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Human carboxyl-terminal variant of α-type c-erbA inhibits trans-activation by thyroid hormone receptors without binding thyroid hormone

(gene expression/gene regulation)

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ABSTRACT **Multiple thyroid hormone (T_3; L-3,3',5-triiodothyronine) receptors related to the viral oncogene v-*erbA* have been identified in mammalian tissues and have been shown to mediate certain actions of T_3. The role of a carboxyl-terminal variant of α-type c-erbA (c-erbAα-2) is controversial, since the human form has been reported to be a T_3 receptor, while the rat form has been shown to not bind T_3. In fact, the rat homolog of c-erbAα-2 has been reported to be an inhibitor of T_3 action. We have compared the properties of human c-erbAα-2 with those of its rat homolog and with other forms of c-erbA. Neither form of c-erbAα-2 binds T_3 with high affinity whether synthesized in reticulocyte lysates or by transient expression in mammalian cells. Also, neither form increases the expression of a T_3-responsive gene. However, human c-erbAα-2 inhibits T_3 action mediated by a T_3-receptor form of c-erbA to an extent similar to that of rat c-erbAα-2. Our data strongly suggest that human c-erbAα-2 has a biological function similar to that of its rat homolog. Thus, the modulation of T_3 action by an endogenous inhibitor related to T_3 receptors is likely a general regulatory mechanism.**

Most cellular actions of thyroid hormone (T_3; L-3,3',5-triiodothyronine) are thought to be mediated via a high-affinity nuclear receptor (1). Cellular homologs of the oncogene v-*erbA* encode T_3 receptors (2, 3). The c-erbA proteins are members of a superfamily that includes receptors for steroid hormones, vitamin D, and retinoic acid in addition to related molecules of unknown ligand specificity (4, 5). There are at least two types of human c-*erbA* genes, β and α, which are located on human chromosomes 3 and 17 and designated *ERBA2* and *ERBA1*, respectively (2), and which encode multiple thyroid hormone receptors. In the rat, two forms of β-type c-erbA that are functional T_3 receptors have been described, r-erbAβ-1 (6, 7) and r-erbAβ-2 (8). These r-erbAβ species differ at their amino termini, but are identical in the putative DNA and T_3-binding regions. Human type β-1 c-erbA (h-erbAβ-1) has been identified and shown to bind T_3 with high affinity (2), while the human homolog of r-*erbA*β-2 has yet to be cloned, perhaps due to its likely pituitary specificity (8).

Two forms of c-erbAα, α-1 and α-2, are encoded by alternative splice products of the c-*erbA*α gene in both the rat and the human. These c-erbAα proteins are identical for the initial 370 amino acids but have completely different carboxyl termini. The c-erbAα-1 species remains highly similar to the c-erbAβ sequences in this region, which is the putative T_3-binding domain (2). Indeed, both r-erbAα-1 (9, 10, 11, 12) and h-erbAα-1 (13) bind T_3 with high affinity, and r-erbAα-1 has been shown to function as a T_3 receptor in a heterologous system (11, 14). In contrast, the literature suggests that h-erbAα-2 (15, 16) binds T_3 with high affinity, whereas r-erbAα-2, which is extremely similar to its human homolog, does not (10, 11, 12). Interestingly, r-erbAα-2 has been shown to inhibit the action of other forms of c-erbA in a dose-dependent manner (14). It has been suggested that the abundance of r-erbAα-2 may explain the adult brain's lack of classical responses to T_3 despite a high quantity of nuclear T_3 receptors (14).

The existence of multiple T_3 receptors suggests that the molecular mechanism of T_3 action is more complicated than had been previously thought. In this context, it is extraordinary that the human and rat homologs of c-erbAα-2 appear to have such different properties. However, this is still controversial, since no single group of investigators has studied both r-erbAα-2 and h-erbAα-2. We now report a direct comparison of T_3 binding of r-erbAα-2 and h-erbAα-2 synthesized in reticulocyte lysates. In addition, we have assessed the ability of h-erbAα-2 to trans-activate a T_3-responsive gene in the presence or absence of hormone. Our data support the hypothesis that the human and rat forms of c-erbAα-2 are functionally similar.

MATERIALS AND METHODS

***In Vitro* Transcription and Translation.** cDNAs encoding r-erbAα-1 (10), r-erbAα-2 (10), r-erbAβ-1 (ref. 6; provided by R. Koenig, University of Michigan), and r-erbAβ-2 (8) were subcloned into pBluescript KS and transcribed into RNA by using phage T7 RNA polymerase. A cDNA encoding h-erbAα-2 subcloned into pGEM3 (ref. 16; provided by A. Nakai and L. deGroot, University of Chicago) was transcribed into RNA by using phage SP6 polymerase. The RNA was translated in reticulocyte lysates in the presence of [^{35}S]methionine, and analysis of proteins present in the lysates by sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis (PAGE) were performed as described (10). T_3-binding activity was measured by the method of Koenig *et al.* (6) with minor modifications. The concentration of [^{125}I]T_3 was 0.1 nM, with additional unlabeled T_3 added in some experiments to bring the final T_3 concentrations to 0.4 nM or 1.0 nM. A 1000-fold excess of unlabeled T_3 was added to parallel binding reactions in order to assess nonspecific T_3-binding.

Transfections. JEG-3 cells were cultured in minimal essential media supplemented with 10% (vol/vol) fetal calf serum and were transfected by the $Ca_3(PO_4)_2$ precipitation method (14). The CDM eukaryotic expression vector and r-*erbA*β-1 in

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Abbreviations: T_3, L-3,3',5-triiodothyronine; rGH and hGH, rat and human growth hormone; CAT, chloramphenicol acetyltransferase; r- and h-*erbA*α and -*erbA*β genes, rat and human types α and β *erbA* genes; TRE, T_3-response element.

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CDM were provided by R. Koenig, P. R. Larsen, and D. D. Moore (Harvard Medical School) (14). cDNAs encoding r-erbAα-1, r-erbAα-2, r-erbAβ-2, and h-erbAα-2 were also subcloned into CDM. Restriction enzyme mapping and DNA sequence analysis by the dideoxynucleotide chain termination method (17) were used to verify that the cDNA inserts were present in the correct orientations and that no sequence changes occurred during the subcloning process. The reporter gene, pTK14AA, containing a tandemly repeated T_3-response element (TRE) related to the rat growth hormone (rGH) TRE inserted just upstream of the herpes simplex virus thymidine kinase (*tk*) promoter in a chloramphenicol acetyltransferase (CAT) expression vector, was provided by R. Koenig, P. R. Larsen, and D. D. Moore. Individual precipitates of 14–16 μg of DNA were split and used to transfect duplicate plates, which were then cultured in medium containing 10% fetal calf serum that had been stripped of T_3 by treatment with activated charcoal and AG1X8 anion-exchange resin (18) and supplemented with 100 nM dexamethasone. Half of the cells were also incubated with 10 nM T_3. After 48 hr the cells were lysed, and CAT activity was measured in the extracts (14). T_3 response was defined as the CAT activity observed in the presence of T_3 divided by the CAT activity present in parallel plates incubated in the absence of T_3. In most experiments the plasmid pTKGH, which expresses human growth hormone (hGH), was used as a control for transfection efficiency. hGH was measured in the medium by radioimmunometric assay (Nichols Institute, San Juan Capistrano, CA). As we have reported (14), hGH expression was slightly decreased when cotransfected with a T_3 receptor in cells treated with T_3. This effect was not seen with r-erbAα-2 or h-erbAα-2, and normalization for hGH expression did not affect the conclusions of these studies.

RESULTS

RNA was transcribed from expression vectors containing the r-erbAα-1, r-erbAα-2, and h-erbAα-2 cDNAs and was translated in rabbit reticulocyte lysates. Fig. 1 shows the structure of these proteins, highlighting the differences between h-erbAα-2 and r-erbAα-2. SDS/PAGE analysis of lysates containing approximately equal amounts of labeled protein revealed that, in each case, the predominant protein product was of the appropriate molecular weight (Fig. 2 *Upper*). T_3-binding assays on identical samples to those subjected to electrophoresis (equal amounts of trichloroacetic acid-precipitable protein except in the case of lysate prepared without input RNA) revealed that r-erbAα-1 specifically bound ≈100-fold more labeled T_3 than was bound in the presence of excess cold hormone (Fig. 2 *Lower*). This level of binding varied linearly with the amount of r-erbAα-1 assayed (data not shown); h-erbAα-2 bound <2% of the T_3 bound by an equivalent amount of r-erbAα-1 protein, similar to the binding observed for r-erbAα-2 (Fig. 2 *Lower*). Unlike the binding of T_3 to r-erbAα-1, this binding did not vary with the amount of h-erbAα-2 assayed.

The low level of specific [^{125}I]T_3 binding to h-erbAα-2 (approximately equal to the nonspecific binding) was no greater than the level of "specific" T_3 binding to reticulocyte lysate unprogrammed with RNA. This binding may be due to the presence of globin or another component of the reticulocyte lysate; however, the affinity of this binding was difficult to quantitate by Scatchard analysis because of the low maximal level of displaceable hormone. Indeed, when the concentration of T_3 was increased to 0.4 nM and 1.0 nM, nonspecific binding to both h-erbAα-2 and reticulocyte lysate alone increased 4- to 6-fold, while displaceable binding increased <2-fold. Again, no significant differences were observed between h-erbAα-2 and unprogrammed lysate. In contrast, T_3 binding to r-erbAα-1 increased saturably with increasing T_3 concentration (to 200-fold that of h-erbAα-2 or lysate alone) in reasonable agreement with previously reported kinetic data (7, 9).

The h-erbAα-2 cDNA was subcloned into the same eukaryotic expression vector that has been used to demonstrate the abilities of r-erbAβ-1 (14), r-erbAα-1 (14), and r-erbAβ-2

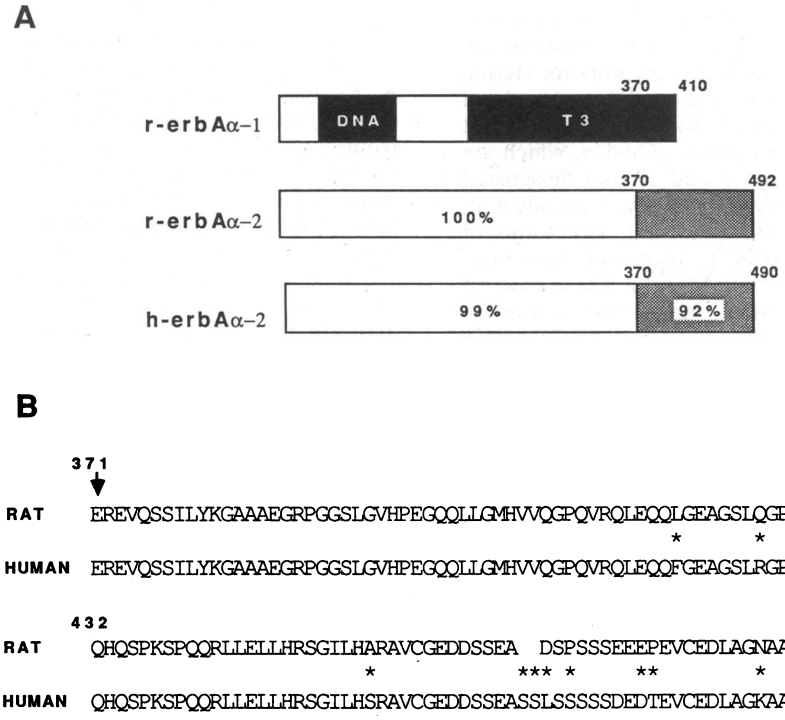

FIG. 1. Comparison of c-erbAα proteins. (*A*) Schematic depiction of r-erbAα-1, r-erbAα-2, and h-erbAα-2. Putative DNA- and hormone-binding domains are indicated. Lightly shaded regions represent areas of no homology to r-erbAα-1. Levels of amino acid identity are indicated as percentages. (*B*) Amino acid sequences of the carboxyl termini of h-erbAα-2 and r-erbAα-2. Differences are highlighted by asterisks.

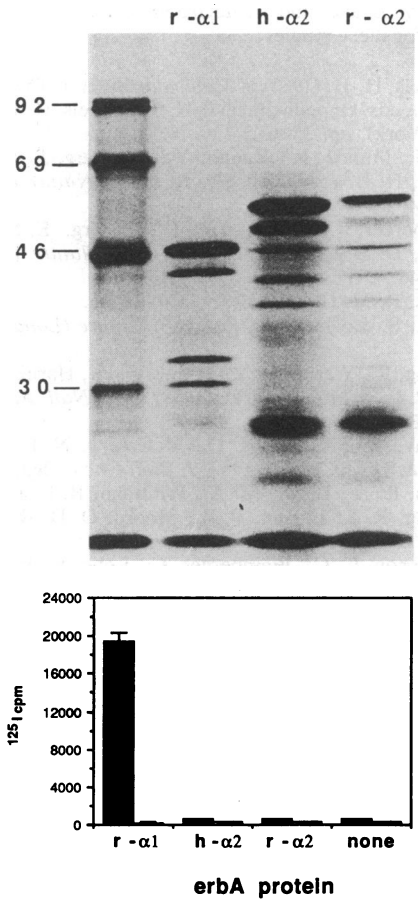

FIG. 2. h-erbAα-2 does not bind T_3 *in vitro*. (*A*) c-erbA proteins translated *in vitro*. r-erbAα-1 (lane r-α1), r-erbAα-2 (lane r-α2), and h-erbAα-2 (lane h-α2) were produced as described and subjected to SDS/PAGE beside standard proteins. (*B*) T_3 binding of c-erbA proteins *in vitro*. The reticulocyte lysates whose SDS/PAGE patterns are shown in *A* were assayed for their abilities to bind [^{125}I]T_3 (0.1 nM) in the presence (□) or absence (■) of 1000-fold excess unlabeled T_3. The difference between [^{125}I]T_3 bound in the absence versus the presence of unlabeled T_3 is referred to as specific binding.

(8) to function as T_3 receptors in heterologous systems. The c-erbA-expressing plasmids were transfected into JEG-3 cells along with the T_3-responsive reporter gene, pTK14AA-CAT. T_3 does not increase CAT expression in this system without the cotransfection of a T_3 receptor (14). As shown in Fig. 3, r-erbAβ-1 was able to mediate trans-activation of pTK14AA-CAT in the presence of T_3 in agreement with earlier work (14). In contrast, h-erbAα-2 was unable to confer T_3 responsiveness to JEG-3 cells. Furthermore, basal expression from pTK14AA-CAT was unaffected by cotransfection with the h-erbAα-2-expressing vector.

T_3 binding was measured in nuclear extracts prepared from JEG-3 cells transfected with 8 μg of the expression vector alone or with 8 μg of the r-erbAβ-1 and h-erbAα-2 expression vectors. Expression of h-erbAα-2 resulted in no significant increase in T_3 binding (91% ± 13% of the binding obtained with vector alone). In contrast, r-erbAβ-1 expression increased specific T_3 binding by 2.9 ± 0.3 fold.

Although expression of h-erbAα-2-CDM had no effect on T_3 binding or on CAT expression from pTK14AA-CAT, the T_3-dependent increase in CAT expression mediated by r-erbAβ-1 was blocked by h-erbAα-2 (Fig. 4). This effect was dose-related and similar in magnitude to that observed with r-erbAα-2. h-erbAα-2 was also able to inhibit the ability of r-erbAβ-2 and r-erbAα-1 to trans-activate the pTK14AA-CAT reporter gene in the presence of T_3 (data not shown).

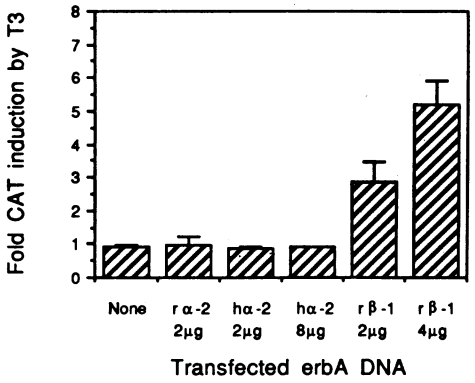

FIG. 3. h-erbAα-2 does not activate the TK14AA reporter gene in the presence or absence of T_3. Expression of CAT from pTK14AA-CAT was measured after cotransfection in JEG-3 cells with various concentrations of plasmids expressing h-erbAα-2, r-erbAα-2, and r-erbAβ-1. CAT activities measured after cotransfection of 2 or 4 μg of r-erbAβ-1 were significantly increased compared with the control ($P < 0.01$ and $P < 0.001$, respectively). No other differences were significant.

DISCUSSION

We have demonstrated that h-erbAα-2 is similar to its rat homolog in three important respects. Both c-erbA proteins, synthesized in reticulocyte lysates, bind T_3 no better than lysate alone, in contrast to the abundant high-affinity binding seen with other forms of c-erbA. In addition, expression of h-erbAα-2 in JEG-3 cells resulted in no increase in T_3 binding, indicating that posttranslational modification does not convert h-erbAα-2 to a T_3 receptor. We cannot explain the T_3 binding to the h-erbAα-2 protein, which has been observed by two groups (15, 16), except to note that we do find a consistent, low level of specific T_3 binding to one or more components of the reticulocyte lysate alone. This binding did not increase when the lysate was programmed with h-erbAα-2 RNA derived from an expression vector that contained the intact h-erbAα-2 cDNA by sequence analysis, despite the presence of a protein (*i*) of the appropriate size as predicted for h-erbAα-2 and (*ii*) in greater (by SDS/PAGE analysis) abundance than a T_3-binding form of c-erbA expressed under parallel circumstances.

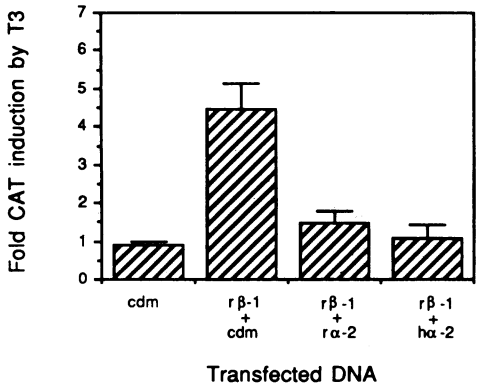

FIG. 4. h-erbAα-2 inhibits activation of the TK14AA reporter gene by the combination of T_3 and r-erbAβ-1, a T_3 receptor. Expression of CAT from pTK14AA-CAT was measured after cotransfection in JEG-3 cells with 2 μg of an r-erbAβ-1-expressing plasmid in combination with 8 μg of CDM alone or with plasmids expressing h-erbAα-2 or r-erbAα-2. CAT activity measured after transfection of r-erbAβ-1 alone resulted in a significant increase in CAT activity versus the control ($P < 0.01$). No other differences were significant.

In JEG-3 cells, expression of h-erbAα-2 is unable to complement T_3 to trans-activate a TRE-containing reporter gene. This is similar to the effect of r-erbAα-2 and distinct from the effects of other forms of c-erbA that function as biologically active T_3 receptors in this system (14).

Furthermore, h-erbAα-2 can inhibit the T_3-dependent activation of the rGH TRE-containing reporter gene by other forms of c-erbA, similar to the inhibition produced by r-erbAα-2 under parallel conditions. The mechanism of this inhibition is unclear and may relate to either competitive binding of the TRE or heterodimer formation between the different c-erbA species.

The discovery that multiple forms of c-erbA are T_3 receptors has had a major impact on our understanding of the molecular mechanism of T_3 action. At present, however, it is unclear how the multiple receptors coordinate to produce such diverse physiological responses to T_3 as regulation of anterior pituitary hormone secretion, increased inotropic and chronotropic states of the heart, and increased basal metabolic rate. Presumably, the unique tissue distributions of the various c-erbA mRNAs are related to their functions. The different c-erbA proteins could interact with different T_3-responsive genes; however, the target genes that have been studied in this regard—rat growth hormone (14, 19), prolactin (19), myosin heavy chains (11), and thyroid-stimulating hormone β (20)—appear to interact with both α and β forms. The only c-erbA species reported to have a unique function at this time has been r-erbAα-2. Whether the ability of r-erbAα-2 to inhibit T_3 action is a curiosity limited to rodent physiology or whether this represents a generalizable mechanism of regulating hormone action is a crucial question. Our data strongly suggest that both the human and the rat forms of c-erbAα-2 do not bind T_3 but can inhibit the transactivation of a T_3-responsive gene by other forms of c-erbA. Thus, the ability of c-erbAα-2 to modulate T_3 action may be an important factor regulating the response to T_3 in humans as well.

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