini *et al.* (32). They showed the presence of PI3K

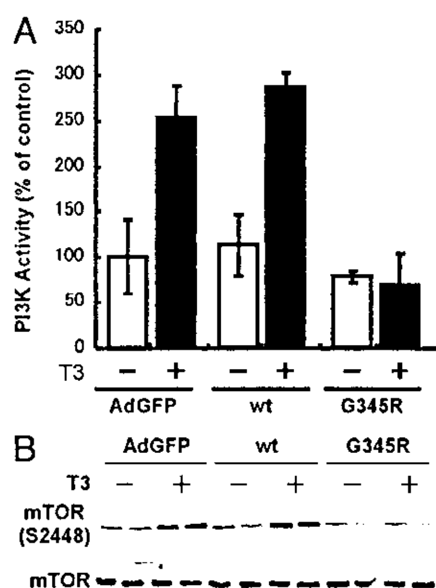

Fig. 5. Expression of a Dominant Negative TR (G345R) Abrogates T_3-Induced Activation of PI3K and mTOR

A, The cell treatment was same as described in Fig. 4 except for control cells, which were infected with AdGFP. Equal amount of proteins (500 μg) were immunoprecipitated with anti-p85α antibody at 4 C overnight. The PI3K activity of each precipitate was then measured by ELISA and expressed as the relative production of $PI(3,4,5)P_3$ by each sample. $PI(3,4,5)P_3$ produced by control cells was set as 100, and others were compared with the control. The assay was done in triplicate. B, The whole-cell extracts were subjected to immunoblot analysis probed with antiphospho-mTOR (S2448) antibody.

activity in the anti-TR antibody immunoprecipitate prepared from T_3-treated human endothelial cells, whereas little activity was observed in cells not treated with T_3.

In concordance with T_3-dependent PI3K activation, S2448 phosphorylation of mTOR was also similarly regulated (Fig. 5B). T_3-dependent mTOR phosphorylation was not further enhanced by overexpressing wild-type TRβ1, whereas that of TR G345R inhibited it. The inhibition by TR G345R is not due to squelching of T_3, because it does not bind T_3. Because both wild-type TRβ1 and TR G345R form a complex with p85α, as shown in Fig. 4, it indicates that mutant TR competes with endogenous TR to bind with p85α, and this complex fails to activate PI3K due to the lack of T_3 binding activity.

T_3 Promotes Nuclear Translocation of S473-Phosphorylated Akt/PKB

A variety of nuclear proteins have been shown to be substrates for Akt/PKB (47–49). However, a limited number of reports have shown the nuclear translocation of Akt/PKB (50, 51). We therefore investigated T_3 effects on subcellular localization of Akt/PKB and

mTOR. Human skin fibroblasts maintained in serum-free medium were treated with T_3, and nuclear and cytosolic fractions were subjected to Western blot analysis, using antibody directed against Akt/PKB, not against phosphorylated Akt/PKB. As shown in Fig. 6A, Akt/PKB was detected in nuclear fractions as early as 5 min after T_3, and the amount gradually increased. Wortmannin prevented this nuclear accumulation. In contrast, cytosolic Akt/PKB gradually decreased after T_3 treatment. The total amounts of nuclear and cytosolic Akt/PKB appear not to be altered during T_3 treatment, in agreement with the result shown in Fig. 2B. These results clearly demonstrate that T_3 induces nuclear translocation of Akt/PKB in a PI3K-dependent

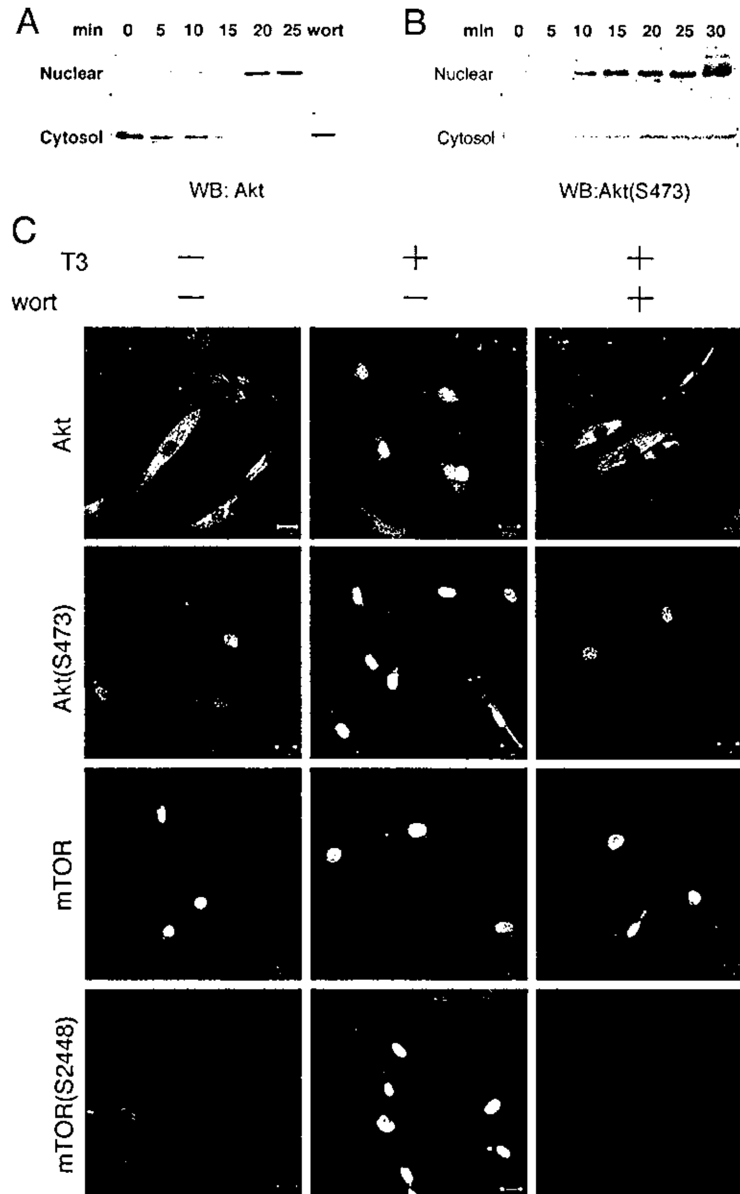

Fig. 6. T_3 Induces Activation of Akt/PKB and Its Nuclear Migration

A, Nuclear migration of Akt/PKB activated by T_3. After incubation of the fibroblasts in serum-free DMEM for 1 d, they were treated with T_3 and harvested at intervals. Wortmannin (wort, 100 nM) was added 30 min before T_3. Nuclear and cytosolic fractions were subjected to Western blot (WB) analysis using a polyclonal antibody against Akt/PKB. B, An antibody against phospho-Akt (S473) was used. C, Localization of Akt/PKB after T_3 treatment. Human skin fibroblasts were incubated in serum-free DMEM for 1 d and stimulated with T_3 (10^{-10} M) for 30 min. Wortmannin (wort, 100 nM) was added 1 h before T_3 addition. Fibroblasts were then fixed and incubated with the rabbit polyclonal antibodies against Akt, phospho-Akt (S473), mTOR, and phospho-mTOR (S2448) at 4 C overnight. The cells were subsequently incubated with Alexa Fluor 488-coupled secondary antibody. The representative laser confocal scanning images were shown at a magnification of ×400.

manner. The translocated Akt/PKB is likely to be a S473-phosphorylated, active form, because the time course of appearance of Akt/PKB in nuclear fractions is similar to that of S473 phosphorylation shown by Western blot analysis using whole-cell lysates (Fig. 2B). In accordance with these results, Western blot analysis using the antibody against S473-phosphorylated Akt/PKB revealed phosphorylated Akt/PKB in the nucleus was markedly increased by T_3, whereas in cytoplasm, the increase was moderate (Fig. 6B). The different degree of increase of phospho-Akt/PKB in nucleus and cytoplasm is likely due to nuclear translocation of newly phosphorylated Akt/PKB.

To further clarify the intracellular localization of Akt/PKB and mTOR, we performed immunocytochemical analysis (Fig. 6C). The serum-starved cells were treated with T_3 for 30 min. In unstimulated cells, Akt/PKB was detected mainly in the cytoplasm, and Akt/PKB phosphorylated at S473 was barely detected in the nucleus. T_3 treatment resulted in marked translocation of Akt/PKB into the nucleus. This was concordant with an increase in the phosphorylated Akt/PKB in the nucleus. T_3-dependent phosphorylation and nuclear translocation of Akt/PKB were abrogated by wortmannin. These observations confirm T_3- and PI3K-dependent phosphorylation and nuclear translocation of Akt/PKB.

In contrast, both mTOR and S2448-phosphorylated mTOR were detected only in the nucleus. S2448-phosphorylated mTOR was hardly detected in the unstimulated cells. T_3 markedly increased the amount of the phosphorylated mTOR in the nucleus, and this was inhibited by wortmannin. However, there was no change in total mTOR (Fig. 2 and Fig. 6C). The result strongly suggests that Akt/PKB-dependent activation of mTOR occurs in the nucleus. Nuclear localization of mTOR was also reported by Zhang *et al.* (52). They showed that mTOR is predominantly present in the nucleus of human fibroblasts as well as several cell lines, with human embryonic kidney 293 cells being the only exception. The nuclear mTOR might be an active form, because they used cells cultured with serum not depleted of T_3. However, the predominant existence of activated mTOR in the nucleus appears curious, because $p70^{S6K}$ and 4E-BP1, the best characterized downstream targets of mTOR, are present mainly in the cytoplasm. It was shown recently that mTOR is a cytoplasmic-nuclear shuttling protein, and that inhibition of nuclear export of mTOR reduces $p70^{S6K}$ and 4E-BP1 activation (53). Therefore, a part of activated mTOR might be exported from the nucleus and activate $p70^{S6K}$ in the cytoplasm after T_3 treatment.

T_3-Mediated ZAKI-4α Regulation Is Initiated by the Sequential Activation of PI3K-Akt/PKB-mTOR Cascade

Although it has been shown that PI3K, Akt/PKB, and mTOR can be independently activated by a variety of stimuli, in this study we show that T_3 activates Akt/PKB, mTOR, and $p70^{S6K}$ only through PI3K, because the activation of the former three kinases is prevented by PI3K inhibitors, wortmannin and LY294002, and by $\Delta p85\alpha$. In addition, time course studies demonstrated sequential phosphorylations of serine and threonine residues of Akt/PKB, mTOR, and $p70^{S6K}$, which are critical for their kinase activity. These findings clearly indicate that the activation of these kinases by T_3 is not a parallel, but hierarchical event, which involves PI3K, Akt/PKB, and mTOR kinase cascade. The present study also demonstrates that activation of mTOR is required for T_3-dependent ZAKI-4α expression. To confirm the involvement of PI3K-Akt/PKB cascade, the effects of PI3K inhibitor LY294002 and the dominant negative mutant $\Delta p85\alpha$ on T_3-induced ZAKI-4α expression were examined by Northern blot analysis. Human skin fibroblasts cultured in the medium containing T_3-depleted serum were incubated with T_3 for 12 h in the presence or absence of LY294002 or with adenovirus expressing $\Delta p85\alpha$. As shown in Fig. 7, A and B, both LY294002 and $\Delta p85\alpha$ abrogated T_3-induced ZAKI-4α expression, demonstrating the involvement of PI3K-Akt/PKB.

The PI3K-Akt/PKB and mTOR signaling pathways may regulate ZAKI-4α expression by controlling downstream effectors such as $p70^{S6K}$. However, CHX-sensitive regulation of ZAKI-4α by T_3 suggested requirement of *de novo* protein synthesis (11), and thus the downstream effectors could not regulate ZAKI-4α expression directly. The physiological target of $p70^{S6K}$ is the 40S subunit of the S6 ribosomal

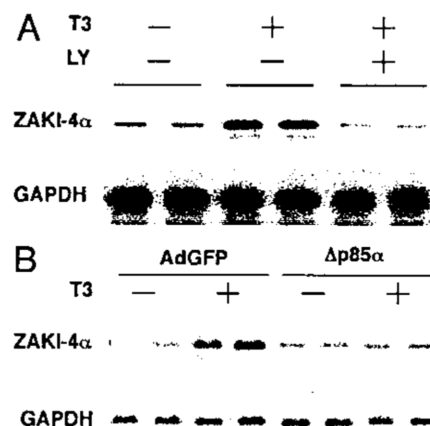

Fig. 7. Inhibition of T_3-Dependent ZAKI-4α Expression by PI3K Inhibitor and $\Delta p85\alpha$

A, Cells were incubated in DMEM with 5% T_3-depeleted FBS for 2 d and treated with T_3 (10^{-8} M) for 12 h. PI3K inhibitor LY294002 (LY, 50 μM) was added 30 min before T_3 addition. B, Adenovirus expressing either green fluorescent protein (AdGFP, $\Delta p85\alpha$ minus) or $\Delta p85\alpha$ was infected at a M.O.I of 200 for 1 h. Fibroblasts were then incubated in DMEM with 5% T_3-depleted FBS for 2 d and treated with T_3 (10^{-8} M) for 12 h. Total RNAs were subjected to Northern blot analysis and hybridized with ZAKI-4α-specific probe.

protein. Phosphorylation of S6 subsequently initiates translation of 5′-terminal oligopyrimidine mRNAs, which encode components of the translational machinery such as elongation factors, ribosomal proteins, and poly(A)-binding protein and thus plays a key role in modulating translational efficiency (27). A certain protein(s) under the control of such mTOR-$p70^{S6K}$-initiated translational machinery might be involved in ZAKI-4α expression. A search for such protein(s) will elucidate the mechanism of T_3-mediated ZAKI-4α regulation.

It is noteworthy that T_3 inhibits endogenous Cn (protein phosphatase 2B) through regulating ZAKI-4α (15). The present study demonstrating T_3-dependent activation of mTOR raises a possibility that T_3 may also regulate protein phosphatase 2A because activated mTOR has been shown to phosphorylate protein phosphatase 2A and to prevent the dephosphorylation of 4E-BP1 and $p70^{S6K}$ (54).

T_3 Possibly Modulates Divergent Cellular Functions through Regulation of PI3K-Akt/PKB-mTOR-$p70^{S6K}$ Cascade

Thyroid hormone plays important roles in growth, development, metabolism, and differentiation. These functions were considered to be mediated mainly by nuclear TR, which regulates the transcription of target genes after T_3 binding. On the other hand, nongenomic action of thyroid hormone has recently been recognized at the molecular levels. It was suggested to control Ca^{2+} entry, intracellular protein trafficking, and regulation of protein kinase C, MAPKs, and cytoskeleton (55). Involvement of PI3K in the nongenomic action was also suggested by using PI3K inhibitors. They inhibited thyroid hormone-dependent activation of Na^+/H^+ exchange and amino acid transport in chick embryo hepatocytes (56) and activation of certain class of voltage-gated potassium channel in a rat pituitary cell line (57). The present study demonstrates, for the first time, a novel nongenomic action of T_3 that requires TRβ1 bound to p85α and causes a rapid activation of PI3K-Akt/PKB-mTOR-$p70^{S6K}$ cascade in human skin fibroblasts.

Activation of this cascade has been shown to be crucial in multiple biological processes such as cell growth (58), neuronal cell survival (59), glucose uptake (60), and cardiac hypertrophy (61). Ubiquitous distribution of TR suggests that the nongenomic action of T_3 mediated by this cascade may modulate different cellular functions cooperatively with genomic actions.

MATERIALS AND METHODS

Antibodies and Reagents

The following rabbit polyclonal antibodies were used: anti-Akt and antiphospho-Akt (S473), anti-mTOR and antiphospho-mTOR (S2448), and antiphospho-$p70^{S6K}$ (T389). An immunohistochemistry-specific antiphospho-Akt (S473) was used for staining Akt (S473). All the antibodies were purchased from Cell Signaling Technology (Beverly, MA). Monoclonal antibody specific to TRβ1 isoform was purchased from Affinity BioReagents (Golden, CO). A part of the antibody was immobilized on CNBr-activated Sepharose 4 Fast Flow (Amersham Biosciences Corp., Piscataway, NJ) according to the manufacturer's instructions and used for immunoprecipitation. An immobilized anti-p85α antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used for immunoprecipitation, whereas a monoclonal anti-p85α antibody (BD Biosciences, San Jose, CA) was used for immunoblot analysis. Rabbit polyclonal antibody against mTOR (Santa Cruz Biotechnology) was used for immunocytochemistry. Enhanced chemiluminescence detection reagents were obtained from Pierce Biotechnology, Inc. (Rockford, IL). Wortmannin, LY294002, rapamycin and CHX were purchased from Sigma Chemical Co. (St. Louis, MO). FK506 is a gift from Fujisawa Pharmaceutical Co. (Tokyo, Japan).

Recombinant Adenoviruses

Constructions of recombinant viruses expressing wild-type TRβ1 and a mutant TRβ1 (TR G345R), exhibiting a strong dominant negative action *in vitro* and *in vivo*, were described previously (62). TR G345R was identified in a family with resistance to thyroid hormone (63). A recombinant adenovirus expressing green fluorescent protein (AdGFP) was constructed using AdEasy System and was used to determine the suitable multiplicity of infection (M.O.I.). The virus expressing a dominant negative form of p85α-regulatory subunit of PI3K (Δp85α) was a gift from Dr. Kasuga (31). Substitution of residues 479–513 with Ser and Arg abolished the binding to the p110 catalytic subunit.

Cell Cultures and Treatments

The source of human skin fibroblasts and their growth conditions have been described previously (15). Adenoviral vectors were delivered to cells grown to 90% confluence by incubation for 1 h in serum-free DMEM. Preliminary experiments revealed that nearly 100% of cells could be infected at a M.O.I. of 200. They were then incubated in the medium with 5% T_3-depleted fetal bovine serum (FBS) for 2 d. The inhibitors such as FK506 (1 μM), rapamycin (1 μM), wortmannin (2 μM or 100 nM), LY294002 (50 μM), and CHX (10 μg/ml) were added 30 min before treatment with T_3 (10^{-8} M). Cells were harvested at intervals for determination of mRNA levels, Western blot analysis, or immunoprecipitation.

Northern Blot Analysis

RNA extraction and the construction of specific probes for ZAKI-4α and GAPDH (glyceraldehyde-3 phosphate-dehydrogenase) were described previously (15). Images were analyzed by BAS2000 bioimage analysis system (Fuji Photo Film Co., Tokyo, Japan). The experiments were performed in duplicate dishes and repeated at least three times.

Western Blot Analysis

The nuclear, cytosol, or whole-cell extracts were separated by 7.5% SDS-PAGE. Equal amounts of protein per lane were loaded and transferred onto polyvinylidene difluoride membrane (Amersham Biosciences Corp.). The blots were probed with the antibodies described above (diluted in Tris-buffered saline, 0.1% Tween 20 with 5% (wt/vol) skim milk at 1:1,000) followed by incubation with horseradish peroxidase-conjugated antirabbit IgG (1:2500 diluted). The protein was then visualized by enhanced chemiluminescence detection reagents.

Immunoprecipitation

Human skin fibroblasts were lysed in the buffer containing 50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40 (NP-40), 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM Na_3VO_4, and 1 mM NaF. The lysate was then centrifuged at 4 C for 15 min at 14,000 × *g*, and the supernatant was incubated with immobilized anti-p85α antibody or anti-TRβ1 antibody. The precipitated proteins were detected by immunoblot analysis with anti-p85α or with anti-TRβ1 antibody.

PI3K Assay

Cell culture and adenovirus infection are the same as described above. PI3K activity was measured using an ELISA kit (Echelon Biosciences, Inc., Salt Lake City, UT) according to the supplier's protocol. In brief, the cells were rinsed with buffer A [137 mM NaCl, 20 mM Tris-HCl (pH 7.4), 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.1 mM sodium orthovanadate] and harvested in lysis buffer (buffer A plus 1% NP-40 and 1 mM phenylmethylsulfonyl fluoride). After centrifugation at 4 C for 15 min at 13,000 × *g*, protein concentrations of the supernatants were measured, and equal amounts (500 μg) were incubated with immobilized anti-p85α antibody overnight. Immune complexes were washed three times with buffer A plus 1% NP-40; three times with buffer containing 100 mM Tris-HCl (pH 7.4), 5 mM LiCl, and 0.1 mM sodium orthovanadate; and twice with buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 0.1 mM sodium orthovanadate. The complexes were then incubated with a reaction mixture containing PtdIns(4,5)P_2 substrate and ATP. The reaction mixtures were first incubated 3 h later with antibody to PtdIns(3,4,5)P_3 and then added to the PtdIns(3,4,5)P_3-coated microplate for competitive binding. A peroxidase-linked secondary antibody and colorimetric detection are used to detect anti-PtdIns(3,4,5)P_3 binding to the plate. The colorimetric signal is inversely proportional to the amount of PtdIns(3,4,5)P_3 produced by activated PI3K. Finally, the PtdIns(3,4,5)P_3 production was calculated according to the standard curve. PtdIns(3,4,5)P_3 produced by the control cells without T_3 treatment and infected with AdGFP was set as 100, and those of others were compared with control cells.

Immunocytochemistry

Human skin fibroblasts, incubated in serum-free DMEM for 24 h, were treated with wortmannin (100 nM) and 1 h later were stimulated with T_3 (10^{-10} M) for 30 min. Samples were washed twice in cold PBS and fixed with freshly prepared 4% paraformaldehyde (30 min at room temperature) and permeabilized with 0.3% Triton X-100 in PBS (10 min at room temperature). Nonspecific binding of the antibodies was blocked with 3% BSA before incubation with the antibodies (diluted 1:100 in blocking solution) at 4 C overnight. The cells were subsequently incubated with 1000-fold diluted Alexa Fluor 488-conjugated antirabbit IgG (Molecular Probes, Inc., Eugene, OR). Laser confocal scanning images were obtained using a Zeiss LSM510 Laser Scan Microscope (Carl Zeiss, Tokyo, Japan) under the same conditions.

Acknowledgments

Received March 3, 2004. Accepted September 14, 2004.

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This work was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology (Monbukagakusho) of Japan (nos. 13470217 and 16390269) (to H.S.); by the 21st Century COE program "Integrated Molecular Medicine for Neuronal and Neoplastic Disorders" of Monbukagakusho (to H.S. and X.C.); and by National Institutes of Health Grant DK15070 (to S.R.). L.C.M. is a recipient of a grant (Mo 1018/1-1) from the Deutsche Forschungsgemeinschaft (DFG).

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