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A Conserved Lysine in the Thyroid Hormone Receptor–α1 DNA-Binding Domain, Mutated in Hepatocellular Carcinoma, Serves as a Sensor for Transcriptional Regulation

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Abstract

Nuclear receptors are hormone-regulated transcription factors that play key roles in normal physiology and development; conversely, mutant nuclear receptors are associated with a wide variety of neoplastic and endocrine disorders. Typically, these receptor mutants function as dominant negatives and can interfere with wild-type receptor activity. Dominant-negative thyroid hormone receptor (TR) mutations have been identified in over 60% of the human hepatocellular carcinomas analyzed. Most of these mutant TRs are defective for corepressor release or coactivator binding *in vitro*, accounting for their transcriptional defects *in vivo*. However, two HCC-TR mutants that function as dominant-negative receptors in cells display near-normal properties *in vitro*, raising questions about the molecular basis behind their transcriptional defects. We report here that a single amino acid substitution, located at the same position in the DNA-binding domain of both mutants, is responsible for their impaired transcriptional activation and dominant-negative properties. Significantly, this amino acid, K74 in TRα, is highly conserved in all known nuclear receptors and seems to function as an allosteric sensor that regulates the transcriptional activity of these receptors in response to binding to their DNA recognition sequences. We provide evidence that these two human hepatocellular carcinoma mutants have acquired dominant-negative function as a result of disruption of this allosteric sensing. Our results suggest a novel mechanism by which nuclear receptors can acquire transcriptional defects and contribute to neoplastic disease. *Mol Cancer Res; 8(1); 15–23. ©2010 AACR.*

Introduction

Thyroid hormone receptors (TR) are members of the nuclear receptor family of ligand-regulated transcription factors (1, 2). TRs bind to specific DNA sequences (denoted T3 response elements; TRE) and regulate the expression of adjacent target genes in response to T3 and T4 thyronine (1-3). Notably, TRs can either repress or activate transcription by alternatively recruiting corepressors or coactivators. These auxiliary proteins mediate the actual molecular events responsible for the suppression or activation of gene expression. The direction of the transcriptional response to hormone depends on the nature of the target gene (4, 5). Positive response genes are repressed in the absence of hormone agonist and are activated in its presence. Negative response genes exhibit the opposite effects. Multiple loci and alternative mRNA splicing produce a series of interrelated TR isoforms, with TRα1 and TRβ1 representing the predominant isoforms in most mammalian tissues (1-3).

Mutations in nuclear hormone receptors are associated with a wide variety of human endocrine, metabolic, and neoplastic diseases (6). Interestingly, many of these diseases are heterozygotic disorders and are caused by genetic lesions encoding dominant-negative mutant receptors that can interfere with the actions of the corresponding wild-type (WT) receptors. Inherited TR mutations are associated with the human endocrine disorder, resistance to thyroid hormone syndrome, whereas spontaneous TR mutations are found at high frequency in a variety of human cancers, including hepatocellular carcinoma (HCC), renal clear cell carcinoma, and several forms of thyroid and pituitary neoplasia (7-16).

We previously reported a study of the molecular basis behind the dominant-negative properties of TR mutants isolated from human HCCs (17). We showed that the dominant-negative TRβ1 mutants studied possessed amino acid substitutions that disrupted their ability to bind hormone, and/or altered their recruitment and release of corepressors or coactivators (17). In contrast, the two TRα1 HCC mutants analyzed behaved as strong dominant-negative inhibitors in cells, but displayed near-WT biochemical properties *in vitro*, leaving the molecular basis of their transcriptional defects unresolved (17). We report here that these two TRα1 mutants fail to activate transcription, and instead interfere with WT receptor function,

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due to amino acid substitutions at lysine 74, a highly conserved amino acid found in the DNA-binding domain (DBD) of virtually all known nuclear receptors. This amino acid makes crucial nucleotide-specific contacts with the DNA response element, and has been proposed to be a sensor by which interaction of the receptor with DNA is communicated allosterically to regulate the receptor's transcription response. We provide evidence that this is true for the HCC-TRα1 mutants analyzed here, and that the transcriptional defects/dominant-negative properties of these mutants represent a failure, at least in part, to correctly interpret whether they are bound to DNA or not. These results reveal a novel basis by which mutations in nuclear receptors can contribute to defective transcriptional regulation and to human neoplasia.

Materials and Methods

Molecular Clones and Cell Lines

The pSG5 human TRα1 WT and mutant constructs were generated as described previously using a QuikChange protocol (17). The Gal4-binding domain (GBD) TR fusions were created by subcloning each mutant into a pSG5 vector containing the GBD (pSG5-GBD; ref. 18). The GBD-TRα1-ΔABC fusion was generated by PCR to amplify the appropriate region of TRα1 using the following primers: (sense) ggtac cgcgg ccgcc accat ggact tggtt ctaga tgac, and (antisense) agatc tgcta gctta gactt cctga tcctc aaag. The PCR products were subcloned into pSG5-GBD using NotI (5′) and NheI (3′). The DR4-tk promoter luciferase reporter, AP-1 collagenase luciferase reporter, and pRSV-c-jun constructs were described previously (17, 19). The Gal4-UAS-tk luciferase reporter was created by inserting a 2× reiterated Gal4-UAS sequence, CCGGA GGACA GTCCT CCGGC CGGAG GACAG TCCTC CGG, into a pGL4.10 luciferase reporter plasmid containing the thymidine kinase minimal promoter. The TRE-tk promoter luciferase reporter was created by inserting the following CGAG GTCGA GCAAG GACCT GAAAT GACCC TGTCG A sequence into a pGL4.12/thymidine kinase minimal promoter construct. CV-1 cells were originally the generous gift of K. R. Yamamoto (University of California at San Francisco, San Francisco, CA); the cells have not been further authenticated.

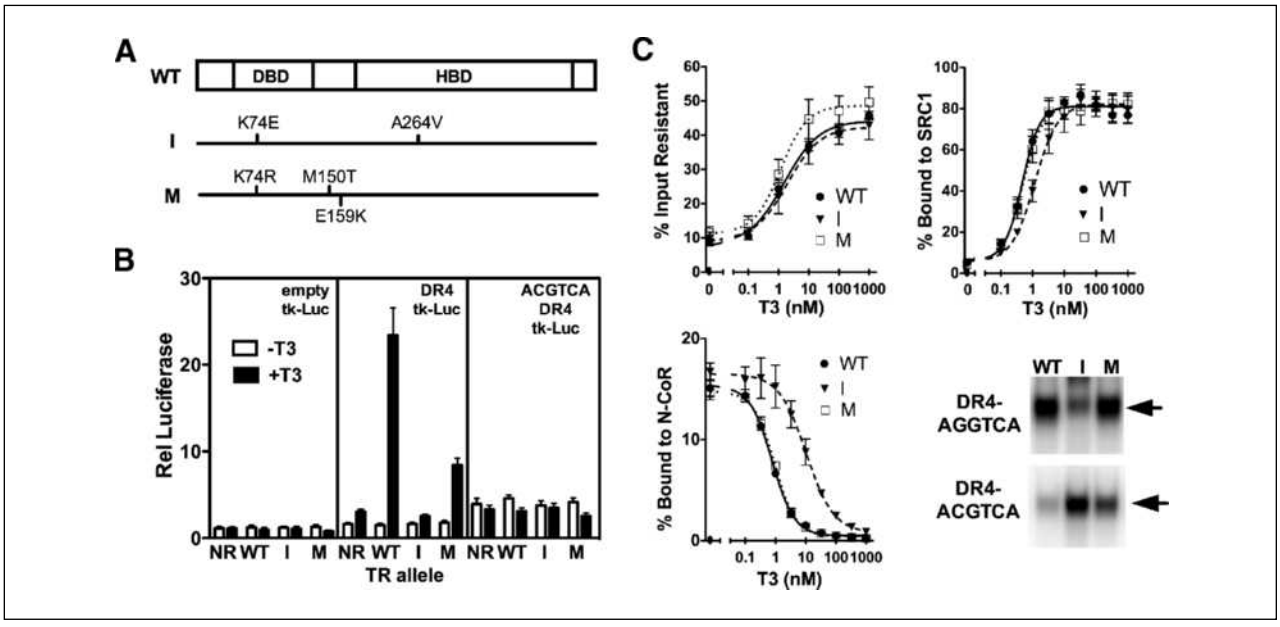

FIGURE 1. The TRα1-I and TRα1-M mutants are defective for transcriptional activation in cells, but display relatively unimpaired biochemical properties *in vitro*. **A.** Schematics of the WT and HCC mutant TRα1 proteins. Horizontal bar, the TRα1-WT protein, with the DBD and hormone-binding domain (*HBD*) indicated. The TRα1-I and TRα1-M mutant proteins are represented below (*horizontal lines*) with the location of the genetic lesions in each mutant indicated by codon number. **B.** T3-induced activation of a DR4 luciferase reporter by mutant and WT TRs. Expression vectors for the mutant or TRα1-WT alleles were introduced by transient transfection into CV1 cells together with the DR-4-tk-Luc reporters indicated and a pCH110 lacZ internal control. After 24 h, 100 nmol/L T3 hormone (or carrier only) was added to the cells; the cells were harvested 24 h later; and relative luciferase activity (normalized to the β-galactosidase control) was calculated. Columns, mean and of two or more experiments; bars, SD. **C.** T3 binding, coactivator recruitment, corepressor release, and DNA binding by mutant and WT TRs *in vitro*. For the T3-binding assay, the percentage of TR resistant to elastase at different T3 concentrations, a measure of hormone binding, is shown; points, mean of three independents experiments are presented; bars, SEM. For the coactivator and corepressor-binding assays, radiolabeled mutant or WT TRs were incubated with an immobilized GST-SRC1 or immobilized GST-NCoR construct at the T3 concentrations indicated. After washing, the percentage of receptor bound to the GST-coregulator (input, 100%) was determined. Points, mean of at least three independent experiments; bars, SEM. For assaying TRα1 binding to AGGTCA and ACGTCA DR4 response elements, the WT and mutant TRα1 proteins were mixed with retinoid X receptor α and incubated with radiolabeled DR4 response element composed of AGGTCA or ACGTCA repeats, as indicated. The resulting TRα1/DNA complexes (*arrows*) were resolved by native gel electrophoresis and were visualized by phosphorimager analysis. The results in **C** are derived from experiments in ref. 17.

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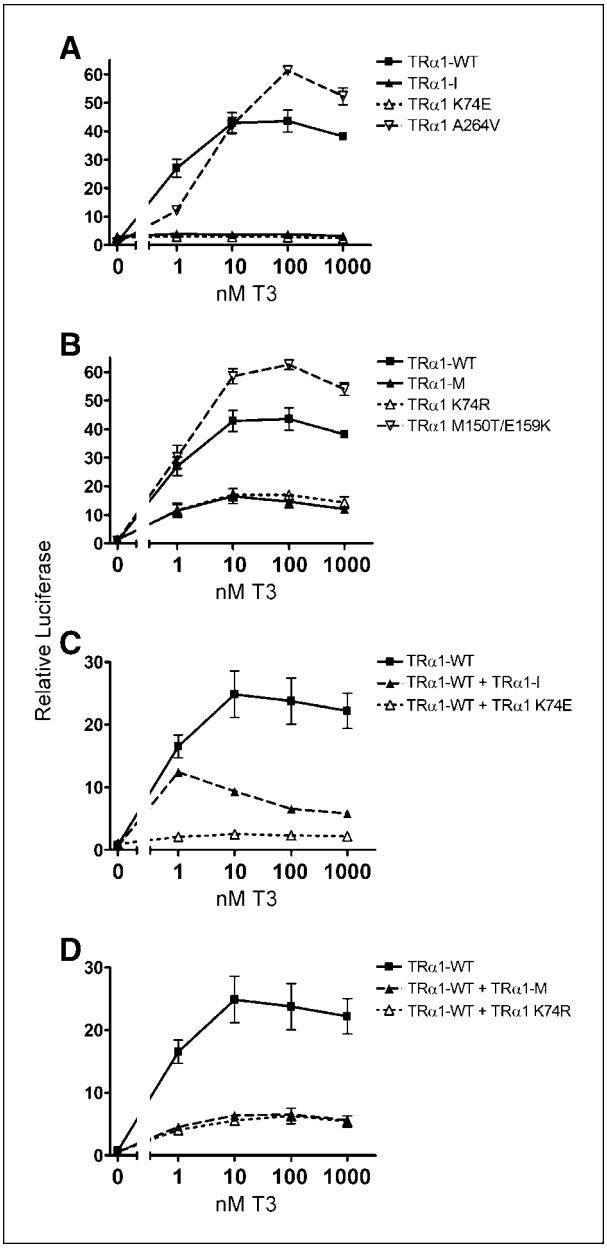

FIGURE 2. The K74 substitution is responsible for the regulatory defects and dominant-negative properties of the TRα1-I and TRα1-M mutants on a positive response element. **A.** Positive response gene regulation by TRα1-WT, TRα1-I, or mutants derived from TRα1–I. Expression vectors for the TRα1 alleles indicated were introduced by transient transfection into CV1 cells together with a DR-4-tk-Luc reporter and a pCH110 lacZ internal control. After 24 h, T3 hormone (or carrier only) was added to the cells to the concentrations indicated; the cells were harvested 24 h later; and relative luciferase activity (normalized to the β-galactosidase control) was calculated. Points, mean of two or more experiments; bars, SD. **B.** Positive response gene regulation by TRα1-WT, TRα1-M, or mutants derived from TRα1-M. The same method was used as in **A**. **C.** Inhibition of TRα1-WT function by TRα1-I or mutants derived from TRα1-I. The same method was used as in **A**, except a 5:1 mixture of TRα-1 mutant to TRα1-WT was used in each transfection. **D.** Inhibition of TRα1-WT function by TRα1-M or mutants derived from TRα1-M. The same method was used as in **B**, except using a 5:1 mixture of TRα-1 mutant to TRα1-WT in each transfection.

Transient Transfection Assays

CV-1 cells (3×10^4) were seeded into 24-well plates, and cell transfections were done using 2 μL of Enhancer, 2.5 μL Effectene, 50 ng of pCH110-lacZ, sufficient pUC18 to bring the total DNA to 250 ng, and additional plasmids as follows: for TRE reporter assays, 50 ng of the appropriate luciferase reporter and 5 ng of the appropriate pSG5-TR; for DR4 dominant-negative assays, 50 ng of DR4-tk-luciferase reporter, 5 ng of WT pSG5-TR, and 25 ng of the appropriate mutant pSG5-TR; for c-Jun reporter assays, 50 ng of the collagenase promoter-luciferase reporter, 10 ng of the appropriate pSG5-TR, and 10 ng of the pRSV-c-jun construct; for c-Jun dominant-negative reporter assays, 50 ng of the collagenase promoter-luciferase reporter, 5 ng of WT pSG5-TR, 25 ng of the appropriate mutant pSG5-TR, and 10 ng of the pRSV-c-jun construct; for GBD fusion assays, 50 ng of the Gal4-UAS-tk-luciferase reporter and 10 ng of the appropriate GBD-TR fusion construct. Medium was replaced 24 h posttransfection with fresh medium containing either the T3 concentration indicated or equivalent ethanol carrier. Luciferase and β-galactosidase activities were determined 24 h later (20).

In vitro DNA and Protein Binding Assays

T3 binding (using a protease protection method), electrophoretic mobility shift DNA binding, NCoR corepressor binding, and SRC1 coactivator-binding assays were done as describe in ref. 17. A GST-NCoR (1817-2453) and a GST-SRC1 (568-895) construct were used for the latter, GST-pulldown methods.

Results

The Transcriptional Activation Defects Exhibited by the HCC TRα1 Mutants Are Due to Genetic Lesions at K74

We analyzed a series of WT and mutant TRα1 alleles (Fig. 1A) by transfection into CV-1 cells, which express very low levels of endogenous TRs and therefore represent a clean background (17, 20). Introduction of a TRE-tk-luciferase reporter into these cells in the absence of an ectopic TR produced a low-level basal expression of the reporter gene that was relatively unaffected by T3 (Fig. 1B). Cointroduction of this reporter together with an expression vector for WT TRα1 (TRα1-WT) produced a strong induction of luciferase in response to T3 (Fig. 1B). The TRα1-I mutant, in contrast, was unable to activate the luciferase reporter in response to T3, and the TRα1-M mutant was severely attenuated in this regard compared with TRα1-WT (Fig. 1B; ref. 17). Both mutants bind as retinoid X receptor heterodimers to the consensus TRE *in vitro* (a direct repeat of AGGTCA with a four base spacer, a DR4), although TRα1-I does so at reduced levels compared with TRα1-WT (Fig. 1C). Notably, TRα1-I and TRα1-M also failed to activate a reporter gene bearing an alternative TRE (an ACGTCA DR4) that these mutants bind more strongly than does TRα1-WT (Fig. 1B and C).

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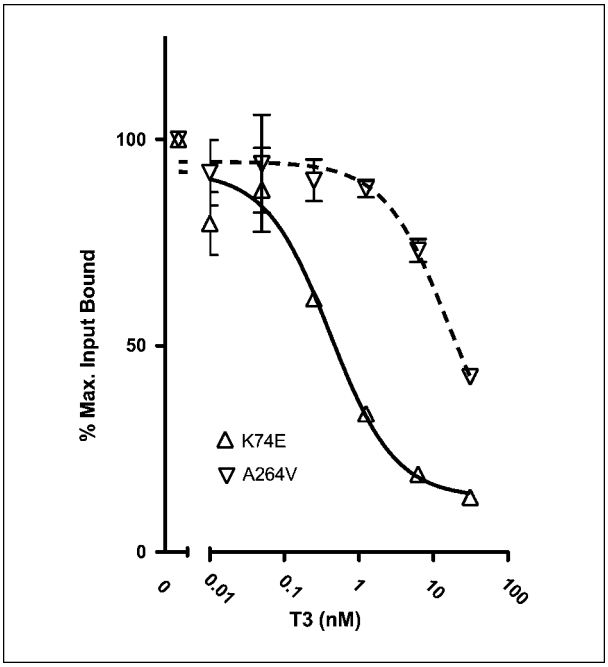

FIGURE 3. The A264V substitution is responsible for the delayed release of the corepressor by the TRα1-I mutant. Radiolabeled TRα1-K74E or TRα1-A264V mutant proteins were assayed for the ability to bind to GST-NCoR under different T3 conditions, as in Fig. 1C. Points, mean of two independent experiments; bars, SEM. Error bars smaller than the graph symbols may not be visible.

Furthermore, both the TRα1-I and M mutants bind T3 hormone efficiently, and release corepressor and recruit coactivator in response to T3 *in vitro* (although the TRα1-I mutant requires a slightly higher T3 concentration to do so than does either TRα1-WT or TRα1-M; Fig. 1C). We conclude that although the TRα1-I and TRα1-M HCC mutants are impaired for transcriptional activation *in vivo*, these defects are not readily explained by their near WT biochemical properties *in vitro*.

To better understand the molecular basis for the TRα1-I and TRα1-M transcriptional defects, we dissected the underlying genetic lesions in more detail. The TRα1-I mutant contains two different mutations: a K74E mutation located in the DBD and an A264V mutation within the hormone-binding domain (Fig. 1A). We therefore created and analyzed TRα1 mutants containing these mutations individually as single genetic lesions. In prior studies of resistance to thyroid hormone syndrome and renal clear cell carcinoma TR mutants, the dominant-negative activity has mapped to mutations in the receptor ligand–binding domain. Unexpectedly, the TRα1-K74E single mutant fully reproduced the transcriptional defects and dominant-negative function displayed by the original TRα1-I double mutant (Fig. 2A). In contrast, the TRα1-A264V mutant displayed a slightly reduced response to low levels of T3, but mediated WT or better transcriptional activation at higher hormone concentrations (Fig. 2A).

Although derived from an unrelated HCC tumor, the TRα1-M mutant also contains a substitution at K74 (to an arginine, rather than a glutamate) plus two mutations, M150T, and E159K, within the hinge domain that joins the DNA and hormone-binding domains of the receptor (Fig. 1A). Dissection of these individual mutations showed that the K74R mutation alone was sufficient to fully convey the transcriptional defects exhibited by the original TRα1-M triple mutant (Fig. 2B). Conversely, a TRα1 bearing the M150T and E159K mutations induced a somewhat greater than WT T3 response on the luciferase reporter (Fig. 2B). We conclude that the mutations at K74 are the primary basis for the defective transcriptional properties of both the TRα1-I and TRα1-M HCC mutants.

The K74 Substitutions in TRα1-I and TRα1-M Also Confer Dominant-Negative Properties on These Mutant Receptors

Significantly, TRα1-I and TRα1-M are not simple null alleles, but instead function as dominant negatives and inhibit TRα1-WT function when coexpressed (Fig. 2C and D). When tested in the same manner, TRα1 single mutants bearing either the K74E or K74R mutation also functioned as strong dominant-negative inhibitors of TRα1-WT (Fig. 2C and D). In fact, the K74E single mutant conferred a somewhat stronger dominant-negative ability than the original TRα1-I double mutant, whereas the K74R single mutant exhibited identical dominant-negative capabilities as did the original TRα1-M triple mutant (Fig. 2C and D). Taken together, our results indicate that the lysine at position 74, in the DBD, plays a critical role in modulating TRα1 transcription, and substitution of either a glutamate or an arginine at this position is sufficient to convert TRα1 into a dominant-negative inhibitor on the positive response DR4-tk-luc reporter.

The A264V Substitution Is Responsible for the Delayed Corepressor Release Observed for the TRα1-I Mutant

As noted, the TRα1-I mutant requires higher levels of T3 to release corepressor (and to bind coactivator) than did the WT receptor, although these *in vitro* properties did not correlate with the *in vivo* defects in transcriptional regulation observed for this mutant. To establish the lesion responsible for this altered T3 release of corepressor by the TRα1-I mutant, we performed glutathione *S*-transferase (GST)–pulldown experiments using the individual K74E and A264V substitution mutants. The K74E mutant readily released from the NCoR corepressor in response to T3, whereas the A264V mutant required higher than normal levels of hormone to do so (Fig. 3). We conclude that the delayed corepressor release by the TRα1-I mutant is caused by the A264V substitution, but is not the primary basis behind the profound transcriptional defects observed for TRα1-I, which map instead to the K74E lesion. The TRα1-M multiple mutant exhibited normal corepressor release and was therefore not dissected further in our experiments.

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The Lysine 74 Mutations Are Responsible for the Altered Regulation Observed for the HCC-TRα1 Mutants on a Negative Response Element

Certain TRα1 target genes, such as collagenase, display a negative response to hormone and are repressed, rather than activated, by T3 (21). For collagenase, this is apparently mediated by combinatorial interactions operating at an activator protein (AP-1) site in the promoter (22-27). C-Jun binding to this AP-1 site in the absence of a TR confers basal expression. TRα1-WT interacts with c-Jun at this AP-1 site to further enhance expression in the absence of T3, but, conversely, to repress it in the presence of T3 (Fig. 4A). Both the TRα1-I double mutant and the TRα-1-K74E single mutant were inactive in this assay, neither inducing expression of the Col-luc reporter in the absence of T3, nor repressing it the presence of this hormone (Fig. 4A). The TRα1-M triple mutant displayed a partially impaired ability to activate the Col-reporter in the absence of hormone, but has no ability to repress this reporter in the presence of T3; the K74R substitution alone was sufficient to manifest the same effects (Fig. 4B).

The ability of the TRα1 HCC mutants to interfere with TRα1-WT function extends to negative response elements (17). Both TRα1-I and TRα1-M prevented TRα1-WT repression of the Col-luc reporter in response to T3, although neither HCC mutant interfered with the activation of this reporter by TRα1-WT in the absence of T3 (Fig. 4C). The K74R single mutant was indistinguishable from the TRα1-M triple mutant in this assay (Fig. 4D). The K74E single mutant, interestingly, displayed an enhanced ability to block WT function on the collagenase promoter than did the TRα1-I double mutant by preventing both activation in the absence and repression in the presence of T3 (Fig. 4C). We conclude that the mutations at lysine 74 in TRα1-I and TRα1-M are responsible for the dominant-negative properties of these mutants on both the negative-acting and positive-acting TRE tested here.

Several other nuclear hormone receptors also use the collagenase AP-1 site as a negative response element,

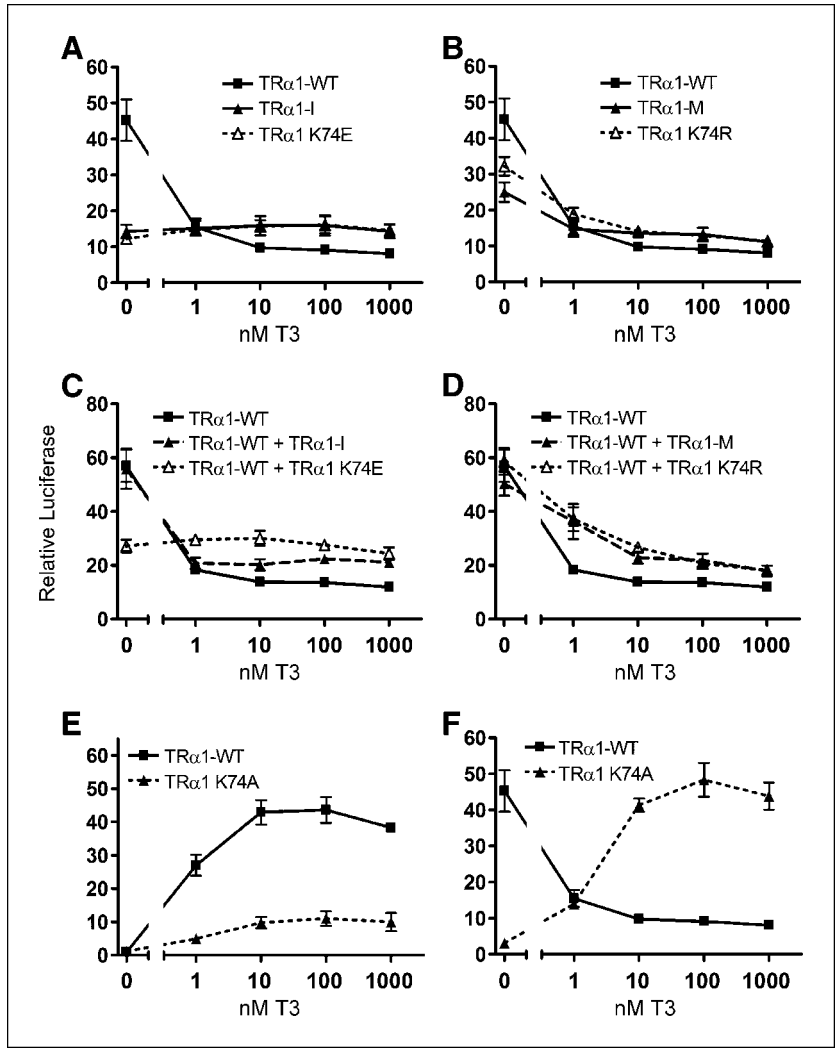

FIGURE 4. The K74 substitution also accounts for the regulatory defects and dominant-negative properties of the TRα1-I and TRα1-M mutants on an AP-1–negative-response element. **A.** Negative response gene regulation by TRα1-WT, TRα1-I, or TRα1-K74E. Expression vectors for the TRα1 alleles indicated were introduced by transient transfection into CV1 cells together with an AP-1 Col-Luc reporter and a pCH110 lacZ internal control. After 24 h, T3 hormone (or carrier only) was added to the cells to the concentrations indicated; the cells were harvested 24 h later; and relative luciferase activity (normalized to the β-galactosidase control) was calculated. Points, mean of two or more experiments; bars, SD. **B.** Negative response gene regulation by TRα1-WT, TRα1-M, or TRα1-K74R. The same method was used as in **A**. **C.** Inhibition of TRα1-WT function by TRα1-I or TRα1-K74E. The same method was used as in **A**, except a 5:1 mixture of TRα-1 mutant to TRα1-WT was used in each transfection. **D.** Inhibition of TRα1-WT function by TRα1-M or TRα1-K74R. The same method was used as in **B**, except a 5:1 mixture of TRα-1 mutant to TRα1-WT was used in each transfection. **E.** Positive response gene regulation by TRα1-WT or an artificial TRα1-K74A mutant. A DR4-Luc reporter was used using the same method as in Fig. 2A. Points, mean of two or more experiments are presented; bars, SD. **F.** Negative response gene regulation by TRα1-WT or an artificial TRα1-K74A mutant. An AP-1 Col-Luc reporter was used, using the same method as in **A**. Points, mean of two or more experiments are presented; bars, SD.

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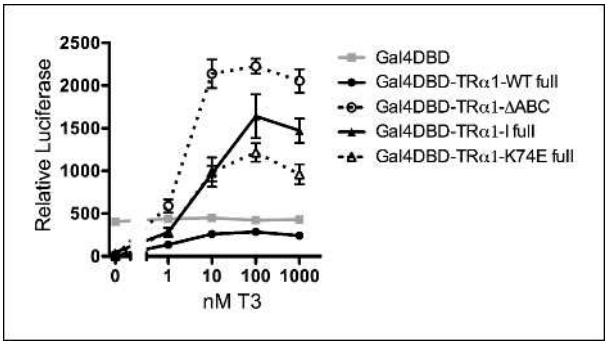

FIGURE 5. The TRα1-WT DBD inhibits positive response gene regulation; the K74E mutant or deletion of the DBD reverses this inhibition. The Gal4DBD-domain was fused to TRα1-WT full-length, TRα1-I full-length, TRα1-K74E full-length, or TRα1-WT with the DBD deleted (*TRα1-ΔABC*), and the constructs were introduced into CV-1 cells together with a Gal17-mer luciferase reporter and the pCH110 lacZ internal control. T3 was added as indicated 24 h after transfection; the cells were harvested an additional 24 h later; and relative luciferase was determined as in Fig. 2. The GalDBD-domain alone was also tested.

including glucocorticoid and retinoic acid receptors (28). Prior dissections of the DBD of these receptors showed an unexpected result: an artificial alanine substitution at the lysine equivalent to TRα1-K74 reversed their response on the AP-1 element from a negative into a positive one (28). To relate these observations to our own results with the HCC mutants, we introduced a K74A substitution into TRα1 and assayed its activity by transient transfection. The artificial TRα1-K74A mutant activated the positive-response TRE-luciferase reporter in the presence of T3, although at a reduced level compared with TRα1-WT (Fig. 4E). More dramatically, the TRα1-K74A mutant displayed a reversed transcriptional response on the AP-1 Col-luc reporter: repressing in the absence and strongly activating in the presence of T3 (Fig. 4F). These findings agree with those previously published (28) and, taken together with our analysis of the K74E and K74R mutations, show that the amino acid at this highly conserved position in the DBDs of the nuclear receptors exerts strong, context-specific effects on transcriptional regulation.

The WT K74 Inhibits Transcriptional Activation in the Absence of a Cognate DNA Binding Site; the K74E Mutation or a Deletion of the Entire DBD Reverses This Inhibition

To further explore the role of DNA recognition in the transcriptional defects displayed by our HCC-TRα1 mutants, we created Gal4DBD fusions of these receptors and assayed their transcriptional properties on a Gal4-17mer luciferase reporter. In this manner, we could recruit TR to a reporter gene through the ectopic GAL4DBD, leaving the native DBD of the receptor unengaged by cognate nucleic acid. Interestingly, whereas the WT GAL4DBD-TRα1 fusion repressed reporter gene expression in the absence of T3, addition of T3 resulted in only a very modest reversal of this repression (Fig. 5). The K74R mutation was indistinguishable from WT in this assay (data not shown). In contrast, the Gal4DBD-TRα1-K74E fusion mediated a much greater T3 response on the GAL-17mer luciferase reporter than did the Gal4DBD-TRα1-WT construct (Fig. 5). In fact, a deletion of the entire native TR DBD from the GalDBD-TRα1-WT fusion produced an effect similar to the K74E substitution mutant: inducing the Gal17mer reporter much more strongly than did the full-length Gal4DBD-TRα1-WT (Fig. 5). These results suggest that the presence of the native DBD of TRα1 inhibits transcriptional activation by the receptor when it is not engaged with a cognate DNA element, whereas lysine 74 binding to a cognate DNA element (such as on the TRE-tk reporter) or its substitution with a glutamate can reverse this inhibition. Although the precise mechanism is unknown, we propose that there is an allosteric change in the receptor in response to whether lysine 74 is bound to a cognate DNA response element or not, and that this allosteric change can propagate through the receptor to influence the ability of the receptor to mediate transcription up or down (please see the Discussion).

Discussion

Two TRα1 Mutants Isolated from Human HCCs Reveal a Novel Mechanism by Which Disease-Associated Nuclear Receptors Acquire Dominant-Negative Function

The majority of the mutant TRs analyzed from human HCCs are impaired for transcriptional activation and inhibit WT TR function (11, 17). As such, the HCC mutants fall into a larger grouping of nuclear receptor mutants that function in a similar manner and contribute to a wide range of human endocrine, metabolic, and neoplastic diseases (6). In the vast majority of cases studied, the loss of transcriptional activation and the acquisition of dominant-negative properties map to genetic lesions within the receptor hormone–binding domain; these lesions inhibit the binding of hormone agonist, the release of corepressor, or the recruitment of coactivators (9, 11, 16, 20, 29-35). The TRβ1 HCC mutants we previously analyzed fit this model, yet the two TRα1 HCC mutants studied here, TRα1-I and TRα1-M, do not (17). Despite their strong, dominant-negative inhibitory properties in cells, both TRα1-I and TRα1-M display near-normal T3 binding and mostly unimpaired coregulator interactions *in vitro* (17). This prompted us to analyze in more detail the molecular basis behind the transcriptional properties of these two TRα1 mutants.

Although both TRα1-I and TRα1-M contain mutations in their hormone-binding/hinge domains, the transcriptional defects of these mutants unexpectedly mapped to their DBDs, and reflected the substitution of the WT lysine at codon 74 to glutamate or arginine, respectively. These K74 mutations disrupted transcriptional activation and conferred dominant-negative inhibition of WT TR activity, and these effects could be observed both on a positive and on a negative response element.

The TRα1-I and TRα1-M mutants therefore reveal a newly recognized mechanism by which dominant-negative

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function can be acquired by nuclear receptors. Given that TRα1-I and TRα1-M mutants were isolated from unrelated HCC tumors, lysine 74 seems to represent a genetic hotspot in this regard. Consistent with this idea, an analogous lysine to glutamate mutation has been identified in the vitamin D receptor (VDR) in a patient with inherited rickets (36). The mutant vitamin D receptor is defective for transcriptional activation, although the basis for this defect has not been investigated in detail. Significantly, a lysine is highly conserved at the equivalent position in almost all members of the nuclear receptor family, and both helps define the specificity of these receptors for their cognate DNA-binding sites, and (as discussed below) functions as a sensor by which DNA binding can modulate the transcriptional output. It is likely that additional examples of dominant-negative mutations mapping to this (or adjacent) amino acid sites and operating through similar mechanisms will be discovered as more nuclear receptors are isolated and analyzed from different human diseases.

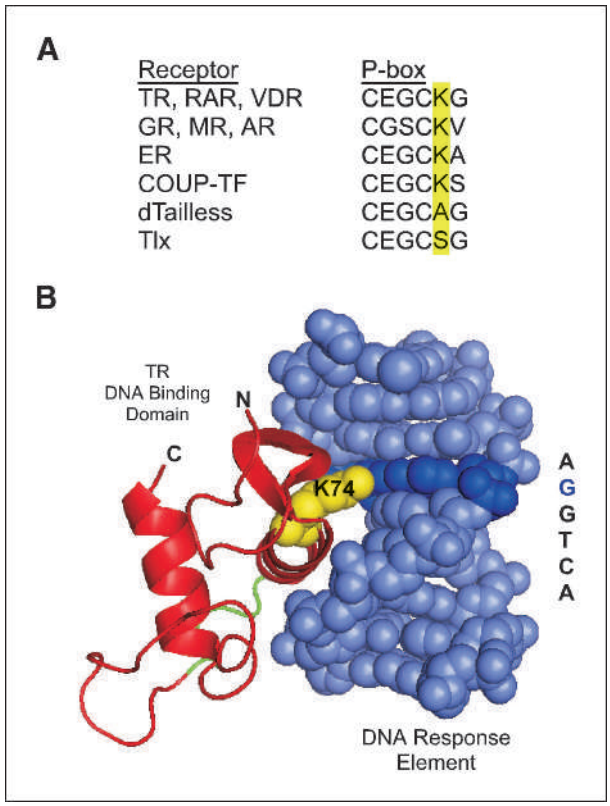

FIGURE 6. A conserved lysine in the P-box of multiple nuclear receptors makes important contacts within the response element half-site. **A.** The amino acid sequence of the P-box of the DBD of different nuclear receptors. Sequences include TR, retinoic acid receptor (*RAR*), vitamin D3 receptor (*VDR*), glucocorticoid receptor (*GR*), mineralocorticoid receptor (*MR*), androgen receptor (*AR*), estrogen receptor (*ER*), COUP-TFI, *Drosophila* Tailless (*dTailless*), and a mammalian Tailless ortholog (*Tlx*). **B.** The crystal structure of a portion of the DBD of TRα1 bound to a DNA response element. The TR protein backbone is presented as a ribbon/helix/strand schematic; the DNA and K74 are shown as space fill (46). Right, the half-site sequence of the DNA; the contact between the second bp in the DNA and K74 is highlighted in blue and yellow. Green, a lever arm identified in glucorticoid receptors is shown in the TR structure (40).

Lysine 74 Seems to Function as a Sensor by Which the Transcriptional Properties of the Nuclear Receptors Can Be Regulated in Response to DNA Binding

Lysine 74 in TRα1-WT forms part of the P-box recognition α-helix by which nuclear receptors contact, and read off the bp sequence, in the major groove of their cognate DNA-binding sites (37, 38). A lysine is invariant at this position in virtually all known nuclear receptors, and contacts a similarly highly conserved G present in the second position of the consensus DNA recognition sites for these receptors (either an A*G*GTCA or an A*G*AACA; Fig. 6A and B; refs. 37, 38). Recognition of the importance of this lysine for transcriptional recognition came first from experiments designed to disrupt DNA binding by the glucocorticoid receptors by mutating this lysine to an alanine (28). Unexpectedly, this K to A mutation only modestly inhibited DNA binding/transcriptional activation on a positive response element but, more remarkably, converted the GR from a repressor to an activator of AP-1 function (28, 39). Similar results were observed with a variety of nuclear receptors (refs. 26, 28 and verified here for TRα1).

These results suggest that the P-box lysine plays a dual role, both helping to define the base recognition properties of the nuclear receptors and, once bound, acting as a sensor that communicates contact with a cognate DNA-binding site has been achieved. The lysine to alanine mutations seem to correctly interpret receptor binding to a cognate DNA sequence as a signal for transcriptional activation, but also inappropriately communicate a transcriptional activation signal when these receptors are tethered indirectly to DNA through protein-protein contacts with c-Jun.

The HCC TRα1-M mutant analyzed here, a K74R substitution, maintains the positive charge at this position, but inserts a more highly branched arginine for the native lysine. The K74R mutation partially impeded transcriptional activation on a positive response element (much as did the K74A), but did not reverse TRα1 function on the AP-1 reporter. Instead, the K74R mutant functioned as a dominant-negative inhibitor of TRα1-WT activation on the DR4 TRE and of TRα1-WT repression on the AP-1 element in response to T3. Interestingly, the K74R mutation retained the ability of TRα1-WT to activate the AP-1 element in the absence of T3. This mixed phenotype of the K74R substitution likely reflects the relatively conservative nature of this substitution. The K74E mutant was, in contrast, incapable of activating the DR4 element in response to T3, and unable to either activate or repress the AP-1 element. Interesting, the K74E mutant was a strong dominant-negative inhibitor of TRα1-WT function in all contexts. The more profound effects of the K74E mutation are probably due to the more radical nature of the amino acid

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substitution in this allele, which replaces a positively charged amino acid with a negatively charged one.

These results suggest that the native K74 position may also serve broadly to inhibit the transcriptional activity of the receptor when not in direct contact with a cognate response element. In fact, this is consistent with what we observed when TRα1 was artificially tethered to a noncognate DNA-binding site by fusion with an ectopic Gal4DBD. The Gal4DBD-TRα1-WT was severely limited in its ability to activate a Gal-17mer reporter in response to T3. Introduction of the K74E mutation, or deletion of the entire DBD, reversed this inhibitory effect.

It should be noted that the effects of these codon 74 mutations on TRα1-transcriptional regulation can be distinguished from their effects on target DNA-binding. Both TRα1-I and TRIα1-M can bind detectably to the DR4 AGGTCA element *in vitro*, despite being severely impaired in their ability to activate from this type of sequence *in vivo* (17). Similarly, TRα1-I and TRα1-M bind to a DR4 A*C*GTCA element *in vitro* more strongly than does the WT receptor, yet still fail to activate a DR4-luciferase reporter bearing this A*C*GTCA repeat.

Despite this evidence that K74 serves as a sensor of cognate DNA binding, the precise mechanism remain unclear. Presumably, contact of the lysine with the conserved G in the positive-acting DNA-binding site generates a change in the conformation of the P-box helix that is propagated allosterically to transcriptional regulatory surfaces elsewhere in the receptor. These transcriptional regulatory surfaces then permit or prevent corepressor and coactivator recruitment in response to hormone ligand. These concepts are consistent with studies indicating that the nature of a DNA response element can influence the transcriptional properties of a nuclear receptor bound to it (e.g., ref. 40). Structural analysis has, in fact, identified a lever arm within the DBD of the glucocorticoid receptor that undergoes conformational changes when these receptors are bound to different response elements possessing different transcriptional properties (40); we envision that this or analogous conformational couplings may be responsible for the phenomenon we report here for TRs (Fig. 6B, the corresponding region in TR is depicted in green).

Intriguingly, there is a known exception to the otherwise near-universal presence of a lysine in the P-box of WT nuclear receptors; the Tailless/Tlx orphan receptors encode an alanine or serine instead (Fig. 6A; ref. 41). Notably, Tailless/Tlx receptors recognize AAGTCA half-sites (41), and the P-box alanine or serine presumably helps Tailless/Tlx receptors to bind to the AAGTCA half-sites. We further suggest that the P-box alanine or serine, by binding to the A in the second position of the half-site, may confer on Tailless/Tlx the information that it is bound to a cognate DNA element, in much the same manner we propose that other nuclear receptors use lysine to sense the G in AGGTCA or AGAACA consensus half-sites. Tailless/Tlx seem to operate primarily as repressors, and it is also possible that the atypical P-box and half-site sequence used by these receptors are adaptations involved in mediating transcriptional repression. The actions of Tailless/Tlx at AP-1 sites have not been reported.

The Same Mutations That Confer Dominant-Negative Inhibition on TRα1-I and on TRα1-M Also Alter Their Target Gene Repertoire

The majority of the mutant TRs isolated from human cancers bear multiple genetic lesions, including mutations in both their DNA and hormone-binding domains, whereas the TR mutants isolated from human endocrine disorders contain single mutations restricted to their hormone-binding domain (12). We have previously proposed that the mutations in the DBD of the neoplasia-associated TRs alter their target gene specificity, and that this novel target gene repertoire is crucial in mediating their oncogenic properties (17, 33, 42-45). In most of the cases we have analyzed, the mutations that confer dominant-negative function on these receptors map to their hormone-binding domain and operate in addition to the mutations in the DBD (17, 33). The TRα1-I and TRα1-M mutants studied here appear instead to represent a scenario where a single mutation confers both of the properties we have associated with neoplasia: dominant-negative function and altered target gene recognition. It is important to note therefore that the TRα1-I and TRα1-M mutants may contribute to neoplasia not only by functioning as repressors on WT TR target genes, but alternatively by binding to and regulating, (perhaps in some cases positively) genes that are not normally regulated by WT TRs; these genes may include targets of non–nuclear receptor transcriptional factors, such as AP-1. Extranuclear and transcriptional squelching mechanisms may also play a role. It will also be important to determine if the K74 mutations alone can mediate a neoplastic phenotype, and/or if the additional mutations in the hormone-binding domains of TRα1-I and TRα1-M also contribute to disease initiation or progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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