Thyroid hormone receptors are cellular homologues (c-erbAs) of the v-erbA oncoprotein of the avian erythroblastosis virus. Exclusive of the viral gag region, v-erbA differs from the chick c-erbA-a receptor by two amino acid changes N-terminal of the DNA binding domain, two amino acid changes in the DNA binding domain, nine amino acid changes in the C-terminal region corresponding to the ligand binding domain of c-erbA, and a nine-amino acid deletion near the C terminus. v-erbA does not bind thyroid hormone and when expressed in cells inhibits the activity of wild-type thyroid hormone receptors. We reported previously that mutants of chick c-erbA/thyroid hormone receptor which lack the DNA binding domain (DBD-) inhibit transcriptional activation by wild-type thyroid hormone and retinoic acid receptors (Forman, B. M., Yang, C.-R., Au, M., Casanova, J., Ghyssael, J., and Samuels, H. H. (1989) Mol. Endocrinol. 3, 1610-1626). This dominant negative activity mapped to a series of hydrophobic heptad motifs which are conserved in the C terminus of these receptors and have been suggested to play a role in receptor dimerization. In this study we show that unlike DBD- c-erbA, DBD- v-erbA does not block receptor activity, suggesting that v-erbA acts by competing for DNA response elements rather than by formation of nonfunctional v-erbA/c-erbA heterodimers. This difference in activity was localized to a single Pro to Ser change in v-erbA just N-terminal of the last heptad motif. Introduction of this Pro to Ser change into DBD- c-erbA resulted in a protein which was inactive both functionally and in blocking receptor dimer formation in vitro.

Regulation of gene expression by thyroid hormone 3,5,3'-triiodo-l-thyronine (l-T3) is mediated by intrinsic nuclear receptors which are DNA binding proteins (1, 2). Cloning of these receptors was facilitated by the observation that the cellular homologues (c-erbAs) of the v-erbA oncoprotein bound hormone with affinities similar to those found for thyroid hormone receptors in a wide variety of cells and tissues (3, 4). These receptors are encoded by two genes (α and β) on separate chromosomes, and the proteins show about 85% similarity at the amino acid level (4, 5). The α and β c-erbAs are members of a subfamily of related nuclear receptors which mediate transcriptional activation by thyroid hormone, retinoic acid, and 1,25-dihydroxyvitamin D (6, 7). These receptors contain a 68-amino acid DNA binding "C" domain, which dictates target gene specificity (6), and a C-terminal region (>200 amino acids "D," "E," and "P" domains) (Fig. 1) which determines ligand specificity (6-8). Analysis of N- and C-terminal deletion mutants of chick c-erbA (α form) which lack the DNA binding domain (DBD-) indicated that the C-terminal region could block trans-activation of wild-type α and β c-erbAs and retinoic acid receptor (RAR) (9). This dominant negative activity mapped to amino acids 278-372 which contains a series of 9 leucine zipper-like hydrophobic heptad motifs which are conserved among the c-erbAs and RARs (7, 9). The inhibitory activity of DBD- chick c-erbA proteins containing these heptad motifs suggest that these mutations may act to block homodimer formation by wild-type receptors or heterodimeric interactions of receptor with other transcription factors (7, 9).

The v-erbA oncoprotein differs from the chick c-erbA/thyroid hormone receptor by two amino acid changes N-terminal of the DNA binding domain, two amino acid changes in the DNA binding domain, nine amino acid changes in the C-terminal region corresponding to the ligand binding domain of c-erbA, and a nine-amino acid deletion at the most C-terminal region (Fig. 1) (3). As a result of these changes, v-erbA does not bind thyroid hormone (10, 11) and acts as a dominant negative regulator of wild-type c-erbAs (12-14). Such inhibition could result from competition for binding to DNA response elements (12, 15) and/or from the formation of non-functional v-erbA/c-erbA heterodimers mediated by leucine zipper-like hydrophobic heptad motifs found in the C-terminal regions of both proteins (7, 9). In this study we show that in contrast to DBD- chick c-erbA-(120-408), which strongly inhibits l-T3-dependent stimulation by wild-type chick c-erbA-(1-408) (9), DBD- v-erbA-(122-402) lacks dominant negative activity. This suggests that wild-type v-erbA inhibits c-erbAs by competing for DNA response elements rather than by formation of inactive heterodimers. The lack of dominant negative activity in DBD- v-erbA-(122-402) was localized to a single Pro to Ser change at amino acid 366. Introduction of a Pro to Ser change in the equivalent position of DBD- chick c-erbA (amino acid 364) eliminated its domi...
Dominant Negative Activity and Receptor Dimer Formation

EXPERIMENTAL PROCEDURES

Plasmids—The reporter plasmid MTV-TREpCAT contains one copy of a palindromic thyroid hormone and retinoic acid response element (TREp) (AGGTCTAGACCT) cloned upstream of a mouse mammary tumor viral LTR-CAT vector lacking glucocorticoid response elements (16). The plasmid vectors expressing wild-type v-erbA (12), wild-type chick c-erbA (1-408) (9), DBD- chick c-erbA (120-408) (9), and the human retinoic acid receptor (alpha form) (17) were described previously and are regulated by the Rous sarcoma viral (RSV)LTR. To construct DBD- v-erbA, DBD- c-erbA was excised from the RSV vector by NcoI and Asp718, and the analogous region of v-erbA (corresponding to amino acids 120-402) was inserted into the NcoI and Asp718 sites cloned into the expression vector (9). The v-erbA/NcoI-SacI construct (designated v-erbA(ss)) was constructed by cleaving wild-type v-erbA with NcoI and SacI (Fig. 1). This fragment was blunt-ended by the Klenow fragment of Esche- richia coli DNA polymerase. The v-erbA SacI-NcoI fragment was blunt-ended-ligated into a blunt-ended RSV expression vector (9) which had been digested with NcoI and Asp718 to remove c-erbA sequences. The blunt-ended NcoI site in the vector contains an ATG codon which is in an open reading frame with the SacI-NcoI fragment of v-erbA and expresses a protein which eliminates the viral gag region along with five amino acids at the C-terminal end of v-erbA. Restriction sites common to v-erbA and chick c-erbA and an Asp718 site introduced immediately 3' of the termination codon for both proteins are indicated. These sites define regions designated I, II, and III. Deletion and/or chimeric constructs CCC, VVV, CVC, and VCC which lack the N-terminal (A/B) and DNA binding (C) domains were created by selectively exchanging these regions.

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RESULTS AND DISCUSSION

The C-terminal Region of v-erbA Does Not Mediate Dominant Negative Activity—To analyze whether the C-terminal region of v-erbA might play a role in mediating its dominant negative effect, we compared the functional properties of chick c-erbA and v-erbA using a variety of deletion and chimeric constructs (Fig. 1). The DBD- v-erbA (120-408) and the full-length wild-type chick c-erbA (1-408) have been described previously (9). The effects of the v-erbA and chick c-erbA constructs and chimeras (Fig. 1) on the L-TREp reporter was MTV-TREpCAT (16), which contains one copy of a palindromic thyroid hormone and retinoic acid response element (TRE) (AGGTCTAGACCT) cloned upstream of a mouse mammary tumor virus promoter lacking glucocorticoid response elements.

HeLa cells contain very low levels of endogenous thyroid hormone receptors, and transfection with MTV-TREpCAT alone gives low basal CAT activity which was unaffected by 100 nM L-T3 (Fig. 2A). Cotransfection with 5 µg of the wild-type

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chick c-erbA expression vector had no effect on basal CAT activity but resulted in a 120-fold stimulation by L-T3. Stimulation by L-T3 was reduced about 50% when a vector expressing wild-type v-erbA was co-transfected at a 4-fold molar excess over the wild-type chick c-erbA vector. Similar inhibition was observed with a v-erbA construct (v-erbA/ScalSacI), in which the viral gag region and the F domain were deleted. Although Damm et al. (12) observed that lower amounts of the v-erbA vector could completely inhibit stimulation by c-erbA, studies by Sap et al. (13) found that high levels of v-erbA expression vector were necessary for inhibition which is consistent with our results. These differences may in part be explained by the different reporter promoters used in these studies: Herpes simplex virus thymidine kinase (12), Moloney leukemia viral LTR (13), and MTV (this study).

Remarkably, the DBD− v-erbA-(122–402) deletion mutant (VVV in Figs. 1 and 2A) showed no inhibitory activity, whereas the DBD− c-erbA-(120–408) mutant (CCC) fully inhibited L-T3-stimulation of wild-type chick c-erbA. DBD− v-erbA-(122–402) was also found to lack dominant negative activity in GH4C1 cells which contain endogenous thyroid hormone receptors (not shown) which are inhibited by DBD− c-erbA-(120–408) (9). Since DBD− v-erbA does not inhibit wild-type chick c-erbA, the moderate inhibition by wild-type v-erbA and by v-erbA/Scal-SacI, which both contain the DNA binding domain, probably results from competition for the response element rather than from interactions via their C-terminal domains leading to formation of nonproductive v-erbA/c-erbA heterodimers.

A Single Amino Acid Difference in the C Terminus of Chick c-erbA and v-erbA Influences Dominant Negative Activity and Receptor Dimer Formation — The inactive DBD− v-erbA-(122–402) differs from the dominant negative DBD− c-erbA-(120–408) in nine individual amino acid positions and in a nine-amino acid deletion in the F region near its C-terminal end (Fig. 1). To identify which of these differences contributes to the different functional properties of these two mutants, several DBD− c-erbA/v-erbA chimeras were constructed using unique restriction sites present in both coding sequences and an Asp718 site just 3′ of the termination codon for both proteins. As illustrated in Fig. 1, region I (NcoI to BssHII) contains four of the c-erbA/v-erbA amino acid differences, region II (BssHII to SacI) contains five amino acid differences and all the heptad repeats, and region III (SacI to Asp718) includes the C-terminal end containing the nine-amino acid deletion in v-erbA. Based on these three regions we refer to the DBD− c-erbA-(120–408) as CCC, the DBD− v-erbA-(122–402) as VVV, and the chimeric constructs as CVC and VCC. Co-transfection studies indicated that the DBD− VCC chimer retained strong dominant negative activity, whereas DBD− CVC showed a weak dominant negative effect (Fig. 2A). These results suggest that the four amino acid changes in region I do not significantly affect dominant negative activity and that one or more of the five amino acids changes in region II are critical for inhibition of wild-type c-erbA.

The five amino acids differences in region II (c-erbA versus...
v-erbA) are: Thr¹⁶⁵ to Ser¹⁶⁶ in amino acid position 7 in heptad 7, Pro²⁶⁴ to Ser²⁶⁶ just N-terminal of heptad 9, Thr²⁷³ to Ala²⁷³ in amino acid position 6 of heptad 9, and Cys²⁸⁷ to Tyr²⁸⁷ and Phe²⁸⁵ to Ser²⁸⁶ changes C-terminal of heptad 9 (7). The Pro²⁶⁴ to Ser²⁶⁶ difference was of particular interest because it lies a few amino acids N-terminal of the ninth heptad repeat. This repeat region is highly conserved in the c-erbA and RAR subtypes (7, 9) and is thought to play a role in homo- and heterodimeric interactions (7, 9, 22). Pro²⁸⁷ in chick c-erbA is also conserved in the α and β rat and human c-erbAs as well as the α, β, and γ human RARs (7, 9). The change in v-erbA of Pro to Ser could alter the conformation of the last heptad repeat relative to other regions of the protein and thus might contribute to the lack of dominant negative activity of DBD⁻ v-erbA-(122-402).

Functional studies with site-directed mutants showed that changing Ser²⁶⁶ to Pro in CVC restored full dominant negative activity, whereas changing Pro²⁶⁴ to Ser abolished the dominant negative activity of CCC (Fig. 2B). The dominant negative activities of CCC and CVC(Ser to Pro) were similar in chick c-erbA and in human c-erbA as well as the α, β, and γ human RARs (7, 9). The change in v-erbA from Pro to Ser altered the conformation of the last heptad repeat relative to other regions of the protein and thus might contribute to the lack of dominant negative activity of DBD⁻ v-erbA-(122-402).

Binding reactions for gel mobility shift assays contained 30,000 dpm of ³²P-labeled TREp without (lanes 7-9) or with 10 fmol of wild-type chicken c-erbA (T3 RECEPTOR) (lanes 1-6). Binding reactions also included 100 or 500 fmol of CCC (lanes 2 and 3, respectively) or CCC(Pro to Ser) (designated CVCs) (lanes 5 and 6, respectively). Binding reactions without L-T3 receptor but with 500 fmol of CCC or CCC(Pro to Ser) or without added protein (−) are also shown (lanes 7-9). The purification and quantitation of E. coli expressed wild-type and DBD⁻ chick c-erbA proteins and conditions for the gel mobility shift assay are described under "Experimental Procedures."

Labeling of intact cells with L-[³⁵S]methionine followed by immunoprecipitation of labeled proteins with antisera (antibody 28) raised against a conserved region of v-erbA and c-erbA (9, 19) indicates that all of the mutants used in these studies were expressed at similar levels (Fig. 3). Thus, loss of dominant negative activity in CCC(Pro to Ser) does not result from poor expression of this mutant. The equilibrium dissociation constant (Kα) for L-T3 binding of DBD⁻ c-erbA-(120-408) (CCC) is similar to wild-type chicken c-erbA (Kα about 0.5 nM) (11). To exclude the possibility that loss of dominant negative activity in CCC(Pro to Ser) might result from a marked reduction in ligand binding, we studied the binding of L-[³²P]T3 to CCC and to CCC(Pro to Ser) expressed in E. coli using a T7 phage promoter vector (20, 21). The Kα values for L-T3 binding of CCC and CCC(Pro to Ser) were not markedly different (1.2 and 4.0 nM, respectively).

Results from our laboratory indicate that wild-type chick c-erbA binds as a monomer to a six-nucleotide half-site (AGGTCA) and as monomers and dimers to the TREp inverted repeat (AGGTCACTGACCT) (21).² Formation of homodimers by wild-type chick c-erbA purified from E. coli is
blocked in vitro by DBD–c-erbA-(120–408) produced in a baculovirus expression system. Gel shift studies with 32P-labeled TREp were performed to determine whether the different functional activities of CCC and CCC(Pro to Ser) might be related to differences in their ability to inhibit wild-type chick c-erbA binding to the TREp (Fig. 4). CCC, CCC(Pro to Ser), and wild-type chick c-erbA were expressed in E. coli and purified. As expected, CCC and CCC(Pro to Ser) which lack the DNA binding domain did not bind to the 32P-TREp (lanes 8 and 9). Wild-type chick c-erbA (10 fmol) binds to the TREp as a monomer (lower complex) and as a dimer (upper complex) (lanes 1–6). A 10-fold molar excess of CCC (lane 2) partially inhibited the formation of wild-type chick c-erbA dimers, whereas a 50-fold excess (lane 3) completely blocked dimer formation and somewhat reduced monomer binding to a half-site. In contrast, a 10- or 50-fold molar excess of CCC(Pro to Ser) (lanes 5 and 6) had no effect on the binding of wild-type chick c-erbA to the TREp.

Implications—Some of the amino acid changes in v-erbA relative to chick c-erbA would be predicted to mediate significant differences in the biological activity of these proteins. One of the two amino acid changes in the DNA binding domain of v-erbA (Gly to Ser corresponding to amino acid 73 in chick c-erbA) is at the C-terminal region of the first zinc finger (P box) which is thought to influence DNA binding specificity (23). Indeed, v-erbA showed a lower affinity than chick c-erbA for a TRE in the LTR of the Moloney murine leukemia virus (13). The nine-amino acid deletion at the C terminus of v-erbA removes a potential amphipathic helix which has been suggested to play a role in hormone-dependent trans-activation by c-erbA (14). A chick c-erbA construct (amino acids 120–392) which lacks this amphipathic helix, however, acts as a dominant negative regulator of wild-type c-erbAs (9). Dominant negative activity was mapped to a series of hydrophobic heptad motifs located between amino acids 260–372 of chick c-erbA (9). The last C-terminal heptad (ninth) is the most conserved among v-erbA and the c-erbA and RAR subtypes (7, 9) and is part of a region which has been suggested to play a role in homo- and heterodimer interactions of c-erbA and RAR (7, 9, 22). This suggestion is supported by our results which reveal the critical role of a Pro to Ser change in v-erbA just N-terminal of the last heptad. This change appears to modify the dominant negative activity of DBD–mutants by altering protein-protein interactions and thus may contribute to some of the fundamental differences in the activity of v-erbA and c-erbA on gene expression.

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