Residues K128, 132, and 134 in the Thyroid Hormone Receptor-α Are Essential for Receptor Acetylation and Activity

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The thyroid hormone receptor (TR)-α is a nuclear receptor that mediates both transrepression and ligand-dependent transactivation. Here we show that TRα is posttranslationally modified by acetylation in response to its own ligand (T_3). Acetylation increases binding to DNA. Using mutagenesis, we identified three conserved lysine residues in the carboxi-terminal extension (CTE) of the DNA binding domain that are targets of the cAMP-response element-binding protein acetyltransferase. Substitution of these lysines by arginines in TRα decreased ligand binding affinity and precluded ligand-dependent release of corepressors and recruitment of coactivators. The acetylation TRα mutant lost the ability to transactivate even at high T_3 concentrations and acts as a dominant-negative inhibitor of wild-type TR activity. In addition, whereas native TRα interferes with AP-1 function, the mutant is unable to mediate transrepression. Finally, TRα suppresses NIH-3T3 fibroblast transformation by the Ras oncogene both in a ligand-dependent and -independent manner, but the CTE mutant is unable to mediate ligand-dependent repression of transformation. These results reveal a key role for the CTE region on acetylation, ligand affinity, transactivation, transrepression, and antitransforming properties of TRα. **(*Endocrinology* 150: 5143–5152, 2009)**

The gene regulatory actions of the thyroid hormone T_3 are mediated through the binding to nuclear thyroid receptors, termed thyroid hormone receptor (TR)-α and TRβ, which are encoded by genes located on human chromosomes 17 and 3, respectively. Transcripts of each of these genes undergo alternative splicing generating the TRα1 and -α2 and TRβ1 and -β2 receptor isoforms (1). The nuclear receptors have a modular structure with several regions (A/B, C, D, and E). The C region contains the DNA binding domain (DBD), composed of two zinc fingers and a α-helical C-terminal extension (CTE) that makes auxiliary contacts with DNA. Transcriptional regulation by the receptors is achieved through autonomous activation functions (AFs): a constitutive N terminal AF-1 in the A/B region and a ligand-dependent AF-2 located in the C-terminal E region of the ligand binding domain (LBD) (1, 2). TRs can regulate gene expression by binding, preferentially as heterodimers with the retinoid X receptors (RXRs), to specific DNA sequences [thyroid hormone response elements (TREs)] located in regulatory regions of target genes (3) and by positive or negative interference with the activity of other transcription factors such as activator protein-1 (AP-1), cAMP response element-binding protein (CREB) or nuclear factor-κB, a mechanism referred to as transcriptional cross talk (4–10). When bound to TREs the unliganded TR recruits corepressors and re-

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Abbreviations: ACTR, Activator of thyroid and retinoic acid receptor; AF, activation function; AP-1, activator protein-1; AR, androgen receptor; CAT, chloramphenicol acetyl transferase; CBP, cAMP-response element-binding protein acetyltransferase; CREB, cAMP response element-binding protein; CTE, C-terminal extension; DBD, DNA binding domain; ER, estrogen receptor; GST, glutathione-S-transferase; HAT, histone acetylase activity; HDAC, histonedeacetylase; LBD, ligand binding domain; RXR, retinoid X receptor; SAHA, suberoylanilide hydroxamic acid; SMRT, silencing mediator of retinoid and thyroid receptors; TR, thyroid hormone receptor; TRE, thyroid hormone response element.

presses transcription, whereas ligand binding induces a conformational change in the receptor C-terminal α-helix (helix 12) that leads to the release of corepressors and the recruitment of coactivators (11, 12). Some nuclear receptor coactivators such as cAMP-response element-binding protein acetyltransferase (CBP)/p300 and the p160 proteins possess histone acetylase activity (HAT) that cause chromatin decompaction. CBP/p300 proteins acetylate histones but also p160 coactivators (13) and multiple transcription factors such as p53 (14), cellular transcription factor involved in adenovirus early region 1A (E1A) (E2F1; Ref. 15) , p65 nuclear factor-κB (NF-κB; Ref. 16), or some nuclear receptors (17–23). Acetylation can regulate transcription factor activity by modulating DNA binding, protein-protein interactions, protein stability or subnuclear localization (24). The finding that the sirtuins deacetylase nuclear receptors provides a new level of complexity in the control of nuclear receptor activity (25, 26).

To date, three nuclear receptors have been shown to be CBP targets: the androgen receptor (AR) (20), the estrogen receptor (ER)-α (22, 23), and TRβ (27). AR acetylation occurs in the DBD CTE in a KLKK motif corresponding to K630/K632/K633 residues, and mutation on these sites decreases corepressor binding, augments association with coactivators, and increases ligand-dependent transactivation (19). ERα acetylation that occurs in K266 and K268 sites corresponding to K632 and K633 in the AR, enhances DNA binding activity, and correlates with an increase in transcriptional activity (22). ERα contains additional sites of acetylation by p300 in K299/ K302/K303 residues, and the mutant K302/K303 shows an increased estradiol-dependent activation, suggesting a role of ERα acetylation in ligand sensitivity (28).

TRs contain a KRV/LAKRK sequence equivalent to the KLKK motif in AR corresponding to K184/K188/K190 in TRβ and K128/K132/K134 in TRα, but in contrast to AR and ERα, little is known about the functional consequences of TRα or TRβ acetylation. There is one report describing thyroid hormone-dependent TRβ acetylation, but residues responsible for acetylation have not been identified (27).

Here we show that the CTE region of the TRα DBD is also a target for acetylation by CBP and that TRα acetylation increases binding to DNA. A mutant in the acetylation region shows loss of ligand-dependent transactivation and transrepression capacity and acts as a dominant-negative on positive TREs. In addition, in contrast with the native receptor (29), the CTE mutant has lost the ability to show increased inhibition of fibroblast transformation by oncogenic Ras in the presence of hormone. Collectively, our results suggest an important role for CTE region of TRα in the transcriptional and the antioncogenic actions of this receptor.

Materials and Methods

Plasmids

pSG5-CTE, pSG5K128R, and pSG5K132/134R were generated from chicken TRα by site-directed mutagenesis with the Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA) by using the double stranded oligonucleotides: 5′-GACGACTCGAGGCGC-GTAGCCAGGCGGAGGCTGATCGAG-3′, 5′-GACGACTC-GAGGCG CGTAGCCAAGCGGAAGCTGATCGAG-3′, and 5′-GACGACTCGAAGCGCGTAGCCAGG CGGAGGCTGAT-CGAG-3′, respectively. PCR products were digested with *Dpn*I. The mutations were confirmed by sequencing. pSG5-K232I was previously described in (29).

Transient transfection assays

HeLa or HEK 293T cells were grown in DMEM containing 10% fetal bovine serum. Cells were transfected with calcium phosphate. One microgram of the mouse mammary tumor virus luciferase reporter plasmid containing a thyroid-responsive element (MMTV-TREpal-LUC), DR4-tk-LUC (30), −39/+12 GH-CAT that contains a fragment of the rat GH gene (−39/+12) fused to chloramphenicol acetyl transferase (CAT) (31), or −73col-luc together with 0.6 μg pRL-TK (renilla) were transfected with 0.5 μg of expression vectors (pSG5-0, pSG5-TRα, pSG5-CTE, pSG5-K128R, or pSG5-K132/134R). Twelve hours after transfection, the cells were preincubated in DMEM supplemented with 10% AG1-X8 resin and charcoal-stripped newborn calf serum and then treated with T_3 for 36 h. Luciferase and renilla activity were assayed with a dual-luciferase assay system (Promega, Madison, WI). CAT activity was determined as previously described (31).

Synthesis and purification of recombinant proteins

Glutathione-S-transferase (GST)-fusion proteins were purified from *Escherichia coli* on glutathione-Sepharose and analyzed by SDS-PAGE. Alternatively, ^{35}S-labeled proteins were *in vitro* generated with the TNT-T7 Quick kit (Promega).

In vitro acetylation assays

GST-fusion proteins or *in vitro*-translated proteins were incubated with the HAT domain of CBP (1098-1877 fragment) fused to GST in the presence of [^3H]acetyl-CoA in HAT buffer (15). The reactions were incubated at 30 C for 60 min and were analyzed in 10% SDS-PAGE. Gels were exposed with X-OMAT films (Kodak, Rochester, NY) for 2 d. Acetylation reactions with unlabeled acetyl-CoA were carried out under similar reaction conditions.

In vivo acetylation assays

GH4C1 cells that express endogenous TRα and TRβ were grown in DMEM supplemented with 10% fetal serum. Twelve hours prior to T_3 and suberoylanilide hydroxamic acid (SAHA) treatment, cells were incubated with DMEM containing 10% AG1-X8 resin and charcoal-stripped newborn calf serum. Cells were treated for 24 h with 5 nM T_3 and 2 μM SAHA and then lysed in lysis buffer [50 mM HEPES (pH 8), 600 mM KCL, 0.5% Nonidet P-40, 1 mM Na_3VO_4, 1 mM dithiothreitol, protease inhibitors]. Two milligrams of whole-cell lysates were precleared and incubated overnight with 2 μg of rabbit IgG or antiacetyl lysine-

specific antibody. The immunoprecipitated proteins were detected by Western blotting.

Western blot assays

For Western blot assays, cell extracts were prepared in lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 0.5% sodium dodecyl sulfate, 30 mM sodium pyrophosphate (PPi), 0.5M NaF, protease inhibitors], and 5–10 μg were loaded into 12% sodium dodecyl sulfate gels. Membranes were probed with the following antibodies: anti-TRα (Santa Cruz Biotechnology, Santa Cruz, CA), anti-H3 (Millipore, Tennecula, CA), and antiacetylated lysine (α-LysAc; Cell Signaling, Beverly, MA).

In vitro pull-down assays

^{35}S-labeled proteins were used in pull-down assays with 1 μg of GST or GST-fused proteins as described previously (9).

Band-shift assays

In vitro-translated TRα was either unmodified or acetylated with CBP. One microliter of *in vitro*-translated RXR together with 1 μg of GST-silencing mediator of retinoid and thyroid receptors (SMRT) or GST-activator of thyroid and retinoic acid receptor (ACTR) was added to TR on ice for 15 min before incubation with a [^{32}P]-end-labeled double-stranded oligonucleotide (5′-AGCTCAGGTCACAGGAGGTCAG-3′) containing a consensus TRE sequence for 30 min at room temperature in the presence or absence of 1 μM T_3. Similar assays were performed in the absence of RXR. The samples were analyzed on nondenaturing 5% polyacrylamide gels run in 0.5 × Tris-borate EDTA. Alternatively, 0.5 μg GST-TRα or GST-CTE with GST-RXR were used.

Protease resistance assay

In vitro-translated TRα or CTE mutant were incubated in absence or presence of 5 nM of T_3 with 0.02, 0.01, and 0.5 U trypsin (Sigma, St. Louis, MO).

Foci formation assays

NIH-3T3 fibroblasts were transfected with calcium phosphate and 500 ng of pCEFL-Ha-Ras^{val12} and 4 μg of expression vectors for TRα or with the same amount of empty vector (32). Cultures were fed with fresh medium containing 10% T_3-stripped donor calf serum every 2 d in the presence or absence of 5 nM T_3. At 14 d after transfection, the foci were stained with Giemsa and scored visually.

^{125}I-T_3 binding assays

Binding assays were essentially performed as described (33, 34).

Statistical analysis

All data are expressed as means ± SD. Student's *t* test was performed using the SSC-Stat software (version 2.18; University of Reading, Reading, UK).

Results

TR is acetylated by CBP in the CTE region

A previous report has shown that TRβ can be acetylated (27). To examine whether TRα is also a target for acetylation, GH4C1 cells were treated for 24 h with 5 nM T_3 alone or in combination with the histone deacetylase (HDAC) inhibitor SAHA (2 μM). Acetylated proteins were immunoprecipitated from nuclear extracts with an antibody for acetylated lysines, and the acetylated receptor was detected with a TRα-specific antibody. Figure 1A shows that T_3, and to a lesser extent SAHA, increased acetylated TRα (lanes 6–7), whereas acetylated histone H3 was increased only in the presence of SAHA or SAHA plus T_3 (lanes 7–8). To examine the acetylation sites of TRα, we generated three lysine mutants, one in the DBD

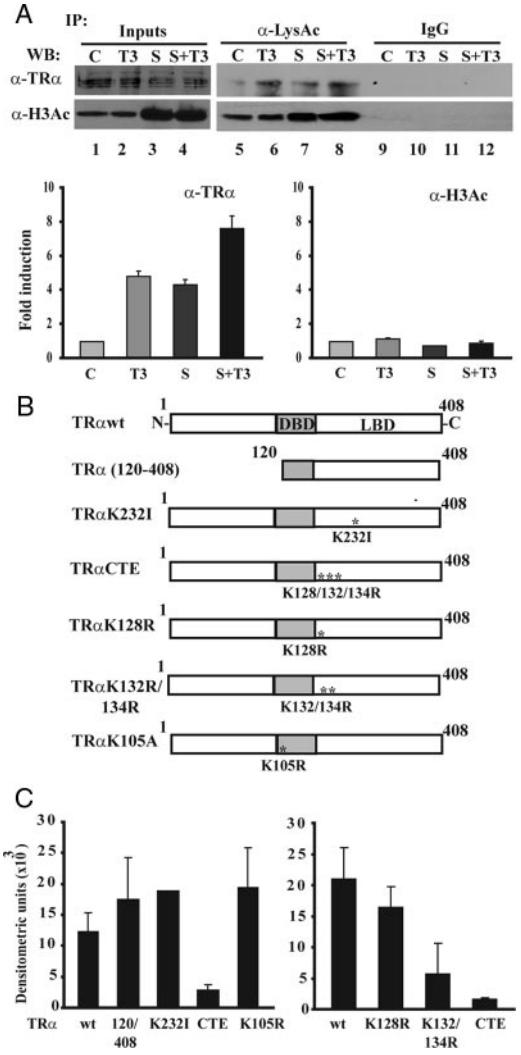

FIG. 1. TRα is acetylated *in vivo* and *in vitro*. A, Acetylated proteins were immunoprecipitates (IP) from GH4C1 cells previously treated in the presence or absence (C) of 5 nM T_3, 1 μM SAHA (s), and SAHA plus T_3 for 24 h. Western blot (WB) assays were developed with TRα and acetylated H3 antibodies. The input amount is the 10% of the total extract protein. Quantifications of acetylated TRα and H3 (H_3Ac) levels are represented on the graphic. B, Schematic representation of the location of TRα mutations (indicated by *asterisks*) used in this study. The DBD and LBD are indicated. C, Quantifications of acetylation assays with the point and deletion mutants shown in B. GST-TRwt and mutants were incubated with GST-CBP in the presence of [^{3}H]acetyl-CoA and CBP. The reactions were analyzed by SDS-PAGE with subsequent fluorography. wt, Wild type.

(K105A mutant) equivalent to K128A mutant that is acetylated in TRβ, other in the consensus motif KLKK described for AR and ERα in the C-terminal extension of the receptor DBD (K128/132/134R or CTE mutant), and other in the conserved K232 residue (K232I) in the LBD (Fig. 1B). We examined by *in vitro* acetylation assays the acetylation of TRαwt, these mutants, and one additional TRα mutant corresponding to a deletion of the AF-1 domain and the DBD (120-408TRα). Quantification of the results (Fig. 1C) shows that TRwt was acetylated *in vitro* by CBP and that the deletion of the N′-terminal domain that contains the AF-1 domain; the mutation K232I in helix 3 or the mutation of the K105 residue did not prevent TRα acetylation by CBP. However, triple substitution of lysines 128, 132, and 134 by arginines abolished TRα acetylation. To determine whether one or two residues in the CTE were essential for acetylation, we generated two additional mutants, TRαK128R and TRαK132/134R. Both mutants show a decrease in acetylation with respect to TRwt, although this decrease was only significant for the TRαK132/134R mutant. However, total abrogation of TRα acetylation was achieved only with the CTE mutant. These data demonstrate that TRα acetylation by CBP occurs in the CTE, in accordance with previous reports for ERα and AR (20, 28).

Acetylation of TRα increases DNA binding

Given that the acetylated residues are adjacent to the DBD, we next tested by band-shift assays whether acetylation affected the ability of TRα to bind DNA. In these experiments, *in vitro*-translated TRα was acetylated in the presence of CBP and nonradiactive acetyl-CoA in the absence or presence of T_3. Each reaction was used in bandshif assays with a ^{32}P-labeled double-stranded oligonucleotide containing a consensus TRE sequence (DR4). In the absence of hormone, TRα binds to the DR4 as a heterodimer with RXR with the same affinity independently of its acetylation state (Fig. 2A, lanes 1 and 4). However, in the presence of T_3 (1 μM) and at low receptor concentrations, the acetylated receptor binds to the TRE with a higher affinity than the nonacetylated TRα (lanes 2 *vs.* 5). Also GST-TRα, in the presence of T_3, bounds to the DNA with higher affinity after incubation with acetyl-CoA, but with T_3 and without acetyl-CoA, the binding of TR to DNA was not increased (Fig. 2B). Next, we performed band-shift assays with the wild-type receptor and the acetylation mutant. Again, acetylation increased DNA binding of the TRαwt in a T_3-dependent manner, whereas the hormone did not increase binding affinity of the receptor that contains a triple mutation in the acetylation region (CTE) (Fig. 2C), confirming that TRα acetylation enhances binding of the heterodimer to a consensus TRE. Because TRα can also bind as a homodimer to this element, we performed the assay under similar conditions but in the absence of RXR. Binding of homodimers to the TRE was not modified after TRα acetylation (Fig. 2D).

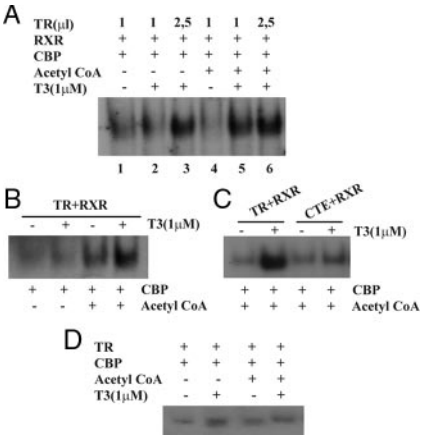

FIG. 2. Acetylated TRα shows increased DNA binding activity in the presence of T_3. A, One and 2.5 μl of TRα nonacetylated or acetylated *in vitro* with CBP and acetyl-CoA were used in band-shift assays together with 1 μl of *in vitro*-translated RXR in the absence or presence of T_3. B, TRα binding was analyzed after incubation with CBP, and the presence or absence of acetyl-CoA C, GST-TRα, or GST-CTE was acetylated *in vitro* and the binding to the TRE as heterodimers with RXR was analyzed in the absence and the presence of T_3. D, TRα (2 μl), nonacetylated or acetylated, was incubated with or without T_3 in the absence of RXR and used in band-shift assays.

The CTE of TRα is important for the interaction with coactivators and corepressors

In the absence of ligand, TR is bound to corepressor proteins [SMRT or nuclear receptor corepressor (NCoR)] associated with HDACs (35, 36), repressing gene transcription. Receptor occupancy by T_3 results in dissociation of corepressors and derepression, followed by recruitment of coactivators and associated cofactors, some of which exhibit HAT activity, leading to transcriptional activation. We next examined the ability of TR-RXR and CTE-RXR heterodimers to recruit coactivators when bound to the consensus direct repeat TRE (Fig. 3A). Wild-type TRα-RXR complexes recruited a GST-fusion protein containing the receptor-interacting domain of the p160 coactivator ACTR (GST-ACTR, residues 621-821) in a T_3-dependent manner (lane 4). In this assays the amount of ACTR used was sufficient to observe binding of ACTR to the wild-type heterodimer, even in the absence of hormone (lane 3). In contrast to TRαwt-RXR, recruitment of GST-ACTR by the CTE-RXR heterodimer was not detected either in the absence or presence of hormone (lanes 9 and 10). These results were also analyzed in pull-down assays with S^{35}-methionine *in vitro*-translated receptors (Fig. 3B). TRα bound detectably ACTR in the absence of hormone and this interaction increased after T_3 treatment,

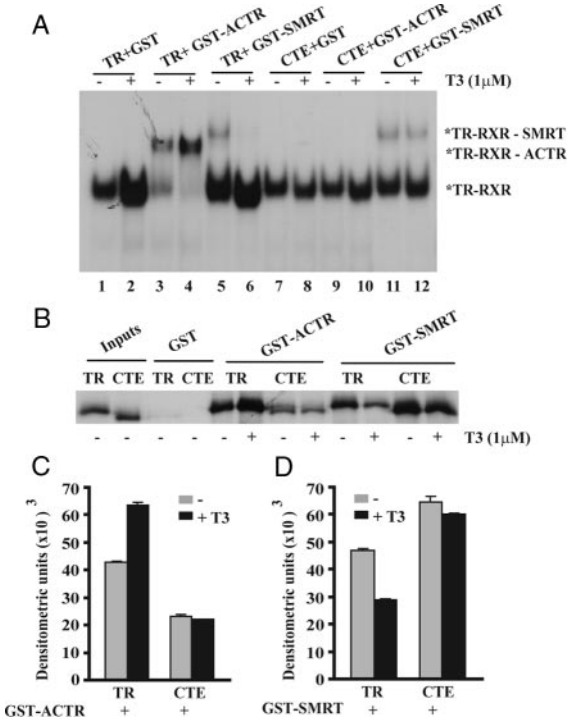

FIG. 3. Altered coactivator interaction and impaired release of corepressors by the CTE mutant. A, A labeled oligonucleotide spanning the DR4 element was incubated with *in vitro*-translated TRwt/RXR or CTE/RXR heterodimers in the presence of GST-0, GST-ACTR, or GST-SMRT. When indicated, the binding reactions were carried out in the presence of 1 μM T_3. The mobility of TR/RXR, TR/RXR/ACTR, and TR/RXR/SMRT complexes is indicated. B, GST-0, GST-ACTR, and GST-SMRT were incubated with S^{35}-labeled TRwt or CTE mutant. C and D, Pull-down quantification corresponding to the TR/ACTR, TR/SMRT, CTE/ACTR, and CTE/SMRT complexes in the presence of 1 μM T_3. Data were corrected with the corresponding inputs and are expressed relative to the values obtained with the unliganded wild-type receptor. Data (means ± SD) are the result of three different experiments.

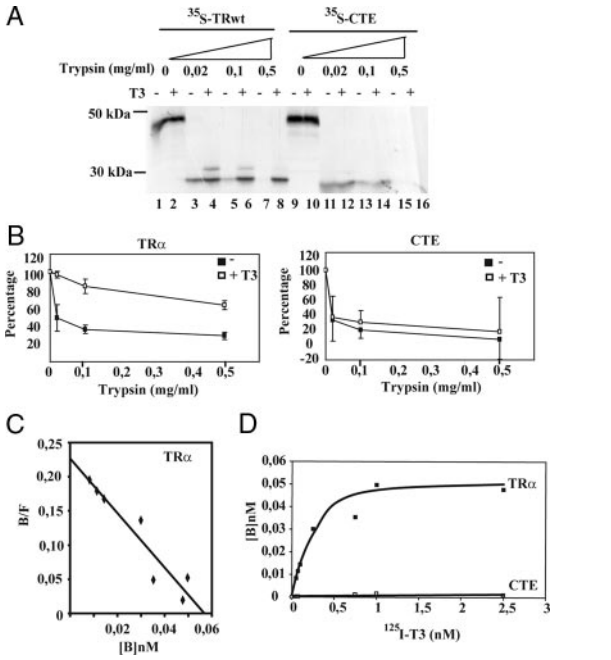

FIG. 4. The CTE mutant shows reduced T_3 binding. A, T_3 binding by wild-type (wt) and CTE mutant receptors was determined using hormone-mediated protease resistance assays. B, The percentage of input TR remaining after digestion with increased amounts of trypsin at 1 μM T_3 concentration was quantified. The data are the mean values corresponding to three different experiments. C, Scatchard plot of [^{125}I]T_3 binding to TRαwt protein synthesized *in vitro*. Aliquots of the lysates were incubated overnight at 4 C with different concentrations of [^{125}I]T_3. Nonspecific binding was measured in the presence of a 500-fold excess of T_3. The ratio between the amount of [125-I] T_3 bound (B) and free (F) is represented in y-axis (B/F). D, Kinetic curves of TRαwt and CTE mutant.

whereas the CTE mutant showed less interaction with coactivators than the wild-type receptor, and this interaction did not increase in the presence of hormone. Pull-down quantification (Fig. 3C) showed again the reduced binding of coactivators by the unliganded mutant receptor as well as the lack of response to T_3. The low interaction between the CTE mutant and ACTR was not sufficient for detection as a supershift in the band shift.

We also compared the binding of wild-type and CTE mutant receptors to corepressors. We observed that in the absence of T_3, the CTE mutant and the wild-type receptor bound the corepressor SMRT with similar affinity (Fig. 3A, lanes 5 and 11). However, whereas TRαwt was able to release SMRT very efficiently after T_3 incubation, the CTE mutant remained bound to SMRT, even in the presence of 1 μM T_3 (lanes 6 and 12). In pull-down assays, the CTE mutant bound the corepressor with even higher affinity than the native TRα but in coincidence with the results obtained in band-shift assays was not able to release SMRT in the presence of hormone (Fig. 3, B–D).

Protease and binding assays

Although the CTE mutations are outside the LBD, the fact that in the presence of hormone, the CTE mutant did not show increased coactivator recruitment and was not able to release corepressors suggested that the primary defect of this mutant might be in hormone binding affinity. To check that possibility, we used two experimental approaches, a protease protection assay in which T_3 binding is measured through its ability to generate a protease-resistant conformation in the receptor (Fig. 4, A and B) and binding assays with I^{125}-T_3 to determine receptor affinity (Fig. 4, C and D). *In vitro*-translated TRαwt or CTE mutant were digested in the presence of increasing amounts of trypsin. In the absence of T_3, both wild-type and mutant TRα were efficiently degraded by protease digestion. Incubation of TRαwt with T_3 resulted in formation of a protease-resistant polypeptide that progressively disappeared in function of increasing amounts of trypsin. However, the CTE mutant in the presence of hormone was totally digested, even at the lowest amount of protease used. Figure 4B shows the quantification of three experiments. To confirm the results obtained in the protease assays, the capacities of both receptors to bind thyroid

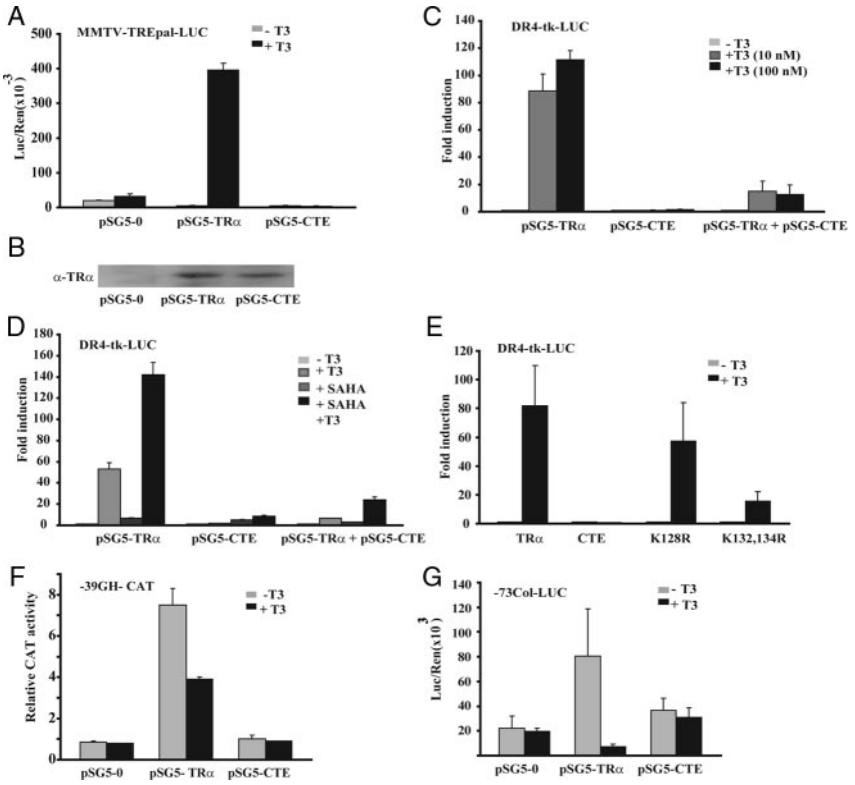

FIG. 5. TRα acetylation mutant exhibits altered transcriptional activation and repression and acts as a dominant negative. A, An empty pSG5-0 vector, pSG5-expressing wild-type TRα, or pSG5-expressing CTE mutant was transfected in HeLa cells together with a MMTV-TREpal-LUC construct. After transfection the cells were treated with medium alone or 100 nM T_3 (*black bars*) and after 36 h were harvested for luciferase assay. All experiments were repeated at least three times in duplicate, and all data are presented as the mean ± SD. B, After transfection, the expression of TR wild-type or CTE mutant levels was analyzed by Western blot. C, DR4tk-LUC was cotransfected with equal amounts of pSG5-TRwt or pSG5-CTE mutant in HEK 293T cells. After transfection cells were treated with 10 and 100 nM T_3. Data are represented as fold of induction with respect to basal levels in untreated cells. D, HEK293T cells were cotransfected with the reporter construct and expression vectors for TRwt or the CTE mutant, and luciferase activity was determined in cells incubated with 10 nM T_3 and/or 1 μM SAHA, as indicated. E, HEK293T cells were transfected with DR4tk-LUC and expression vectors for TRwt or the CTE, K128R, and K132,134R mutants. F, HeLa cells were transfected with the reporter plasmid containing 39 bp 5′ from the start of transcription of the GH promoter (−39GH-CAT). The plasmid was cotransfected with an expression vector for TRα or for the CTE mutant and CAT activity determined in control cells and in cells treated with 100 nM T_3. G, Luciferase activity was determined in HeLa cells transiently transfected with a collagenase-3-LUC reporter together with TRwt or the CTE mutant in absence or presence of 100 nM T_3. Luciferase activity was always corrected by renilla activity.

hormone were assessed. The same amounts of *in vitro*-translated receptors, as determined by Met-S^{35} incorporation, were tested for their ability to bind hormone by incubation with increasing concentrations of ^{125}I-T_3 in the presence and absence of a 500-fold molar excess of unlabeled hormone. To determine accurately the effect of the mutations on hormone binding, we performed Scatchard and kinetic analyses (Fig. 4, C and D). The dissociation constant obtained for TRα was 0.25 nM, whereas the CTE mutant exhibited no detectable binding activity. These results indicate that the same residues in the CTE of TRα that are the target of acetylation by CBP appear to be also important for ligand binding.

The TRα acetylation sites regulate TR transactivation and transrepression

Because the acetylation of TRα increases DNA binding activity and the CTE mutant does not recruit coactivators in a DNA element, we investigated the effect of mutation of the acetylation residues on transactivation. HeLa cells that do not express endogenous TRs were transiently transfected with a luciferase reporter plasmid that contains a palindromic TRE (MMTV-TREpal-LUC) together with expression vectors for TRαwt or the CTE mutant (Fig. 5A). As expected, TRα induced reporter activity in the presence of T_3. Conversely, the CTE mutant was not able to mediate T_3-dependent transactivation, showing that K128/132/134 residues play an important role in regulating the transcriptional activity of TRα. Interestingly, the native and mutant receptors were equally able to repress basal reporter activity in the absence of T_3, in agreement with the finding that the CTE mutant binds corepressors constitutively. Western blot analysis showed that wild-type and mutant TRα were expressed at similar levels after transfection (Fig. 5B). Collectively our results demonstrate a good correlation between the decreased DNA binding and coactivator recruitment with transactivation activities of the CTE mutant when compared with wild-type TRα. Because promoter-selective effects have been observed for the AR acetylation site mutants (17), HEK293T cells that do not express endogenous TR were transiently transfected with both receptor expression plasmids in the presence of a different TR receptor target, the DR4-tk-LUC construct (Fig. 5C). The results were similar to those observed in HeLa cells with the MMTV-TREpal-LUC reporter gene, showing that the lack of CTE transactivation activity was not dependent on cell or promoter type as has been previously shown for AR acetylation mutants. Trying to compensate for reduced binding affinity of the CTE mutant, we treated cells with a higher amount of T_3 (Fig. 5C). Even after incubation with 100 nM T_3, the CTE mutant was unable to mediate ligand-dependent transactivation. In addition, when cotransfected with equimolecular amounts of TRαwt, the

acetylation mutant acted as a dominant negative, even at high hormone concentrations. HEK293T cells were also treated with 1 μM SAHA in the presence or absence of T_3 after transient transfection with the DR4-tk-luc construct (Fig. 5D). SAHA significantly induced transcriptional activation of the reporter gene by T_3 in cells expressing TRαwt, whereas it had no effect in cells expressing the CTE mutant. Additionally, we compare transactivation by TRwt and the three mutant receptors in the CTE region (Fig. 5E). Whereas activity of the DR4-tk LUC reporter gene was strongly increased in the presence of hormone after expression of TRwt or the K128R mutant, the TRK132/134R mutant exhibited a significantly decreased reporter activity in response to T_3, and this response was completely suppressed with the CTE mutant.

TRs can repress transcription through binding to negative TREs present in the promoter region of T_3-regulated genes, or through transcriptional interference with other transcription factors such as AP-1. To assess the effect of the CTE mutant on repression, we used a CAT reporter construct comprising the −39/+12 sequence of the GH promoter that contains a negative TRE element (31, 37). A common finding is that on negative TREs the unoccupied receptor increases transcription and the ligand reverses this stimulation (38, 39). As shown in Fig. 5F, TRα cotransfection induced basal activity that was reverted by hormone treatment. In contrast, the CTE mutant did not induce the basal activity and was unable to respond to T_3. Given the important role of AP-1 complexes in the control of cell proliferation, we also checked the transrepression properties of the CTE mutant on activity of the AP-1 containing collagenase promoter (Fig. 5G). TRαwt is known to enhance AP-1 activity in the absence of hormone and repress in the presence of T_3 (7, 40, 41). As expected, expression of native TRα induced basal activity of the collagenase promoter that was reverted in the presence of hormone. In contrast, the CTE mutant displayed a very modest induction of basal transcription and T_3 treatment had not a significant effect. Thus, the CTE mutant is defective both in the ability to enhance AP-1 function in the absence of hormone and to suppress in the presence of hormone.

The CTE mutant does not mediate ligand-dependent inhibition of *ras*-mediated fibroblast transformation

We previously described that TRs can inhibit Ras-mediated cellular transformation (32). Therefore, we checked whether TRα acetylation is also important in this process. For this purpose, we performed foci formation assays with NIH-3T3 fibroblasts transfected with H-ras^{val12} alone or in combination with TRα or the CTE mutant (Fig. 6). As

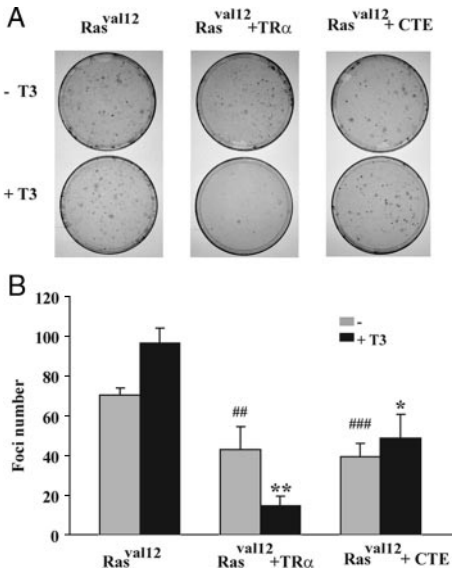

FIG. 6. TRwt but not the CTE mutant is able to reduce foci formation in the presence of T_3. A, Foci formation by NIH-3T3 cells transfected with ^{val12}H-ras and pSG5-TRα or pSG5-CTE expression vectors. Cells were treated for 15 d with 5 nM T_3. B, Quantitation of foci number. Ras^{val12}, Valine 12 rat sarcoma. The statistical significance of difference between untreated cells groups was expressed by #, and that of the difference between T_3-treated cells groups was shown by * (#,*, $0.01 < P < 0.05$; ##,**, $0.001 < P < 0.01$; ###,***, $P > 0.01$).

expected, the transforming ability of the *ras* oncogene was greatly reduced in cells coexpressing the native receptor. TRα reduced formation of transformation foci in the absence of exogenously added T_3, and this reduction was stronger in T_3-treated cells. In contrast, although in the absence of hormone the CTE mutant also caused reduction of foci formation, T_3 was unable to induce a further inhibition.

Discussion

Our results show that TRα is acetylated *in vitro* by CBP at residues Lys128, 132, and 134 (Fig. 1). The acetylation residues are part of a KRV/LAKRK motif in the CTE of the DBD also present in several members of the steroid receptors family. In fact, it is described that both ARα and ERα are acetylated in the same region (19, 22). *In vivo* the acetylation of the TRα receptor is induced by its own ligand (T_3). It is known that this nuclear receptor recruits coactivators in a ligand-dependent manner. Thus, after T_3 treatment the recruitment of CBP or other coactivators with HAT activity could induce TRα acetylation. In coincidence with TRβ, the K105A mutant in TRα, equivalent to K128A mutant in TRβ that is acetylated *in vivo* (27), was also efficiently acetylated *in vitro* by CBP. TRβ acetylation sites have not been defined, and we cannot rule out that TRβ could be acetylated in different regions as has been demonstrated for ERα (22, 28). In contrast from TRβ

that is transiently acetylated in response to thyroid hormones (27), we observed induction of TRα acetylation, even after 36 h incubation with T_3. In general, our results reinforce the idea that specific ligands seem to induce acetylation of their cognate nuclear hormone receptor.

The acetylation of proteins such as cellular transcription factor involved in adenovirus early region 1A (E1A) (E2F1; Ref. 15), MyoD, or ERα augments DNA binding (15, 22, 42), although other proteins such as yin yang (YY1) and Forkhead box O (FOXO) show a decreased binding when acetylated by CBP (43, 44). In accordance with the results described for ERα, in the presence of ligand, the heterodimeric binding of TRα to DNA increases when the receptor is acetylated (Fig. 2B). Interestingly, homodimeric binding does not appear to be affected by acetylation. In some receptors the CTE of the DBD contributes to DNA binding and dimer stabilization (45). However, in the ternary complex of TRα with RXR and DNA, the CTE region of TR does not participate in the dimer interface and makes nonspecific contacts with the DNA backbone (46). Our results show that in the absence of hormone, the mutation of the acetylation residues K128/132/134R in the CTE does not alter affinity of heterodimeric binding to a consensus DNA element compared with TRαwt. However, binding of the mutant receptor is not increased by T_3 in the presence of CBP and acetyl-CoA, suggesting again that modification by acetylation could enhance TRα binding to DNA and transactivation at a target promoter. Alternatively, the CTE mutant could exhibit altered ligand binding properties.

An unexpected finding in this work was that the CTE mutant, which mimics unacetylated TRα, shows a highly reduced T_3 binding affinity and sensitivity. In the case of ERα and AR, the mutation at the same acetylation region, which is outside the LBD, does not appreciably affect ligand binding affinity. However, a recent report (47) shows that deletion of the 629-RKLKKLGN-636 region in AR, which contains the acetylation residues, enhances the intramolecular interaction between the amino-terminal domain and the LBD increasing ligand sensitivity of the mutant with respect to the wild-type receptor. In this sense, our data show that the CTE domain of the TRα receptor is also crucial for ligand recognition. Additionally, it has been previously shown that residues 120-199 in TRα are required for ligand binding (48) and that the D domain adjacent to the CTE has an important role in stabilizing the overall structure of the LBD (49). It is then plausible that an altered receptor conformation that precludes hormone binding to the LBD could be acquired by mutation of the CTE acetylation residues. Although crystallographic analysis would be necessary to demonstrate this hypothesis, the finding that, as observed in Fig. 3B, the CTE mutant shows a different mobility with respect to TRαwt appears to support this possibility. An important change in receptor conformation by mutation of the lysine residues in the CTE is also indicated by the altered binding of coactivators of the mutant receptor. In *in vitro* assays, the acetylation mutant showed a highly reduced affinity by the ACTR coactivator with respect to wild-type TRα, even in the absence of T_3, and in a DNA element, the CTE mutant is completely unable to recruit ACTR. Probably, as a consequence of the decreased hormone affinity, the mutant receptor CTE also shows a defect in corepressor release. TRα recruits corepressors in the absence of hormone, and after ligand binding the corepressors are released favoring the coactivator binding. The TRα acetylation mutant shows a constitutive binding to corepressors, even in the presence of hormone. A similar behavior has been also observed in other receptors such as AR, in which the acetylation mutant shows enhanced recruitment of nuclear receptor corepressor (NCoR)/phosphorylated mothers against decapentaplegic (Smad)/HDAC-1 complexes and reduced p300 binding compared with the wild-type AR (19).

The AR acetylation site is required for androgen-mediated gene activation (19, 50). The mutant TRα in the CTE lysine residues is also unable to transactivate a TRE-containing reporter gene in a T_3-dependent manner. The decrease in ligand affinity of the TRα acetylation mutant is coincident with the behavior of some naturally occurring mutants detected in human hepatocellular carcinoma cells (51). These mutants also show decreased ligand affinity and failure to transactivate a TRE-containing target gene and act as dominant negatives on TR-mediated transcription. Interestingly, the CTE mutant is not able to transactivate and also acts as a dominant negative of TRαwt mediated transcription. The CTE mutant shows a strong dominant-negative activity despite the finding that acetylation increases DNA binding *in vitro*. Because DNA binding appears to be required for dominant-negative activity, the possibility that TRα acetylation does not enhance DNA binding in the living cell or that little of DNA-bound TRα is acetylated, even in the presence of T_3, cannot be dismissed. On the other hand, due to the inability of the CTE mutant to bind hormone and exchange corepressors by coactivators is not possible to affirm that, as in the case of other nuclear receptors, acetylation is required for ligand-dependent transactivation.

Another interesting property of the CTE mutant is that, unlike the wild-type receptor, the unliganded acetylation mutant does not stimulate the core GH promoter containing a negative TRE and that it has lost the ability of enhancing basal AP-1 activity. The molecular mechanisms underlying the transcriptional effects of the unliganded

receptors are not well understood. There is the possibility that receptor acetylation could be required *in vivo* for this ligand-independent effect, but the finding that the acetylation residues were also required *in vitro* to observe interaction with coactivators again suggests that it could be secondary to the important role of these residues on overall receptor structure and stability.

Besides lacking ligand-independent activation on negative elements or AP-1 motifs, the CTE mutant does not mediate ligand-dependent transrepression. It is well known that AP-1 complexes play a central role in the control of normal cell proliferation, survival, and oncogenesis. Wild-type TRs enhance c-Jun/AP-1 activity in the absence of T_3 but inhibit this activity in the presence of T_3 (5, 7, 52). We have demonstrated that the acetylation mutant has a strongly reduced ability to stimulate AP-1 activity in the absence of hormone and has completely lost the ability to suppress AP-1 function in the presence of T_3. Therefore, this mutant that acts as a dominant-negative receptor of the wild-type TR in transactivation could also interfere with the antiproliferative effects of the liganded TRs and may allow genes involved in cell proliferation and survival to be expressed under T_3 conditions that would normally be growth suppressive. The CTE mutant not only does not inhibit AP-1 activity but also is not able to inhibit fibroblast transformation in the presence of physiological doses of T_3, showing that the lysine residues are also required for ligand-dependent antitransforming effects of TRα, although the mutant receptor maintains the ability to reduce transformation in the absence of ligand.

In summary, our results show that TRα can be acetylated in the CTE and that ligand binding stimulates receptor acetylation and increases heterodimeric binding to its cognate element. Because mutation of the residues that are targets for acetylation also alters ligand binding and association with coactivators and corepressors, a direct functional role of acetylation on ligand-dependent transcriptional activation or repression cannot be unambiguously established in the case of this receptor. More significantly, our results reveal the important and unexpected role of the CTE region on receptor conformation that affects ligand affinity, transactivation, transrepression, and antitransforming properties of TRα.

Acknowledgments

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