Thyroid hormone receptors (TRs) are critical regulators of growth, differentiation, and homeostasis. TRs function by regulating the expression of thyroid hormone (T3) target genes in both ligand-dependent and -independent pathways. Distinct classes of co-regulatory proteins modulate these two pathways. We show here a novel role of cyclin D1 as a T3-independent co-repressor for TRs. Cyclin D1 interacted with TR in vitro and in cells in a ligand-independent manner. Cyclin D1 acted to repress both the silencing activity of the unliganded TR and the transcriptional activity of the liganded TR. The repression was not due to the inhibition of the binding of TR to the thyroid hormone response element but by serving as a ligand-independent bridging factor to selectively recruit HDAC3 to form ternary complexes. The repression was augmented by increasing the expression of HDAC3 but not by HDAC1 and was alleviated by trichostatin A. Thus, cyclin D1 is a novel ligand-independent co-repressor that opens a new paradigm to understand the molecular basis of the silencing action of TR.

Thyroid hormone receptors (TRs) belong to a steroid hormone/retinoic acid nuclear receptor superfamily that plays an important role in cell proliferation, differentiation, and homeostasis (1, 2). Four different thyroid hormone (T3) binding TR isoforms deriving from alternative splicing of two separate genes are identified. They are expressed in tissue-dependent and developmentally regulated fashion (1, 2). TR functions not only as a ligand-dependent transcription factor in the presence of ligand but also acts as a constitutive repressor for their target genes in the absence of ligand. A network of associated proteins identified as co-repressors and co-activators has been shown to facilitate TR in gene regulation. One mechanism for the action of co-activator proteins has been shown to involve acetylation/deacetylation of the lysine residues on the amino terminus of the core histones of chromatin. Several co-activators such as steroid hormone receptor co-activator (SRC) family members pCBP/300 and p/CAF have been shown to harbor intrinsic histone acetyltransferase activity (3–5). In contrast, co-repressors such as N-CoR and SMRT have been shown to be associated with histone deacetylases (HDAC) and mediate the transcriptional silencing by TR (6–9).

Dynamic changes in the state of acetylation of core histones, which are controlled by the opposing actions of histone acetyltransferases and HDACs, lead to transcriptional activation or repression of genes (10). Hyperacetylation of the histone tail relaxes the chromatin structure and leads to transcriptional activation. Conversely, deacetylation condenses chromatin and results in gene repression. In humans and mice, nine HDACs have been cloned and identified to date. They are divided into two distinct classes based on their size and sequence homology to yeast HDACs. The class I enzymes such as HDAC1 (11), HDAC2 (12), HDAC3 (13, 14), and HDAC8 (15) consist of 400–500 amino acids and are homologous to yeast Rpd3. In contrast, class II HDACs, including HDAC4, HDAC5, HDAC6 (16), HDAC7 (17), and HDAC9 (18), are large in size (~1000 amino acids) and possess domains similar to the deacetylase domain of yeast Hda1. Different HDACs are recruited by different transcriptional regulators to regulate the expression of different genes (16, 17, 19, 20).

Various cyclin-dependent kinases are sequentially activated during cell cycle progression. During the transition of cell cycle, the activity of these kinase complexes is activated by specific cyclins and inactivated by cyclin-dependent kinase inhibitors. Cyclin Ds complexing with CDK4 or CDK6 are strong key regulators of progression through the G1 phase of the cell cycle (21, 22). Recently, it has been reported that cyclin D1 also harbors activities that are independent of its role as a cyclin D kinase regulatory subunit (23). Cyclin D1 was found to activate the estrogen receptor (ER)-mediated transcription in breast tumors by direct binding to the hormone binding domain of the ER and by enhancing the binding of ER to their target genes (24). It was further demonstrated that cyclin D1 interacts with members of the SRC family and p/CAF through a motif that is similar to the co-activator binding domain of nuclear receptors in a ligand-independent manner (25, 26). It is suggested that cyclin D1 serves as a bridging factor to recruit SRC and p/CAF co-activators to ER, thereby facilitating the acetylation of histones and promoting the ER-mediated transcription (25, 26).

Previously we demonstrated that T3 stimulates the proliferation of growth hormone producing GC cells (27). The stimulatory effect is mainly due to a shortening of G0/G1 phase. The shortening in G0/G1 phase correlated with T3-induced increases in the mRNA and protein levels of two key regulators of G1 progression, cyclins D1 and E (27), suggesting a potential direct or indirect functional link of these cyclins with TRs. Because ER and TR share many co-regulatory proteins in their...
signaling pathways (28), we sought to understand whether cyclin D1 also acted to stimulate the transcriptional activity of TRs. We found that indeed, cyclin D1 physically interacted with the hormone binding domain of TR. However, unexpectedly we discovered that in contrast to ER, this interaction led to a repression of both T3-dependent and -independent transcriptional activities of TRs. The repression was mediated by cyclin D1-dependent recruitment of HDAC3 to cyclin D1-TR complexes. These findings expand the role of cyclin D1 in transcriptional regulation. Significantly, we study highlights a novel regulatory pathway in mediating both T3-dependent and -independent repression of the transcriptional activity of TRs.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents**—CV-1, GC, and MCF7 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS), 50 μg/ml penicillin, 0.25 μg/ml streptomycin, and 2 mM l-glutamine at 37 °C under 5% CO2. T3-depleted FBS was prepared by incubating FBS with 20 mg/ml activated charcoal and 50 mg/ml anion exchange resin (AGX-8, Bio-Rad) for 24 h before centrifugation and ultrafiltration. T3 (Sigma) was dissolved in 0.1 ml NaOH. Trichostatin A (TSA) was obtained from Sigma and dissolved in dimethyl sulfoxide. Both reagents were stored at −20 °C and diluted immediately before use.

**Plasmids**—The cyclin D1 expression vectors, pCMV-cyclin D1, pCMV-D1-HA, and pGST-cyclin D1 were generous gifts from M. E. Ewen. The reporter pTK-Pal-Luc contains two copies of palindrome TR response element (TRE) in tandem and a luciferase gene under the control of thymidine kinase promoter (a gift from J. L. Jameson). The region −320 to −110 relative to the TATA box was cloned into a modified pBluecript plasmid to create a reporter plasmid driving luciferase expression downstream of the palindrome TR response element (TRE) in tandem and a luciferase gene under the control of thymidine kinase promoter (a gift from J. L. Jameson). Ewen. The reporter pTK-Pal-Luc contains two copies of palindrome TR response element (TRE) in tandem and a luciferase gene under the control of thymidine kinase promoter (a gift from J. L. Jameson). The region −320 to −110 relative to the TATA box was cloned into a modified pBluecript plasmid to create a reporter plasmid driving luciferase expression downstream of the TATA box. Cells were transfected using FuGENE 6 (Roche Molecular Biochemicals) and incubated with 1 μg of TR expression vector (pCLC51) and 200 ng of cell lysate was incubated with anti-HA (Santa Cruz Biotechnology, Inc.) or anti-FLAG M2 (Sigma). The double-stranded oligonucleotide containing the Lys-TRE was labeled with [32P]dCTP similarly as described by Zhu et al. (32). About 0.2 ng of probe (3–5 × 105 cpm) was added to the binding buffer (25 mM HEPES pH 8.0, 2 mM MgCl2, 0.01 mM ZnCl2, 5 mM dithiothreitol, 6% glycerol, 0.01% Triton X-100) and in vitro translated proteins, and 0.2 μg of sheared salmon sperm DNA. Binding reactions were carried out at room temperature for 30 min, and complexes were resolved on 6% polyacrylamide gels in 0.5× TBE (45 mM Tris-HCl, 45 mM boric acid, 0.5 mM EDTA) at 150 V for 2 h. After drying of the gel, the DNA-bound proteins were detected by autoradiography. 

**RESULTS**

**Cyclin D1 Binds Specifically to TRβ1**—We utilized the GST/glutathione (GSH) binding system to assess whether TRβ1 physically interacted with cyclin D1. GST alone was used as a negative control. Fig. 1A shows that TRβ1 bound to GST-D1 in the absence (lane 3) or presence of T3 (lane 4), but not GST (lanes 1 and 2), indicating the specific physical interaction of TRβ1 with cyclin D1. To map the region of TRβ1 to which cyclin D1 bound, we prepared 35S-labeled truncated TRβ1 in which domains were systematically deleted (Fig. 1B). Deletion of domain A/B (lanes 5 and 6, Fig. 1A), domains A/B+C (lanes 7 and 8, Fig. 1A), or domains A/B+C+D (lanes 9 and 10, Fig. 1A) did not significantly affect the binding of cyclin D1 to TRβ1 regardless of whether T3 was present or not. These results indicate that domain E was the essential binding region for cyclin D1 and that this physical interaction was independent of T3.

To demonstrate the interaction of cyclin D1 with TRβ1 in vivo, CV1 cells were transfected with expression vector of TRβ1 or cyclin D1 tagged with hemagglutinin (D1-HA) or transfected with both expression vectors. Fig. 1C shows the results of such experiments from cells cultured in the absence of T3. Western blot analysis of cell lysates using mAbC4 (31) detected the expression of TRβ1 (lanes 1 and 3, Fig. 1C, a) but not in cells transfected only with D1-HA expression vector (lane 2). When cell lysates were first immunoprecipitated with anti-HA antibodies followed by Western blot analysis using mAbC4, TRβ1 was detected in cells transfected with TRβ1 and D1-HA as shown in lane 6 (Fig. 1C, a) but not in cells transfected with either TRβ1 (lane 4) or D1-HA alone (lane 5), indicating an association of TRβ1 with cyclin D1 in vivo.

The association of TRβ1 with cyclin D1 in vivo was further demonstrated by a reverse experiment in which cell lysates were first immunoprecipitated with anti-TRβ1 (mAbC4) followed by Western blot analysis using anti-HA. As shown in lane 6 of Fig. 1C, b, cyclin D1-HA was detected in cells transfected with both TRβ1 and cyclin D1-HA expression plasmids but not in cells transfected only with TRβ1 (lane 4, Fig. 1C, b) or with cyclin D1-HA (lane 5, Fig. 1C, b). The identity of the co-immunoprecipitated cyclin D1-HA was confirmed by direct Western analysis of cell lysates with anti-HA as shown in lanes 2 and 3 of Fig. 1C, b.

Similar results were obtained from cells cultured in the presence of T3 (Fig. 1D). TRβ1 was detected in cells co-transfected with both D1-HA and TRβ1 after first immunoprecipitation of lysates with anti-HA antibodies followed by Western blot analysis using anti-TRβ1 antibody (mAbC4; lane 6, Fig. 1D, a). Using the same protocol, no TRβ1 was detected in cells transfected only with either TRβ1 (lane 4, Fig. 1D, a) or D1-HA (lane 5, Fig. 1D, a).
Fig. 1. Cyclin D1 complexes with TRβ1 in vitro and in vivo. A, pull-down assay of TRβ1 by cyclin D1. Immobilized GST or GST-cyclin D1 fusion protein was incubated with in vitro translated 35S-labeled truncated TRβ1 in the absence (odd-numbered lanes) or presence of 100 nM T3 (even-numbered lanes) as described under “Experimental Procedures.” B, schematic representation of full-length and TRβ1-truncated proteins used in the binding assay. The amino-terminal domain, DNA binding domain, hinge region, and part of the hormone binding domain of TRβ1 are indicated as A/B, C, D, and E, respectively. The numbers indicate the position of amino acids. Association of TRβ1 with cyclin D1 in vivo without T3 (C) or with T3 (D) is shown. CV1 cells were transfected with the indicated combination of TRβ1 and D1-HA expression plasmids (pCLC51 and pCMV-D1-HA). Whole-cell lysates were immunoprecipitated (IP) with anti-HA or anti-TRβ1 as indicated. Immunoprecipitates were separated by SDS-PAGE followed by Western blotting with anti-TR and anti-HA antibodies. Input represents 10% of the total lysate used in the immunoprecipitation. Proteins were detected by Western blot analysis using antibody against TR (mAbC4) or HA epitope. E, GC cells were transfected with pCMV-D1-HA (10 μg) or empty vector using FuGENE 6. Cell lysates (500 μg) were immunoprecipitated with anti-HA followed by Western blot analysis using mAbC4 (E, a). For E, b and c, direct Western blot analysis was performed on lysates (50 μg) using anti-TR (mAbC4) or anti-HA, respectively.
further confirmed by reversing the antibodies in that anti-TRβ1 antibody was first used to immunoprecipitate TRβ1 followed by Western blot using anti-HA (lane 6, Fig. 1D, b). Lanes 1 and 3, Fig. 1D, a, and lanes 2 and 3 of Fig. 1D, b, show the expression of TRβ1 and D1-HA, respectively, by direct Western blot analysis. Taken together, these data indicate that the association of D1 with TRβ1 in cells was T3-independent.

Cyclin D1 not only associated with the transfected TR but also with the endogenous TR in GC cells (Fig. 1E). As shown in Fig. 1E, a, TRβ1 was detected in GC cells transfected with cyclin D1 (lane 2 of Fig. 1E) by co-immunoprecipitation assay but not in cells without the transfected cyclin D1 (lane 1 of Fig. 1E, a). Fig. 1E, b and c, show the controls by direct Western blot analysis to indicate the expression of endogenous TRβ1 and the transfected cyclin D1 in GC cells, respectively, (lane 2 of Fig. 1E, c). These results indicate that cyclin D1 associates not only with the transfected TRβ1 but, importantly, with the endogenous TRβ1 in GC cells.

**Cyclin D1 Represses the T3-dependent Transactivation Activity of TRβ1**—To assess the functional consequences of the physical interaction of TRβ1 with cyclin D1, we used TRE-containing reporter systems (Fig. 2). CV1 cells were transfected with a constant amount of TRβ1 expression plasmid in the absence or presence of an increasing amount of expression plasmid of cyclin D1. Fig. 2A shows that the transactivation activity of TRβ1 was activated 20-fold by T3 (bar 4, Fig. 2A). Cyclin D1 repressed the T3-dependent transactivation activity of TRβ1 in a concentration-dependent manner. The extent of repression was ~55% (bar 6 versus bar 4) and ~80% (bar 8 versus bar 4) at the cyclin D1:TRβ1 plasmid ratio of 1 and 2, respectively. These results indicate that cyclin D1 negatively regulates the T3-dependent transactivation activity of TRβ1.

Consistent with the T3-independent binding of cyclin D1 to TRβ1 shown in Fig. 1, cyclin D1 also augmented the silencing effect of the unliganded TRβ1 in a concentration-dependent manner (Fig. 2A). The enhancement in the silencing effect was 1.6- and 2-fold when the ratio of the cyclin D1:TRβ1 was increased from 1 to 2 (bars 5 and 7, respectively). Taken together, these results indicate that cyclin D1 not only repressed the T3-dependent transactivation activity but also augmented the T3-independent silencing activity of TR.

The cyclin D1-mediated repression effect was further demonstrated by the transactivation activity of endogenous TRs in GC cells. Fig. 2B shows that transfection of increasing concentrations of cyclin D1 expression plasmid (0.1, 0.2, and 0.5 μg, bars 4, 6, and 8, respectively) into GC cells led to a concentration-dependent repression of the T3-dependent transactivation activity of the endogenous TRs (~50%, ~70, and ~85% repression for bars 4, 6, and 8, respectively). The silencing effect of the unliganded endogenous TR was also augmented up to 1.7-fold at the highest concentration of cyclin D1 (bar 7 versus bar 1). Taken together, these data indicate that cyclin D1 not only repressed the transactivation activity of the transfected TRs but also the endogenous TRs.

To further evaluate the functional consequences of the interaction of cyclin D1 with TR, we co-transfected cyclin D1 and TRβ1 in to a cancer cell line, MCF7 cells (Fig. 2C). Similar to that found in CV1 and GC cells, the T3-dependent transactivation activity of TRβ1 was repressed by cyclin D1 in a concentration-dependent manner (compared bars 6 and 8 with bar 4, Fig. 2C). Furthermore, the silencing effect was also further augmented by cyclin D1 in a concentration-dependent manner (bars 5 and 7 versus bar 3, Fig. 2C). Thus, the repression of the transcriptional activity by cyclin D1 not only occurs in CV1 and GC cells but also in MCF7 cells.

Cyclin D1 Interacts with Other TR Isoforms and Differentially Represses Their Silencing and T3-dependent Transactivation Activities—We further evaluated whether cyclin D1 also interacted with other TR isoforms in cells by carrying out the co-immunoprecipitation assay (Fig. 3, A–C). CV1 cells were transfected with TRα1 or TRβ2 with or without cyclin D1-HA in the absence of T3 (lanes 1–5, Fig. 3, A–C). When lysates from cells transfected with cyclin D1-HA and TRα1 or TRβ2 expression plasmids were first immunoprecipitated with anti-TR (mAbC4) followed by Western analysis using anti-HA, D1-HA was detected as shown in lanes 2 and 3 of Fig. 3A, indicating the association of TRα1 or TRβ2 with cyclin D1-HA, respectively. This association is further supported by a negative control in which no D1-HA was detected in cells without the co-transfection of either TRα1 or TRβ2 (lane 1, Fig. 3A). The lack of detection of D1-HA was not due to the lack of expression because direct Western blot analysis of the same lysates indicates that D1-HA was similarly expressed (lane 1, Fig. 3B) as in cells co-transfected with either TRα1 or TRβ2 (lanes 2 and 3, Fig. 3B). Furthermore, direct Western blot analysis using anti-TR antibody shows that TRα1 and TRβ2 were expressed (lanes 2–5, Fig. 3C). These results indicate that cyclin D1 also physically interacted with TRα1 or TRβ2 in the absence of T3.

Similar association of cyclin D1 with TRα1 or TRβ2 was also detected in cells cultured in the presence of T3 (lanes 7 and 8, Fig. 3A, respectively). Again, this physical association in cells was further confirmed by the negative controls in that when cells were not transfected with either TRα1 or TRβ2, no D1-HA was detected (lane 6, Fig. 3A). The data shown in Fig. 3 indicate that TRα1 and TRβ2 were associated with D1-HA in cells independent of T3.

The functional consequences of the physical interaction of cyclin D1 with each of the TR isoforms were evaluated in the presence or absence of T3 by using TRE-containing reporter system (Fig. 3D). In the presence of T3, the interaction of cyclin D1 with each of the three TR isoforms led to the repression of T3-dependent transactivation of TRs. However, the extent of the repression is TR isoform-dependent in that 60, 80, and 65% repression was observed for TRβ1, TRα1, and TRβ2, respectively.

Fig. 3D also compares the extent of the effect of cyclin D1 on the silencing effect of the un-ligated TRα1 and TRβ2 with that of TRβ1. The augmentation of the silencing effect by cyclin D1 also differed among the TR isoforms in that the extent of the enhancement of the silencing effect was 1.6-, 2.0-, and 1.2-fold for TRβ1 (bar 5 versus 3), TRα1 (bar 9 versus 7), and TRβ2 (bar 13 versus 11).

**Cyclin D1 Does Not Affect the Binding of TRβ1 to TRE**—As a first step to understand the mechanisms by which cyclin D1 repressed the TR-mediated transactivation, we first addressed the question as to whether the binding of TRβ1 to TRE was affected by cyclin D1. We carried out electrophoretic gel mobility shift assay by using the in vitro translated TRβ1 in the absence or presence of in vitro translated cyclin D1. Lane 2 of Fig. 4 was the control, which indicated that no nonspecific band was detected in the unprogrammed lysates. Lanes 1 and 8 were the positive controls to show that TRβ1 bound to TRE as homodimers and heterodimers with the retinoic X receptor β (RXRβ), respectively. Clearly, cyclin D1 itself did not bind to TRE, as no TRE bound was detected in the presence of increasing concentrations of the in vitro translated cyclin D1 (Fig. 4, lanes 3 and 4) nor in the presence of RXRβ (lane 7). The binding of TRβ1 to TRE as homodimers (compare lanes 5 and 6 with lane 1) or as heterodimers with RXRβ (compare lanes 9 and 10 with lane 8) was clearly not affected by the presence of in vitro translated cyclin D1. These data indicate that the repression of
the transactivation activity of TR by cyclin D1 was not due to the inhibition of the binding of TRβ1 to TRE.

The Repression of TR-mediated Transactivation Is Reversed by Histone Deacetylase Inhibitor, Trichostatin A—To investigate whether the cyclin D1-mediated repression of TR involves HDACs, we transfected TRE-containing reporter with or without cyclin D1 into GC cells. The TRE-mediated transactivation activity of the endogenous TR was evaluated in the presence of increasing concentrations of TSA (Fig. 5). As shown in bar 3 of Fig. 5, the T3-dependent transactivation activity of endogenous TRs was activated 12-fold by T3, which was repressed by cyclin D1 (~70% repression; bar 4 versus bar 3). In the presence of 330 nM TSA, the T3-dependent transactivation activity of TR was enhanced (bars 7 versus 3). Importantly, the extent of repression effect on the T3-dependent transactivation activation of TRs by cyclin D1 was reduced from ~70 to ~20% (bar 8 versus 7). When the concentration of TSA was further increased to 660 nM (2-fold), the repression effect on the T3-de-
pendent transactivation of TRs by D1 was totally abrogated (bar 11 versus 12), indicating the TSA reversed the repression effect mediated by D1.

A similar reversal of the repression effect of cyclin D1 on the silencing activity of endogenous unliganded TR by TSA was also observed (Fig. 5). Bar 2 (versus bar 1) shows that cyclin D1 augmented the silencing activity of unliganded TR, which was alleviated by the increasing concentrations of TSA (bar 6 versus...
cyclin D1-HA, and TR show that when lysates from cells transfected with F-HDAC3, the expression levels of TR, FLAG-HDAC1, and HDAC3 were detected, respectively, indicating the association of both HDACs with the cyclin D1 complex. Furthermore, the interaction of cyclin D1 with HDAC3 in cells. The above results prompted us to ask the question as to whether HDACs are physically associated with TR-D1 complexes. Recently, it has been reported that HDAC3 is more distantly related to HDAC1/HDAC2 (13, 33, 34) and, moreover, that HDAC3 has been found in large complexes containing TR (35); we therefore focused our studies on HDAC1 and HDAC3. We transfected FLAG-tagged HDAC (F-HDAC1 or F-HDAC3) together with or without cyclin D1-HA into CV1 cells and analyzed the physical interaction of these proteins by co-immunoprecipitation assay (Fig. 6). Lanes 3 and 9 of Fig. 6A show that when lysates from cells transfected with F-HDAC3, cyclin D1-HA, and TRβ1 were first immunoprecipitated with anti-HA followed by Western blot analysis using anti-FLAG (lane 3) or anti-TRβ1 (lane 9), F-HDAC3 and TRβ1 were detected, respectively, indicating the association of both F-HDAC3 and TRβ1 with D1. However, TRβ1 was not required for the recruitment of F-HDAC3 by cyclin D1 (lane 2, Fig. 6A). Lanes 6–8 were negative controls to show that the absence of transfected cyclin D1-HA, neither F-HDAC3 (lane 6, Fig. 6A) nor TRβ1 (lane 11, Fig. 6A) was detected, indicating the requirement of cyclin D1 to recruit F-HDAC3 and TRβ1 to form complexes.

Significantly, HDAC1 did not associate with cyclin D1 either in the absence or presence of TRβ1 (lanes 4 and 5, Fig. 6A, respectively). Furthermore, the interaction of cyclin D1 with TRβ1 was not affected by the expression of F-HDAC1 (lane 10, Fig. 6A). The lack of interaction shown in lanes 4 and 5 (Fig. 6A) is not due to the lack of expression of cyclin D1 because a similar extent of expression of cyclin D1 was detected when the cells were co-transfected with either F-HDAC3 (lanes 2 and 3, Fig. 6B, c) or with F-HDAC1 (lanes 4 and 5, Fig. 6B, c). In addition, this was not due to the lack of expression of F-HDAC1 because F-HDAC1 was expressed as shown by the direct Western blot analysis (lanes 4 and 5, Fig. 6B, b). These results show that the formation of HDAC3-TRβ1-D1 complexes requires the presence of cyclin D1 and that cyclin D1 selectively interacted with HDAC3.

We further determined whether the enhanced expression of HDAC3 in cells could lead to increases in the cyclin D1-mediated repression of the transactivation activity of endogenous TR. To this end, we transfected TRE reporter, the expression plasmids of cyclin D1 and HDAC3 (or with HDAC1), into GC cells in the presence or absence of T3 (Fig. 7). Comparing bars 2 and 4 of Fig. 7 shows that cyclin D1 repressed the T3-dependent transactivation activity of endogenous TR (~70%). This extent of repression of T3-dependent transactivation activity of endogenous TR was not affected by the co-expression of HDAC1 (no significant differences between bar 8 versus 4, p < 0.01, Fig. 7). Importantly, cyclin D1-mediated repression of the T3-dependent transactivation activity of endogenous TR was further intensified by the co-expression of HDAC3 (bars 12 versus 10).

Similarly, cyclin D1-mediated augmentation of the silencing effect of the endogenous unliganded TR in GC cells (bar 3 versus 1) was not intensified by the co-expression of HDAC1 (bars 7 versus 3, Fig. 7). Significantly, co-expression of HDAC3 further intensified the cyclin D1-mediated augmentation of the silencing effect of the unliganded TR (bar 7 versus 3). Taken together, these data indicate that cyclin D1 recruited HDAC3 to D1-TR complexes, thereby augmenting the

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**Fig. 6. Association of cyclin D1 with TR and HDAC3 in cells.** 

**A.** Immunoprecipitation (IP) and Western blot analysis. 

|        | F-HDAC3: | F-HDAC1: | D1-HA: | TRβ1: |
|--------|----------|----------|--------|-------|
| Western: Anti-Flag | + | + | + | + |
| Anti-TRβ1 | + | + | + | + |

**B. Direct Western Analysis** 

(a) Anti-TRβ1

(b) Anti-Flag

(c) Anti-HA

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5 and 10 versus 9). These findings indicate the involvement of HDACs in the repression effect of transactivation activation of TR by cyclin D1. These data further suggest that the recruitment of HDACs by TR-D1 complex was T3-independent.

**Cyclin D1-dependent Recruitment of HDAC3 by TR**—The above results prompted us to ask the question as to whether HDACs are physically associated with TR-D1 complexes. Recently, it has been reported that HDAC3 is more distantly related to HDAC1/HDAC2 (13, 33, 34) and, moreover, that HDAC3 has been found in large complexes containing TR (35); we therefore focused our studies on HDAC1 and HDAC3. We transfected FLAG-tagged HDAC (F-HDAC1 or F-HDAC3) together with or without cyclin D1-HA and/or TRβ1 into CV1 cells and analyzed the physical interaction of these proteins by co-immunoprecipitation assay (Fig. 6). Lanes 3 and 9 of Fig. 6A show that when lysates from cells transfected with F-HDAC3, cyclin D1-HA, and TRβ1 were first immunoprecipitated with anti-HA followed by Western blot analysis using anti-FLAG (lane 3) or anti-TRβ1 (lane 9), F-HDAC3 and TRβ1 were detected, respectively, indicating the association of both F-HDAC3 and TRβ1 with D1. However, TRβ1 was not required for the recruitment of F-HDAC3 by cyclin D1 (lane 2, Fig. 6A). Lanes 6–8 were negative controls to show that the absence of transfected cyclin D1-HA, neither F-HDAC3 (lane 6, Fig. 6A) nor TRβ1 (lane 11, Fig. 6A) was detected, indicating the requirement of cyclin D1 to recruit F-HDAC3 and TRβ1 to form complexes.

Significantly, HDAC1 did not associate with cyclin D1 either in the absence or presence of TRβ1 (lanes 4 and 5, Fig. 6A, respectively). Furthermore, the interaction of cyclin D1 with TRβ1 was not affected by the expression of F-HDAC1 (lane 10, Fig. 6A). The lack of interaction shown in lanes 4 and 5 (Fig. 6A) is not due to the lack of expression of cyclin D1 because a similar extent of expression of cyclin D1 was detected when the cells were co-transfected with either F-HDAC3 (lanes 2 and 3, Fig. 6B, c) or with F-HDAC1 (lanes 4 and 5, Fig. 6B, c). In addition, this was not due to the lack of expression of F-HDAC1 because F-HDAC1 was expressed as shown by the direct Western blot analysis (lanes 4 and 5, Fig. 6B, b). These results show that the formation of HDAC3-TRβ1-D1 complexes requires the presence of cyclin D1 and that cyclin D1 selectively interacted with HDAC3.

We further determined whether the enhanced expression of HDAC3 in cells could lead to increases in the cyclin D1-mediated repression of the transactivation activity of endogenous TR. To this end, we transfected TRE reporter, the expression plasmids of cyclin D1 and HDAC3 (or with HDAC1), into GC cells in the presence or absence of T3 (Fig. 7). Comparing bars 2 and 4 of Fig. 7 shows that cyclin D1 repressed the T3-dependent transactivation activity of endogenous TR (~70%). This extent of repression of T3-dependent transactivation activity of endogenous TR was not affected by the co-expression of HDAC1 (no significant differences between bar 8 versus 4, p < 0.01, Fig. 7). Importantly, cyclin D1-mediated repression of the T3-dependent transactivation activity of endogenous TR was further intensified by the co-expression of HDAC3 (bars 12 versus 10).

Similarly, cyclin D1-mediated augmentation of the silencing effect of the endogenous unliganded TR in GC cells (bar 3 versus 1) was not intensified by the co-expression of HDAC1 (bars 7 versus 3, Fig. 7). Significantly, co-expression of HDAC3 further intensified the cyclin D1-mediated augmentation of the silencing effect of the unliganded TR (bar 7 versus 3). Taken together, these data indicate that cyclin D1 recruited HDAC3 to D1-TR complexes, thereby augmenting the
silencing effect of the unliganded TR and the T3-dependent transactivation activity of TR.

**DISCUSSION**

The present study identifies cyclin D1 as a novel co-repressor for TR. Cyclin D1 acts as a bridging factor to recruit HDAC3 to augment the silencing activity of the unliganded TR and to mediate the repression of the T3-dependent transcriptional activity. In this regard, cyclin D1 is different from other known co-repressors, such as N-CoR/SMRT, because cyclin D1 interacts with TR in a T3-independent manner. In *vitro* pull-down assays indicate that the binding of TRβ1 to cyclin D1 was independent of T3. Furthermore, the association of cyclin D1 in cells with TR was also T3-independent. Consistent with the findings from the *in vitro* and *in vivo* binding assays, cyclin D1 not only repressed the T3-dependent transactivation activity of TR but also augmented the silencing activity of unliganded TR. This is in contrast to the other known co-repressors such as N-CoR/SMRT, which associate with TR and silence the TR transcriptional activity only in the absence of T3 (6, 9). Thus, cyclin D1 is a novel co-repressor for TR, which acts in a T3-independent manner.

The present studies indicate that the augmentation of the silencing effect of the unliganded TR was mediated by the recruitment of HDAC3. This conclusion was supported by the demonstration of the direct interaction of cyclin D1 with HDAC3 in cells (Fig. 6), augmentation of the unliganded TR when HDAC3 was overexpressed (Fig. 7), and the alleviation of the repression by TSA (Fig. 5). In this regard, cyclin D1 acted similarly as N-CoR/SMRT in that these two co-repressors have recently been shown to bind directly to HDAC3 to mediate the silencing effect of the unliganded TR (33, 35). In the presence of T3, whereas the liganded TR is in a conformation that can no longer bind to N-CoR/SMRT, cyclin D1 assumes a critical role to recruit HDAC3 to mediate the repression of T3-dependent transcriptional activity. Thus, cyclin D1 mediates the repression by recruiting HDAC3 via T3-independent binding to TR.

Our data clearly show that the recruitment of HDAC3, but not the more distantly related HDAC1, is preferred by cyclin D1 to mediate the T3-independent repression. The basis for this preference is not entirely clear. The preferential recruitment of different classes of HDACs by bridging factors is not without precedents. Recently, it has been shown that large complexes containing N-CoR/SMRT and unliganded TR are enriched with HDAC3 (33–36). These findings suggest that the substrate specificity on the chromatin may dictate the preferred subclass of HDACs in the context of the promoter. Alternatively, the preferential recruitment of HDACs may also depend on the structural requirements of the bridging factors for being able to bind physically.

Recently, it was reported that cyclin D1 acts as a co-activator for the estrogen receptor (24, 26) in a ligand-independent fashion. Cyclin D1 functions as a bridging factor between ER and the SRCs to recruit the SRC-family co-activators to ER in the absence of ligand. In contrast, the present studies show that cyclin D1 functions as a bridging factor between TR and HDAC3 to mediate repression of the transcriptional activity of TR. It is remarkable that cyclin D1 has the capacity to bridge different receptors and co-regulatory proteins to mediate different functions. It is conceivable that the receptor cyclin D1 interacts with dictates which co-regulate protein that cyclin D1 recruits. The ER binding site for cyclin D1 is located in the carboxyl-terminal EF region (amino acids 340–595) (24). The binding of cyclin D1 with the carboxyl-terminal EF region of ER leads to the enhancement of the binding of ER to the estrogen response element. The TR binding site for cyclin D1 is located in the second half of the thyroid hormone binding domain. However, cyclin D1 had no effect on the binding of TR to TRE. Together with the findings that cyclin D1 does not activate the progesterone receptor nor a number of other steroid hormone receptors (26), these observations suggest a limited structural requirement of cyclin D1 to bind to receptors. Thus, it is reasonable to propose that the interaction of cyclin D1 with ER or TR leads to a conformation in which cyclin D1 either recruits an SRC-family member or HDAC3, respectively. Therefore, the receptor that cyclin D1 binds to dictates the selectivity of the co-regulatory proteins by the binding surface that cyclin D1 presents to the members of SRC/160 family or HDAC3.

The relative contributions of the N-CoR/SMRT pathway and the cyclin D1 pathway in mediating the transcription repression of the unliganded TR are unclear. One of the deciding factors could be the relative tissue distributions and abundance of cyclin D1 and N-CoR/SMRT co-repressors. Because redundancy of functions is common in biology, these two pathways are less likely to be mutually exclusive. Therefore, one would expect that these pathways could act in conjunction or synergistically, depending on the cellular context.

A distinct picture emerges in the repression of T3-dependent transcriptional activity. Cyclin D1 plays a unique role in preferentially recruiting HDAC3 to mediate the repression not only on the exogenously transfected TR but also on the endogenous TR in GC cells. The relevance of this cyclin D1-dependent pathway is exemplified by identification of the T3 response genes in GC cells. Using cDNA microarrays, we have recently identified 358 distinct genes in response to T3-induced proliferation of GC cells. Among the 228 named genes, 155 genes (43%) are down-regulated by T3 (37). Twenty-six of the down-
regulated genes (17%) are early response genes of which the repression was detected within less than 6 h after T3 treatment. Because treatment of GC cells increases the expression of cyclin D1 protein (27), it is entirely possible that some of these early T3 response genes could be directly repressed by TR via the increased cyclin D1. Moreover, we have recently identified a positive TRE in the promoter of the cyclin D1 gene, suggesting that the increased expression of cyclin D1 by T3 at least in part was by a direct action of TR on the cyclin D1 regulatory activity mediated by cyclin D1 generates a negative feedback loop to maintain the regulatory network in controlling the expression of T3 target genes in cell proliferation. Although the utilization of the cyclin D1-mediated pathway by the downregulated T3-target genes has yet to be tested in future studies, the present studies reveal a novel pathway by which TR represses its target genes.

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