MOLECULAR AND CELLULAR BIOLOGY, Aug. 1997, p. 4687–4695 Vol. 17, No. 8
0270-7306/97/$04.00+0

Mutations in the Conserved C-Terminal Sequence in Thyroid Hormone Receptor Dissociate Hormone-Dependent Activation from Interference with AP-1 Activity

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Received 29 July 1996/Returned for modification 1 October 1996/Accepted 15 May 1997

A short C-terminal sequence that is deleted in the v-ErbA oncoprotein and conserved in members of the nuclear receptor superfamily is required for normal biological function of its normal cellular counterpart, the thyroid hormone receptor alpha (T3Rα). We carried out an extensive mutational analysis of this region based on the crystal structure of the hormone-bound ligand binding domain of T3Rα. Mutagenesis of Leu398 or Glu401, which are surface exposed according to the crystal structure, completely blocks or significantly impairs T3-dependent transcriptional activation but does not affect or only partially diminishes interference with AP-1 activity. These are the first mutations that clearly dissociate these activities for T3Rα. Substitution of Leu400, which is also surface exposed, does not affect interference with AP-1 activity and only partially diminishes T3-dependent transactivation. None of the mutations affect ligand-independent transactivation, consistent with previous findings that this activity is mediated by the N-terminal domain of T3Rα. The loss of ligand-dependent transactivation for some mutants can largely be reversed in the presence of GRIP1, which acts as a strong ligand-dependent coactivator for wild-type T3Rα. There is excellent correlation between T3-dependent in vitro association of GRIP1 with T3Rα mutants and their ability to support T3-dependent transcriptional activation. Therefore, GRIP1, previously found to interact with the glucocorticoid, estrogen, and androgen receptors, may also have a role in T3Rα-mediated ligand-dependent transcriptional activation. When fused to a heterologous DNA binding domain, that of the yeast transactivator GAL4, the conserved C terminus of T3Rα functions as a strong ligand-independent activator in both mammalian and yeast cells. However, point mutations within this region have drastically different effects on these activities compared to their effect on the full-length T3Rα. We conclude that the C-terminal conserved region contains a recognition surface for GRIP1 or a similar coactivator that facilitates its interaction with the basal transcriptional apparatus. While important for ligand-dependent transactivation, this interaction surface is not directly involved in transrepression of AP-1 activity.

The actions of thyroid hormone 3,5,3′-triiodo-L-thyronine (T3) are mediated through binding to receptors (T3Rs) which belong to the nuclear receptor superfamily of transcription factors, encoded by the c-*erbA*α and c-*erbA*β genes (42, 49). Nuclear receptors are ligand-activated transcription factors possessing highly conserved DNA binding domains (DBDs) and moderately conserved ligand binding domains (LBDs) (8, 18). The T3Rs recognize the consensus half site AGGTCA, which is also recognized by the related retinoic acid (RA) and vitamin D3 receptors (8). This sequence can be arranged as a direct repeat, a palindrome, or an inverted repeat, and the spacing between the half sites and their relative orientation determine receptor specificity and the nature of the transcriptional response (33, 46). Unliganded T3Rα can repress promoters that contain T3 response elements (T3REs), and this repression is reversed upon T3 binding, resulting in net activation (14, 43). Unliganded T3Rα can also activate transcription from promoters which contain an inverted repeat of a variant half site, and this activation is reversed upon T3 binding (38). Whereas the ligand-independent repression of basal promoter activity and the ligand-dependent activation functions are mediated by sequences within the C-terminal LBD (5–7, 35, 37), the ligand-independent activation function is mediated through the N-terminal activation domain (38).

A short region in the C terminus of T3Rα is highly conserved among different nuclear receptors (15, 37). This region, which is deleted in the oncogenic derivative of T3Rα, v-ErbA, is critical for both ligand-dependent transactivation and interference with AP-1 activity (37). The conserved C-terminal region is also essential for transactivation by glucocorticoid and estrogen receptors (GR and ER) (15) and the RA and retinoid X receptors (RARs and RXRs) (17, 30). This region has also been implicated in the release of a putative corepressor(s) necessary for transcriptional silencing by T3Rβ (6).

The activity of T3Rα is modulated by interactions with other proteins. In vitro, RXRs form heterodimers with T3Rs that bind to T3REs more efficiently than T3R homodimers (9, 25, 29, 31, 50, 52). In vivo, coexpression of T3Rs with RXRs results in increased ligand-dependent and ligand-independent transcriptional activation (9, 25, 29, 31, 38, 50, 51). Most recently, proteins which act as putative coactivators (11, 23, 27, 28, 34, 41) and putative corepressors (10, 12, 26, 41) and which inter-

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act with the LBD of T3Rs and other nuclear receptors have been identified.

On the other hand, AP-1 complexes, composed of either Jun homodimers or Jun-Fos or Jun-ATF2 heterodimers (for a review, see reference 2), interfere with ligand-dependent transactivation by T3Rs (for a review, see reference 39). Reciprocally, liganded T3Rs and other nuclear receptors, as first described for the GR, interfere with AP-1 activity (for a review, see reference 39). The interference with AP-1 activity is not mediated by direct binding of nuclear receptors to DNA and requires the short conserved region located at their C termini (37, 52). The molecular mechanisms underlying this cross talk have not been definitively established. The difficulty of identifying mutations which dissociate transactivation from transrepression suggested that AP-1 and liganded nuclear receptor may compete for a common cofactor (for a review, see reference 39). Although some mutations were identified in the DBD of GR which discriminate between ligand-dependent activation and interference with AP-1 activity (21, 22), the consequence of these mutations to the overall GR structure and their effect on the interaction of GR with other proteins, such as coactivators, have not been studied to date, and therefore the mechanistic implications of these mutations are limited at present.

In light of its important role for the biological activities of nuclear receptors in general and T3R in particular, we carried out a mutational analysis of the C-terminal conserved region based on information provided by the crystal structure of the LBD of T3Rα (47). We targeted surface-exposed residues located within this region which are likely to form a recognition surface for other proteins that modulate T3R activity. Through this analysis we identified mutations that dissociate ligand-dependent activation from interference with AP-1 activity. We present evidence that GRIP1, originally isolated as a putative coactivator for GR, ER, and androgen receptors (23), acts as a coactivator for T3Rα and interacts with T3Rα in vitro in a manner which correlates very well with the ability of the mutant proteins to carry out ligand-dependent activation. In addition, we show that the short conserved region can activate transcription when fused to the DBD of the yeast transcription factor GAL4. However, mutagenesis of this region has distinctly different consequences in the context of the full-length receptor compared with that of the GAL4 fusion proteins. These data underscore the importance of the conserved region for the biological activities of T3Rα and show that it may consist of two interaction surfaces, one involved in GRIP1 binding and ligand-dependent activation and the other involved in transrepression of AP-1 activity.

MATERIALS AND METHODS

Cell culture, transient transfection, and LUC and CAT assays. CV-1, HeLa, and COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% bovine calf serum and 5 and 10% fetal bovine serum, respectively. The calcium phosphate coprecipitation method was used to transfect CV-1 cells with 1 μg of reporter plasmid, 100 ng of expression vector, and pUC18 to a total of 3 μg of DNA per 60-mm-diameter dish. After 5 to 8 h of incubation with the precipitates, cells were washed once with phosphate-buffered saline and then maintained in DMEM supplemented with 0.55% bovine calf serum in the presence or absence of T3 (10^{-7} M). Chloramphenicol acetyltransferase (CAT) or luciferase (LUC) enzyme activities were determined as previously described (38).

For the transient transfection experiment using mouse mammary tumor virus (MMTV)-T3RE-CAT, HeLa cells were transfected by electroporation as previously described (48). Transfections included 5 μg of reporter plasmid, 0.5 μg of expression vectors, 1 μg of β-actin-galactosidase for internal control, and 0.5 μg of GRIP expression vector. After electroporation, the cells were treated with vehicle (ethyl alcohol) or T3 (10^{-7} M). After 36 h cells were harvested and CAT activities were determined (48).

COS-7 cells were transfected by a liposome-mediated transfection procedure (Lipofectamine; Gibco) with 10 μg of the indicated expression vector per 100-mm-diameter dish. After 6 h, cells were washed with phosphate-buffered saline once and were maintained in DMEM supplemented with 10% fetal bovine serum. Twenty-four hours later, cells were harvested. Whole-cell extracts were prepared and used in the mobility shift analysis or the T3 binding assay.

Plasmids. Reporter plasmids −73 Col-CAT (1), 2XT3RE-CAT (20), RSV_{180}-LUC (32), and MMTV-T3RE-CAT (45) and the expression vector for T3Rα (pSG5-c-ErbA [37]) have been described.

For the generation of the full-length mutant constructs, single-stranded mutagenic primers were synthesized corresponding to the last 15 amino acids of T3Rα with a *Bam*HI site after the stop codon (sequences available upon request). Using a 5′ primer, GGCCCTGCTGCAGGCCGTG (positions 936 to 955), centered around a *Pst*I site within the LBD and the mutant C-terminal primers, we amplified sequences by PCR, digested them with *Pst*I and *Bam*HI, and exchanged them with the corresponding fragment of pSG5-c-ErbA. All mutants were confirmed by sequencing.

For the generation of the wild-type GAL4 fusion construct, a *Hin*dIII-*Sac*I fragment of pSG424 (40), a *Sac*I-*Eco*RI fragment of pSG5-c-ErbA, and a *Hin*dIII-*Eco*RI fragment of pSG6 (16) were ligated to get pSG6-GAL4-CEA-C-WT. For the generation of the mutant fusion constructs, *Sac*I-*Xba*I fragments of pSG5-c-ErbA-M5-11 were exchanged with those of pSG6-GAL4-CEA-C-WT.

GST-GRIP1 was described previously (23).

Mobility shift analysis. Preparation of nuclear extracts, recombinant T3Rα, and mobility shift analysis were as described previously (38). For supershift analysis, recombinant T3Rα or whole-cell extracts from COS-7 cells ectopically expressing the various proteins were incubated with T3Rα-specific antiserum (38) or nonimmune rabbit serum on ice for 15 min prior to the binding reaction.

T3 binding assay. [^{125}I]T3 binding assays were performed on the in vitro-translated wild-type and mutant receptors as described previously (3). The wild-type T3Rα and the mutants were translated in vitro with the TNT expression system (Promega) and used in the T3 binding assay, and K_d values were calculated with the Prism computer program (GraphPad Software, Inc.).

GST pulldown assay. In vitro interactions between T3Rα and GRIP1 were examined by the glutathione *S*-transferase (GST) pulldown assay as described previously for ER-RIP140 interactions (11).

RESULTS

Mutagenesis of the conserved C-terminal sequence in T3Rα. We have generated mutations within the conserved C-terminal region of T3Rα, focusing on three residues that are located at the surface of the protein according to the crystal structure (47) and therefore are candidates for interacting with cofactors: Leu398, Leu400, and Glu401 (Fig. 1A and B). The nonpolar residues, Leu398 and Leu400, were changed either to a smaller nonpolar residue (alanine, M7 and M8) or to an acidic residue (aspartic acid, M9 and M10). The conserved Glu401 was substituted with either a polar residue (glutamine, M5) or a small nonpolar residue (alanine, M6).

We first tested the mutant proteins for proper expression. COS-7 cells were transfected with expression vectors specifying the wild-type or the mutant receptors, and whole-cell extracts were prepared and used in the mobility shift assay with the myosin heavy chain (MHC)-T3RE oligonucleotide as a probe. When extracts prepared from COS-7 cells transfected with wild-type T3Rα were used, two bands were observed corresponding to the T3Rα:T3Rα homodimers and T3Rα:RXRα heterodimers, as previously described (11) (Fig. 1C). Upon coincubation with COS-7 cell extracts containing RXRα, binding to the MHC-T3RE was increased approximately 20-fold and most of the binding was by T3Rα:RXRα heterodimers as shown previously (13). Extracts prepared from cells transfected with the mutant T3Rα expression constructs gave rise to levels of DNA binding activity similar to those exhibited by the wild-type T3Rα-containing extracts when preincubated with RXRα. Extracts containing RXRα alone or nontransfected cell extracts did not display any T3RE binding activity (data not shown) (13). There were no significant differences in DNA binding when smaller amounts of extracts were used (data not shown). These data indicate that all mutant receptors were expressed at levels comparable to those of the wild-type T3Rα, that they have all retained their ability to heterodimerize with RXRα, and that they all bind DNA.

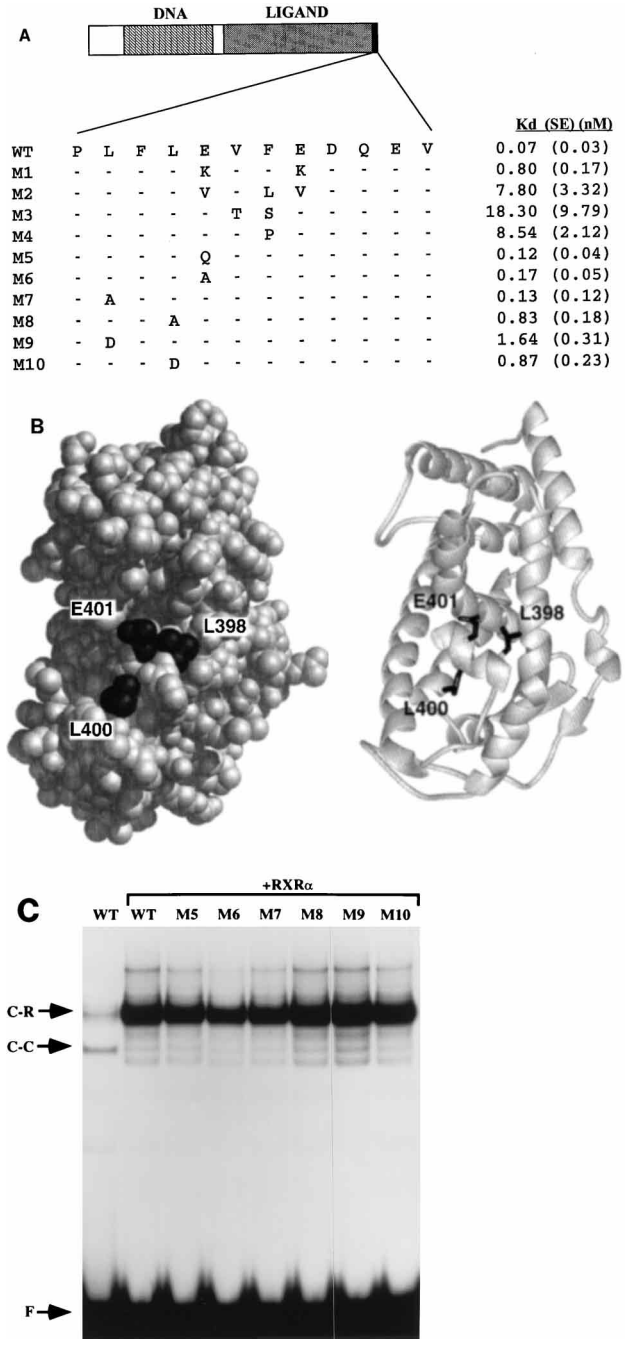

FIG. 1. Detailed mutational analysis of the conserved C-terminal region of T3Rα. (A) The sequences of the conserved C-terminal regions of T3Rα and the various mutants are shown. For the mutants, only those residues which are replaced are indicated. To the right of the alignment are the T3 dissociation constants (Kd) determined as detailed in Materials and Methods. Standard errors (SE) are indicated in parentheses. (B) Representation of T3Rα LBD showing the surface locations of the mutated residues, based on the X-ray structure (47). The side chains of residues L398, L400, and E401 are black. The left panel shows a space-filling model of the complete LBD. The right panel shows a ribbon model of the LBD main chain, along with the side chains of residues L398, L400, and E401. Computer modeling was performed with Midas Plus (Computer Graphics Laboratory, University of California, San Francisco). (C) COS-7 cells were transfected with expression vectors encoding the wild-type (WT) T3Rα, M5 to M10, or RXRα (10 μg each) by the Lipofectamine transfection procedure. After 36 h, whole-cell extracts were prepared. The extracts containing T3Rα were preincubated with extracts containing RXRα and were used in the mobility shift assay with ^{32}P-labeled MHC-T3RE as the probe, as indicated. The migration positions of free probe (F) and homodimeric (C-C) and heterodimeric (C-R) complexes are indicated.

Since all activities of T3Rα described to date—ligand-dependent activation, interference with AP-1 activity, and ligand-independent activation—are modulated by hormone binding, we also examined the ability of the mutant receptors to bind T3. Scatchard analysis showed that the dissociation constants of the mutant receptors M1 and M5 to M10 were in the range of 0.12 to 1.64 nM, compared with 0.07 nM for the wild-type receptor (Fig. 1A). All mutant receptors were expected to be saturated with hormone in the experiments described below, since an approximately 60-fold excess of T3 (100 nM) over the K_d of the weakest binder was used. Therefore, a deficiency in T3 binding is unlikely to account for the differential activities of the mutant receptors that are described below.

Divergent effects of C-terminal mutations on different activities of T3Rα. To determine how the mutations affect the biological activities of T3Rα, we first tested the wild-type and mutant receptors for their ability to stimulate expression of 2XT3RE-tk-CAT (20), in which two copies of the palindromic T3RE upstream to the thymidine kinase (tk) promoter drive expression of the CAT reporter gene. CV-1 cells were cotransfected with 2XT3RE-tk-CAT and either an empty expression vector or expression vectors encoding the wild-type or mutant receptors. After transfection, the cells were either left untreated or treated with T3 for 36 h and CAT activities were determined. As shown in Fig. 2A, in the absence of T3 the wild-type and all mutant receptors repressed basal reporter expression by approximately 50%. In the presence of T3, wild-type T3Rα activated 2XT3RE-tk-CAT expression approximately 10-fold. M8 and M10 also stimulated reporter expression in response to T3, but activation was reduced by 30 and 60%, respectively, compared to wild-type T3Rα. M5 and M6 had approximately 20% of wild-type activity, while M1, M7, and M9 were inactive. These results suggest that among the surface residues in the conserved C terminus of T3Rα, Leu398 and Glu401 are most important for ligand-dependent activation by T3Rα.

We also tested the mutant proteins for their ability to interfere with AP-1 activity. An AP-1-dependent reporter in which a deletion derivative of the collagenase promoter is fused to the CAT reporter gene, −73 Col-CAT (1), was cotransfected into HeLa cells with expression vectors specifying the wild-type T3Rα or M5 to M10. After transfection, the cells were either left untreated or treated with T3 for 36 h. To maximize AP-1 activity, the cells were treated with tetradecanoyl phorbol acetate during the last 12 h, and CAT activities were determined. As we have shown previously (37), in the presence of T3, wild-type T3Rα led to a fivefold decrease in −73 Col-CAT expression (Fig. 2B). All mutants similarly repressed −73 Col-CAT expression except for M1, which has lost this activity (37). Interference with AP-1 activity by M7 and M9 were reduced about 50% compared to that by wild-type T3Rα. Similar results were obtained in CV-1 cells (data not shown). These data suggest that the ability to activate transcription in response to T3 is not required for T3Rα to interfere with AP-1 activity.

We have recently described the ability of T3Rα to activate transcription in a ligand-independent manner from a unique T3RE found in the long terminal repeat of the Rous sarcoma virus (RSV), which is relieved upon T3 binding (38). We tested the ability of the mutant receptors to activate the RSV_{180}-LUC reporter, in which 180 bp of the RSV long terminal repeat drive expression of the LUC gene (32). The wild-type T3Rα increased expression of RSV_{180}-LUC 10-fold, but the increase was lost in the presence of T3 (Fig. 2C). All of the mutants, including the M1 mutant that we have described previously (37), similarly activated transcription in the absence of T3, which was relieved in the presence of T3. However, for M1 the

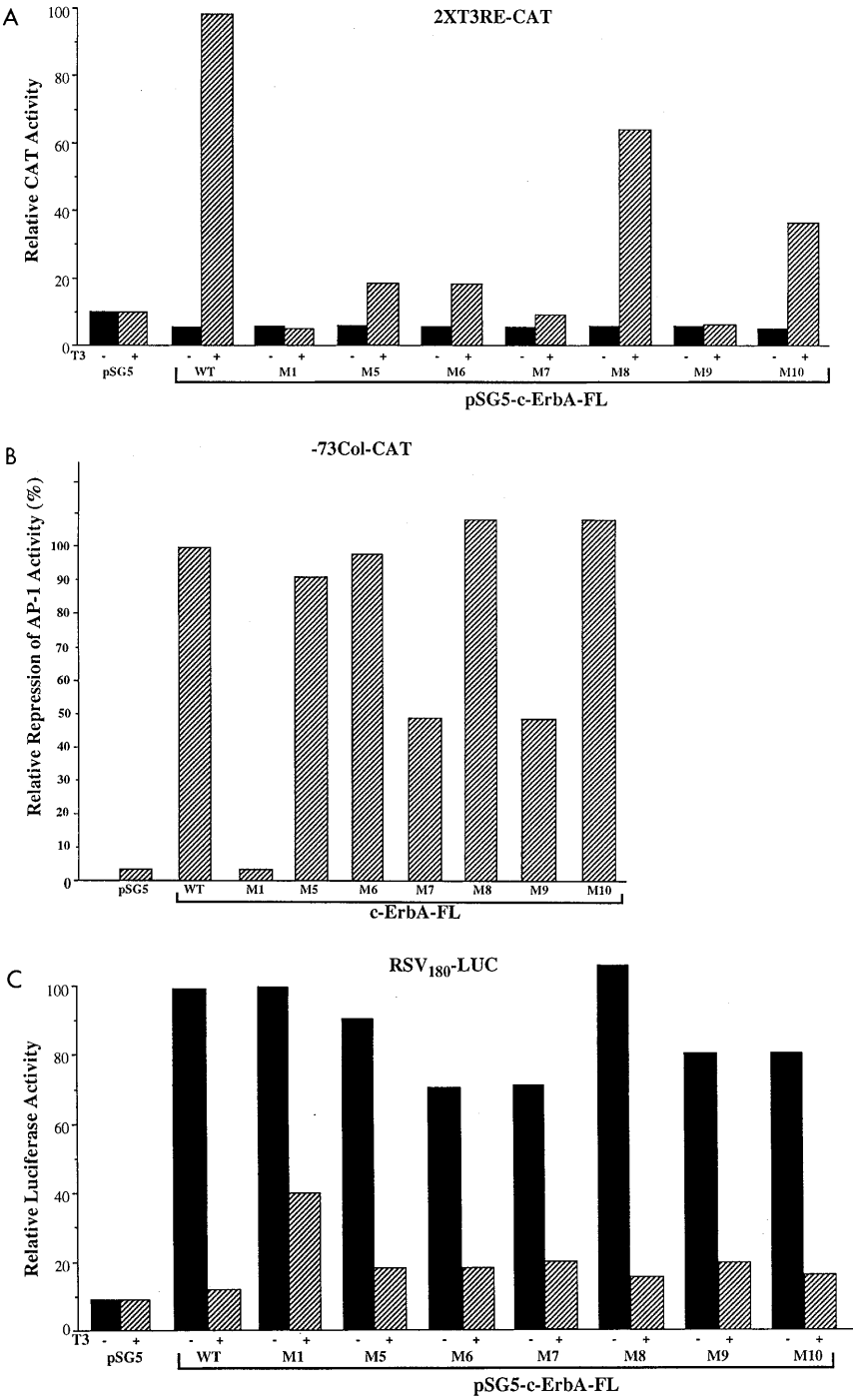

FIG. 2. Biological activities of the C-terminal mutants of T3Rα. (A) CV-1 cells were cotransfected with 2XT3RE-tk-CAT (1 μg) reporter plasmid and expression vectors encoding wild-type (WT) T3Rα, M1, M5 to M10, or the empty expression vector pSG5 (100 ng each) by the calcium phosphate transfection procedure. Cells were either left untreated (−) or treated (+) with T3 (10^{-7} M) as indicated and were harvested after 36 h, and CAT activities were determined. CAT activity in the presence of transfected wild-type T3Rα and T3 is set at 100%. The results represent the averages of at least three experiments. (B) HeLa cells were transfected with the −73 Col-CAT reporter and 250 ng of expression vectors encoding the wild-type or mutant T3Rs to measure their ability to interfere with AP-1 activity. Cells were either left untreated or treated with T3 (10^{-7} M) as indicated, treated with tetradecanoyl phorbol acetate (50 ng/ml) at 24 h, and harvested after 36 h, and CAT activities were determined. Repression of AP-1 activity, which is the ratio of CAT activity in the absence of T3 divided by the CAT activity in the presence of T3 (five times higher), is arbitrarily set at 100% for wild-type T3Rα. Results represent the averages of three experiments. (C) Conditions were the same as for panel A except RSV_{180}-LUC reporter was used to measure the ability of the wild-type or mutant T3Rs to activate transcription in a ligand-independent manner. LUC activity in the presence of wild-type T3Rα and in the absence of T3 is arbitrarily set at 100%.

T3-dependent relief of activation was incomplete. These results confirm our previous findings (38) and suggest that the C-terminal conserved region in T3Rα is not involved in T3-independent activation of RSV_{180}-LUC. Furthermore, these results provide functional evidence that all mutants, with the possible exception of M1, respond to the levels of T3 used in these experiments similarly to the way wild-type T3Rα responds. Therefore, the effects on ligand-dependent activation and interference with AP-1 activity are not due to effects on ligand binding.

Mutations in the conserved C-terminal region have different consequences when it is tested as a chimeric activator. To investigate whether the extreme C terminus of T3Rα can activate transcription on its own, we fused the last 16 amino acids of the wild-type T3Rα to the DBD of the yeast transcriptional activator GAL4 (40) to generate the chimera G4-CEA-C-WT. G4-CEA-C-WT was cotransfected into CV-1 cells with a reporter construct in which five copies of the GAL4 response element control expression of the LUC reporter gene, 5XGAL4-LUC. G4-CEA-C-WT increased 5XGAL4-LUC expression approximately 50-fold more efficiently than the GAL4 DBD (Fig. 3A). When mutant sequences were fused to GAL4 and tested in a similar experiment, variable results were obtained (Fig. 3A). The M1 mutant did not affect the expression of 5XGAL4-LUC, and M2 had only 5% of wild-type activity. All other mutants activated 5XGAL4-LUC at levels ranging between 25 and 85% of wild-type activity, except for M9, which had 2.5-fold higher activity than did the wild type. In particular, M3, M4, M7, and M9, which are completely inactive in the full-length T3Rα context for ligand-dependent transactivation (37) (Fig. 2B), were quite active when fused to the GAL4 DBD. These results indicate that the C-terminal activation surface behaves differently when fused to a heterologous DBD than it does in its natural setting. These differences are likely due to structural alterations induced by ligand binding which only affect the full-length proteins.

To test whether the differences observed in the activation of 5XGAL4-LUC were due to variable expression of the fusion proteins, vectors specifying the expression of the wild-type or mutant GAL4 fusion proteins were transfected into COS-7 cells. After 36 h, whole-cell extracts were prepared and used in the mobility shift assay with the GAL4 response element as a probe. As shown in Fig. 3B and C, extracts prepared from cells transfected with G4-CEA-C-WT gave rise to two major shifted bands in the presence of nonimmune serum. Preincubation with a T3Rα-specific antiserum that was raised against the conserved C-terminal region (38) resulted in the disappearance of the upper band and the appearance of two slower-migrating bands, indicating the presence of the T3Rα epitope in all of the fusion proteins. We do not know the nature of the faster-migrating complex that is not affected by the antiserum, which could be a proteolytic fragment of the fusion construct that has lost the T3Rα epitope. The two supershifted bands may represent alternative conformations of the GAL4 fusion protein plus the antiserum complex. There were no significant differences in DNA binding when smaller amounts of extracts were used (data not shown). These data indicate that all the mutant fusion proteins were expressed at levels similar to that of G4-CEA-C-WT, and thus, variations in activities described above are not due to differences in expression or in DNA binding activities.

T3Rα interacts with GRIP1 in vivo and in vitro: involvement of the conserved C-terminal region. A novel protein, GRIP1, was recently isolated which interacts with the LBDs of the GR, ER, and androgen receptors in a ligand-dependent manner in the yeast two-hybrid system and in vitro (23). Since mutations

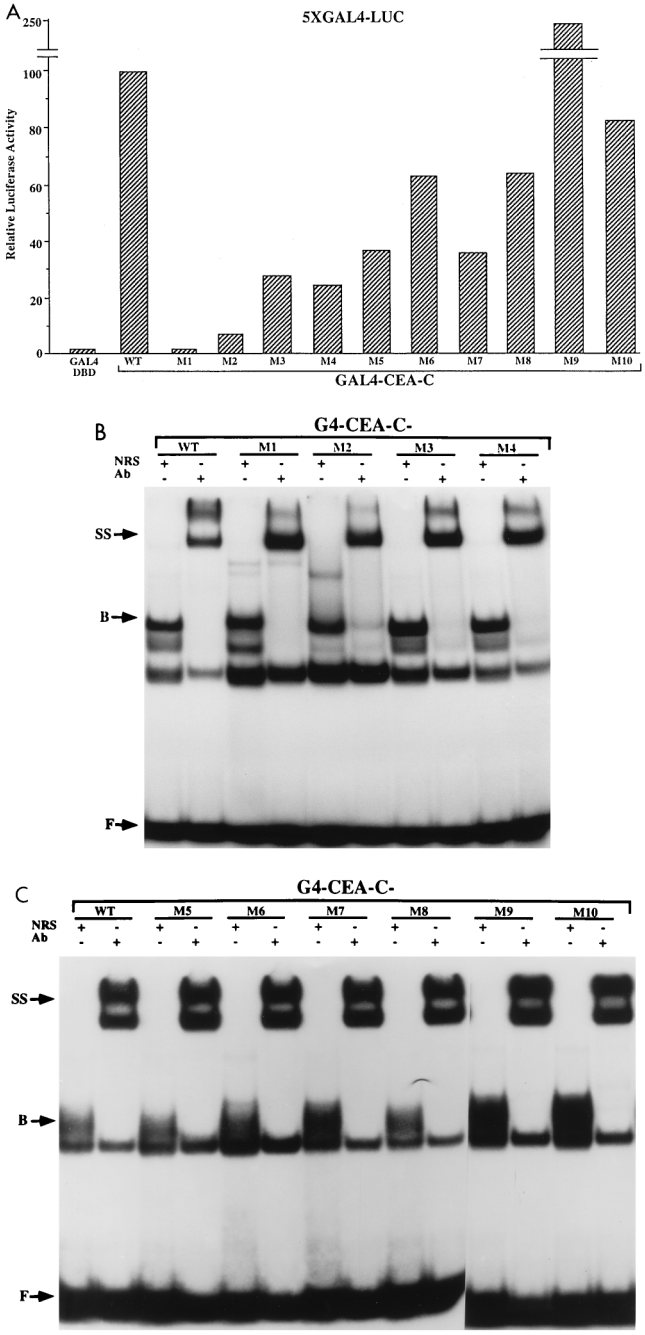

FIG. 3. Transcriptional properties of C-terminal mutants of T3Rα when fused to the GAL4 DBD. (A) The last 16 amino acids of either the wild-type (WT) or mutant T3Rs were fused to the DBD of the yeast transcriptional activator GAL4. CV-1 cells were cotransfected with vectors specifying expression of the fusion proteins and the 5XGAL4-LUC reporter plasmid. After 24 h, cells were harvested and LUC activities were determined. Activation by the wild-type fusion protein was arbitrarily set at 100%. The results represent the averages of at least three experiments. (B and C) The GAL4 fusion proteins are expressed at similar levels. Expression vectors specifying the production of wild-type and mutant proteins were transfected into COS-7 cells with Lipofectamine (Gibco). After 36 h, whole-cell extracts were prepared. Extracts were either preincubated with nonimmune rabbit serum (NRS) or with an antipeptide T3Rα antiserum (Ab) and used in the mobility shift assay with the GAL4 response element as a probe. The migration positions of the free (F), bound (B), and supershifted (SS) complexes are indicated.

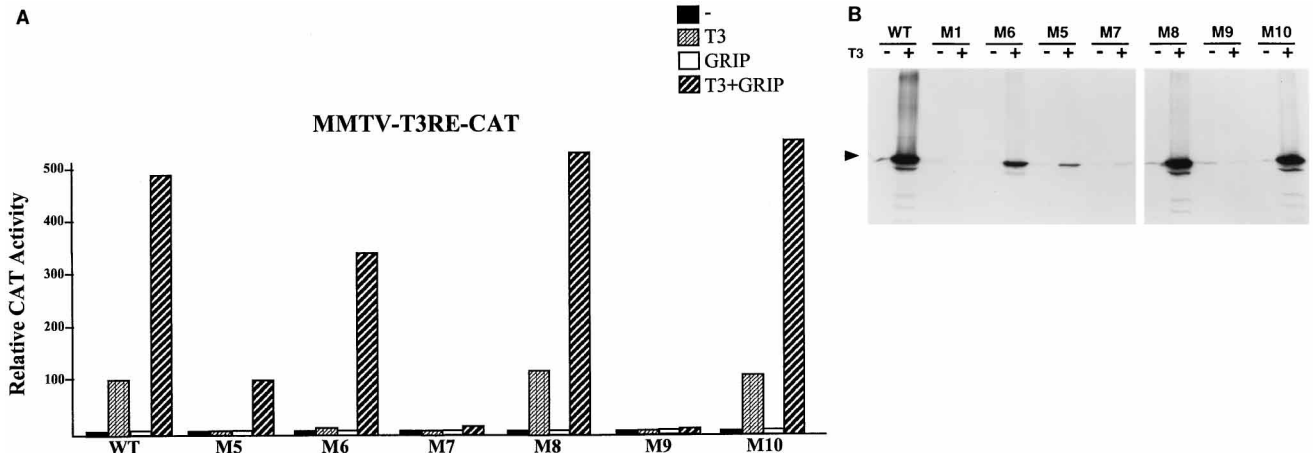

FIG. 4. Ligand-dependent in vivo and in vitro interactions between the wild-type and mutant T3Rα and GRIP1. (A) HeLa cells were cotransfected by electroporation with the MMTV-T3RE-CAT reporter plasmid (5 μg) and expression vectors encoding wild-type (WT) T3Rα, M1, or M5 to M10 (500 ng each) in the presence and absence of a GRIP1 expression vector (500 ng) as indicated. Cells were either left untreated or treated with T3 (10^{-7} M) as indicated and harvested after 36 h, and CAT activities were determined. CAT activity elicited by wild-type T3Rα in the presence of T3 is set at 100%. The results represent the averages of at least three experiments done in triplicate. (B) In vitro interactions between GRIP1 and wild-type or mutant T3Rs, as indicated, were tested in the GST pulldown assay. GRIP1 was expressed in *E. coli* as a GST fusion protein and purified on glutathione agarose beads. This was then used in the GST pulldown assay with the cell-free-translated, ^{35}S-labeled T3Rs in the presence (+) and absence (−) of T3 (10^{-7} M) as detailed in Materials and Methods. The input amounts of ^{35}S-labeled wild-type and mutant T3Rs were the same, as determined by both autoradiography of sodium dodecyl sulfate-polyacrylamide gel electrophoresis and [^{125}I]T3 binding (data not shown). The arrowhead indicates the migration position of T3Rs.

within a conserved C-terminal region of GR and ER which is similar to the C-terminal region in T3R (15, 37) block the ability of these receptors to activate transcription, we examined the possible interaction of wild-type T3Rα and its mutants with GRIP1 in vivo and in vitro. We first used the transient transfection assay to test several reporter constructs representing different classes of T3REs and observed the most reproducible and significant results with the MMTV-T3RE-CAT reporter (45), in which one copy of the palindromic T3RE was inserted in front of a deletion derivative of the MMTV promoter, which drives expression of the CAT reporter gene (30a). After transfection, the cells were either left untreated or treated with T3 for 36 h and CAT activities were determined. In the absence of GRIP1, the wild-type T3Rα activated MMTV-T3RE-CAT expression approximately 10-fold in response to T3, which was further increased in the presence of T3 and GRIP1 (Fig. 4). The transcriptional responses elicited by M8 and M10 in the absence or presence of GRIP1 were similar to those of the wild-type T3Rα. In contrast, the M7 and M9 mutants had completely lost their ability to increase expression of the reporter, which was not significantly affected by GRIP1 coexpression. Interestingly, M5 and M6, which are severely defective in their T3-dependent transactivation function, were significantly stimulated by GRIP1: M5 had 20% (equal to the activity of the wild-type receptor in the presence of T3 alone) and M6 had 70% of the activity elicited by the wild-type T3Rα.

We then tested the wild-type and mutant T3Rs for their ability to associate with GRIP1 in vitro. GRIP1 was expressed in *Escherichia coli* as a GST fusion protein and was used in the GST pulldown assay with cell-free-translated, ^{35}S-labeled wild-type and mutant T3Rs in the presence or absence of T3. As shown in Fig. 4B, GRIP1 bound to wild-type and mutant T3Rα weakly in the absence of T3. However, this binding was significantly increased in the presence of T3 for wild-type T3Rα. GRIP1 binding to GST alone was weak and was not enhanced by the addition of T3 (data not shown). T3-enhanced GRIP1 binding was observed with mutants M8 and M10, similar to that of wild-type T3Rα. In the presence of T3, the M5 and M6 mutants bound GRIP1 approximately 10 and 20% as efficiently as wild-type T3Rα, respectively, whereas the M7 and M9 mutants exhibited the weakest GRIP1 binding activity, which was not considerably different from the background binding to the GST moiety alone (Fig. 4B). The input amounts of ^{35}S-labeled wild-type and mutant T3Rs were the same, as determined by both autoradiography of sodium dodecyl sulfate-polyacrylamide gel electrophoresis and [^{125}I]T3 binding assay (data not shown).

DISCUSSION

In light of its involvement in all biological activities of T3Rα and its conservation among other members of the nuclear receptor superfamily (6, 7, 15, 17, 30, 37), we undertook a detailed mutational analysis of the conserved C terminus of T3Rα based on the crystal structure of the hormone-bound LBD (47). According to the X ray structure, this region contains a polyproline turn followed by a single alpha helix that is seven amino acids long (from Leu398 to Phe403), which is then followed by five unstructured residues (47). The strongly hydrophobic internal face of this alpha helix directly contacts the T3 ligand (47). As shown in Fig. 1B, three of the residues within this region (Leu398, Leu400, and Glu401) are surface exposed and thus are likely to form an interaction surface recognized by cofactors of T3Rα, thereby directly contributing to ligand-dependent transactivation or other activities of T3Rα, such as interference with AP-1 activity. We therefore selected these residues for mutagenesis.

Substitution of Leu398 with either alanine (M7) or aspartic acid (M9) was sufficient to eliminate the T3-dependent transactivation ability of T3Rα. Interestingly, both M7 and M9 retained the ability to interfere with AP-1 activity, albeit at a lower level than that of wild-type T3Rα. On the other hand, substitution of Leu400 with alanine (M8) or aspartic acid (M10) resulted in receptors which could still activate transcription in response to T3 and were fully competent in repressing AP-1 activity. It is interesting to note that Leu398 is much better conserved among other members of the nuclear receptor superfamily than is Leu400 (15, 37). Moreover, the crystal

structure of the ligand-bound RARγ suggests that the homolog of Leu398 (Leu411 in RARγ) is also surface exposed (36). In contrast, Leu400 is less conserved and is substituted more frequently and by more distant amino acids, such as polar residues (arginine and serine in RARγ and progesterone receptor, respectively) or positively charged residues (glutamine, RARα, and RARβ) in other receptors. Substitution of the residue corresponding to Leu398 in T3Rβ (Leu 454) also blocks T3-dependent activation (44). A double mutation in ER, in which residues corresponding to Leu398 and Phe399 (Leu543 and Leu544) are substituted with alanines, is also no longer capable of activating transcription (15), supporting this hypothesis. This suggests that Leu398 in T3Rα and the corresponding residues in other nuclear receptors may have a more important role in hormone-dependent transactivation than does Leu400. It is also interesting to note that according to the crystal structure of the three surface-exposed residues, Leu398 is the one which is most closely associated with helix 3 of the LBD, spanning residues 213 to 236 of the N terminus (47). Helix 3 corresponds to a region of high conservation among members of the nuclear receptor superfamily (18) and contributes directly to ligand binding (47). It is possible that Leu398, but not Leu400, is involved in the interaction of T3Rα with a coactivator that is required for efficient ligand-dependent transactivation and that helix 3 may provide part of the interaction surface.

Mutagenesis of Glu401, which is not only at the surface of T3Rα but extends into the solvent as an ordered residue (47), greatly reduced ligand-dependent activation but did not reduce the ability to interfere with AP-1 activity. It is interesting to note that when Glu401 was substituted along with Glu404, or Phe403 and Glu404, the mutant receptors lost both ligand-dependent transcriptional activation and the ability to interfere with AP-1 activity (37). This suggests that other residues that are C terminal to Glu401 may contribute to interference with AP-1 activity. These residues may form an interaction surface recognized by a cofactor, which is also important for AP-1-mediated transactivation (39).

All of the mutants were fully capable of activating the RSV_{180}-LUC reporter independently of ligand binding. This is consistent with our previous results, which suggested that T3-independent transactivation is mediated by an N-terminal activation domain (38). Ligand-dependent relief of this activation was impaired in M1 but not in other mutants (Fig. 2C). The relief of T3-independent transcription may involve a change in the conformation of T3Rα such that the ability of its N-terminal activation domain to contact the transcriptional initiation complex or the ability of its DBD to interact with the RSV-T3RE is impaired (13, 38).

When a peptide containing the 16 most C-terminal residues of T3Rα was fused to the DBD of the yeast transcription factor GAL4, it was found to function as a potent activation domain both in mammalian cells (Fig. 3A) and the budding yeast (39a). This is consistent with recent findings in which longer portions of the T3Rα C terminus were found to activate transcription, independently of ligand binding, when fused to the GAL4 DBD (6, 7, 15, 17, 30, 44). However, when the different mutations described above were examined in the context of the 16-amino-acid peptide fused to GAL4 DBD, they produced different effects from those produced in the context of the native receptor. For example, M3, M4, M7, and M9, which are incapable of T3-dependent transactivation in the context of the full-length receptor, were efficient activators in the GAL4 fusion context. In fact, M9 was 2.5-fold more efficient than wild-type peptide in transactivation when fused to the GAL4 DBD. Similarly, M5 and M6, which are significantly impaired in T3-dependent transactivation in the full-length context, efficiently activated transcription when fused to GAL4. These data suggest that, although this region may function as an independent activation domain, its conformation is different when expressed as a GAL4 fusion protein than when expressed as the intact receptor. In the latter case, its conformation is likely to be modulated in response to ligand binding such that it can interact with its target only after T3 binding to the LBD. That target is most likely to be different from the one recognized by the 16-amino-acid segment when fused to the GAL4 DBD.

The major physiologically relevant targets for the conserved C-terminal region in T3Rs and other nuclear receptors are yet to be identified. Recent screening of expression libraries or the use of the yeast two-hybrid system resulted in the identification and cloning of candidate cofactors such as Sug1 (28), RIP140 (11), TIF-1 (27), SRC-1 (34), GRIP1 (23) and CBP (24). Since mutations within a conserved C-terminal region of GR and ER which is similar to the C-terminal region in T3Rs (15, 37) block the ability of these receptors to activate transcription in response to hormone (15) and since GRIP1 associates with these receptors in a hormone-dependent manner (23), we examined possible interactions of wild-type T3Rα and its mutants with GRIP1 in vivo and in vitro. We first tested several reporter constructs representing different classes of T3REs and observed that the use of the MMTV-T3RE-CAT reporter and GRIP1 gave the most consistent effects on T3-dependent T3Rα activity, and therefore they were used in this analysis (30a). The reason for the differences observed in the response of T3Rα to GRIP1 with different reporter constructs is not known and requires further investigation. GRIP1 enhanced the ability of wild-type T3Rα to increase transcription from the MMTV-T3RE-CAT reporter approximately fivefold in a transient transfection assay (Fig. 4). M8 and M10 were similarly stimulated by GRIP1. In contrast, the M7 and M9 mutants, which had completely lost their ability to transactivate, were not significantly stimulated by GRIP1. Interestingly, M5 and M6, which are severely defective in their T3-dependent transactivation function, were significantly stimulated by GRIP1: M5 had 20% and M6 had 70% of the activity elicited by the wild-type T3Rα. This suggests that the substitutions in M5 and M6 decrease the affinity of the receptors to interact with GRIP1, which results in a significant loss in their ability to activate transcription. However, when there is an excess of GRIP1 present, this defect is at least in part overcome and the ability of M5 and M6 to activate transcription significantly increases.

We also tested the wild-type and mutant T3Rs for their ability to interact with GRIP1 in vitro. We found excellent correlation between the ability of the various mutants to activate transcription and their ability to interact with GRIP1 in a T3-dependent manner. M7 and M9, which have lost their ability to activate transcription in response to T3, were not affected by GRIP1 coexpression in vivo and did not associate with it in vitro upon T3 binding. Conversely, M8 and M10, which are similar to wild-type T3Rα in their ability to activate transcription in the presence or absence of ectopically expressed GRIP1, associated with GRIP1 in a T3-dependent manner in the GST pulldown assay. M5 and M6, on the other hand, which are severely diminished in their ability to activate transcription in the absence of GRIP1, had detectable, but much weaker, T3-induced binding to GRIP1 compared with that of the wild-type T3Rα. These results suggest that GRIP1 may be an important cofactor for T3-dependent transcriptional activation. Alternatively, another cofactor with recognition properties similar to those of GRIP1 may mediate this function.

The discovery of mutants which are impaired in T3-depen-

dent transactivation function but fully capable of interference with AP-1 transcriptional activity has important mechanistic implications. Based on correlations we and others have drawn from mutations that block both T3-dependent transactivation and interference with AP-1 activity, we have recently suggested that the transcriptional interference between liganded nuclear receptors and AP-1 is due to competition for a cofactor that is required for efficient transactivation by either protein (for a review, see reference 39). Recently, CREB binding protein (CBP), which was previously shown to function as a transcriptional coactivator for the phosphorylated versions of CREB and c-Jun (5), was found to also function as a coactivator for several nuclear receptors (24). It was also shown that overexpression of CBP can restore AP-1 activity which was repressed by liganded GR or RAR (24). However, we found that a nonphosphorylatable mutant of c-Jun, which can no longer bind CBP (4), is fully capable of repressing GR activity (13a). Thus, CBP is unlikely to be the physiologically relevant target for which GR and c-Jun compete. Our present results suggest that the C-terminal activation domain of T3Rα contains at least two separate interaction surfaces: one for GRIP1 or a similar cofactor required for ligand-dependent activation, and the other for a yet-to-be-identified cofactor required for interference with AP-1 activity. Consistent with these conclusions, coexpression of GRIP1 does not relieve the inhibition of AP-1 transcriptional activity by liganded T3Rα, and it is therefore unlikely to be the cofactor mediating this activity (30b). The definitive test of this hypothesis has to await the definition of the interaction surface required for repression of AP-1 activity and the identification of its physiological target.

ACKNOWLEDGMENTS

We thank T. Deng, A. Munoz, M. Pfahl, M. R. Stallcup, M. Zenke, and X.-K. Zhang for the generous gifts of plasmids; R. Wagner for advice on mutagenesis; and J. Shinsako and H. Nguyen for technical assistance.

This work was supported by grants from the National Institutes of Health (CA50528) to M.K., from the Computer Graphics Laboratory at University of California at San Francisco Medical Center (P41-RR01081), and from the University of California AIDS Research Program (K93-SD-014) and the University of Oslo to F.S.

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