Thyroid Hormone Receptor Variant α2

ROLE OF THE NINTH HEPTAD IN DNA BINDING, HETERODIMERIZATION WITH RETINOID X RECEPTORS, AND DOMINANT NEGATIVE ACTIVITY*

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Thyroid hormone receptors bind DNA with highest affinity as heterodimers with retinoid X receptors, and such heterodimers generally are thought to be the biological mediators of thyroid hormone action. An alternative splice product of the thyroid hormone receptor α gene, thyroid hormone receptor variant α2, does not bind thyroid hormone and functions as a weak dominant negative inhibitor of thyroid hormone action. Thyroid hormone receptor variant α2 is missing one-half of the ninth heptad, a region of the bona fide receptor thought to be important for heterodimerization with retinoid X receptors. The role of the ninth heptad in heterodimerization has been evaluated further. Thyroid hormone receptor variant α2-retinoid X receptor heterodimers form on a subset of direct repeat response elements but not on palindromic or inverted palindromic elements. Restoration of the missing ninth heptad sequence is critical for restoring heterodimerization on the palindromic DNA, but either the ninth heptad amino acids or a stretch of alanines is equally able to restore heterodimerization on the inverted palindrome. Thus, the role of the ninth heptad in heterodimerization differs on direct repeat, palindromic, and inverted palindromic response elements, suggesting that the protein-protein interactions differ on each of these elements. The dominant negative activity of thyroid hormone receptor variant α2 requires DNA binding, but the relatively weak nature of the dominant negative activity is only partially explained by the weak DNA binding.

The metabolic actions of thyroid hormone (3,5,3'-triiodothyronine (T₃)) are initiated by the binding of T₃ to nuclear thyroid hormone receptors (TRs), which are members of a large family of zinc finger transcription factors that includes the receptors for all known steroids, retinoids, and vitamin D (see Refs. 1–3 for recent reviews). Thyroid hormone receptors are transcribed by two genes, TRα and TRβ. TRβ produces two functional TRs (TRβ1 and -β2) that contain identical DNA and ligand binding domains but that possess unique amino-terminal domains (Fig. 1). TRα also produces two proteins (Fig. 1), but only one of them (TRα1) is a functional TR. The alternative splice product TR variant α 2 (TRvα2) contains a unique 122-amino acid sequence in place of the carboxyl-terminal portion of the TRα1 ligand binding domain; hence, TRvα2 is not capable of binding T₃ (or any other known ligand). TRvα2 is not a functional TR but, rather, in transfection systems is an inhibitor of thyroid hormone action (4, 5). TRvα2 is widely expressed, and in some tissues such as brain its expression greatly exceeds that of the functional TRs, at least at the RNA level (6). Although the physiological role of TRvα2 is not known, it is plausible that it serves to dampen T₃ regulation of gene expression, perhaps in a tissue- or gene-specific manner.

TRs are unusual in that they regulate gene expression from a wide variety of T₃ response elements (TREs). TREs usually contain two receptor binding sites (half-sites), which are related to the traditional 6-base pair sequence AGGTCA (7) or the higher affinity octamer TAAGTCA (8) and which can be arranged either as a direct repeat (DR), palindrome (Pal), or inverted palindrome (IP) (9–12). Whether there is physiological significance to these different half-site orientations is not known.

In general the functional TRs bind to TREs with highest affinity as heterodimers with retinoid X receptors (RXRs), and it is thought that the TRRXR heterodimer is the biological mediator of T₃ action (13, 14). RXRs are members of the steroid receptor superfamily, and they also serve as heterodimerization partners for other nuclear receptors, such as those for vitamin D and retinoic acid. It appears that multiple domains within the TR ligand binding domain may be involved in heterodimerization with RXRs (15–18). One such domain, located at amino acids 368–374 of TRα1 (LMKVTDL), is known as the ninth heptad. Mutations in this domain can severely impair or abolish TRα1-RXR heterodimerization (16, 17). It is interesting that the alternative splice site for production of TRvα2 is located in the middle of the ninth heptad, between amino acids 370 and 371 (Fig. 1). Since TRvα2 is missing the second half of the ninth heptad, one would predict that it would be unable to heterodimerize with RXR. Indeed, this has been shown to be true on a palindromic or inverted palindromic TRE (19, 20). Surprisingly, however, it has been reported that TRvα2:RXR heterodimers do form on a DR TRE (20). This raises questions about the role of the ninth heptad in TRRXR heterodimerization, as well as questions about the mechanism of the dominant negative activity of TRvα2. Some evidence in the literature supports this (20, 21), but other data do not (22). If DNA binding is required, will the TRvα2 domi-
nificant negative activity be confined to those response elements that support TRv2 heterodimerization with RXR? The goal of these studies was to further evaluate the role of the ninth heptad in TR-RRX heterodimerization, as well as the role of DNA binding in the dominant negative activity of TRv2. Furthermore, it is clear that the dominant negative activity of TRv2 is weak relative to that of mutant TRs that are naturally found in patients with resistance to thyroid hormone (RTH) (23, 24); whether this weakness is due to lack of the full ninth heptad or poor DNA binding has been examined.

EXPERIMENTAL PROCEDURES

Thyroid Hormone and Retinoid X Receptors—Mouse TRα (25), rat TRv2 (6), and mouse RXRα (14) cDNAs were transcribed from pBlue-script plasmids and then translated in rabbit reticulocyte lysate (Pro-beled TRs that remained bound were eluted with 10 mM maltose and their first 370 amino acids but then diverge completely. TRv2 does not bind T3. The DNA and ligand binding domains of the TRs and TR1 are ~85% identical, but their amino-terminal domains are unrelated. Numbers above each protein, amino acid positions. Not shown, TRs encode a second splice form of TRv2 that is similar to TRα2, except it uses a splice acceptor site 117 nucleotides (39 amino acids) downstream of the TRv2 site.

DNA sequences of Tα, response elements used in EMSA and transfection experiments

The sequences shown are the top strands and do not include GATC overhangs at both ends. The sequences are named to denote whether the half-sites are octamers (TAAGGTCA) or hexamers (AGGTCA) and also to denote the relative orientation of the half-sites. Thus, 8DR contains two octamer half-sites as a direct repeat, 8DRD contains a 5’-octamer half-site and a 3’-hexamer half-site as a direct repeat, etc. The AGGTCA hexamers are underlined.

Transient Transfections—JEG-3 human choriocarcinoma cells were maintained and transfected as described previously (31). Receptors were expressed from CDM (32) or Rous sarcoma virus (33) plasmid. Transfections included (as indicated) 10–100 ng of TRα1 vector, 1–3 μg of TRv2 vector, and 3 μg of RXRα vector. Empty vector was added to maintain 6 μg of expression plasmid/60-mm Petri dish transfection. Transfections also included 4 μg of a Tα1-responsive chloramphenicol acetyltransferase (CAT) reporter vector derived from μUTKAT3 (34). The CAT vectors had single copies of the TREs listed in Table I placed 5’ to the basal thymidine kinase promoter. Transfection efficiency was determined using 1 μg of pTKH1 diabetes PETri dish, in which the basal thymidine kinase promoter (without TRE) drives expression of human growth hormone (GH). CAT and GH were assayed as described previously (31). T3 inductions were calculated as (CAT/GH for cells transfected with T3)/(CAT/GH for cells transfected without T3).

Immunoprecipitation—JEG-3 cells were transfected in 100-mm Petri dishes with plasmid amounts scaled accordingly. Two days after transfection the cells were placed in methionine-free media with 10% dialyzed fetal bovine serum and [35S]methionine, 0.15 mCi/ml (4 ml/Petri dish). The cells were cultured for 4 h. Cell harvesting and immunoprecipitation, using a rabbit antiserum directed against the unique carboxyl terminus of TRv2, were performed as described (29), except that incubation with the antibody was for 2 h, and the immunoprecipitates were isolated using protein A-agarose. Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and quantified on a Molecular Dynamics PhosphorImager.

RESULTS

Heterodimerization of TRv2 and RXRα on Direct Repeat TREs—Since TRv2 lacks one-half of the ninth heptad, it was not expected to be able to heterodimerize with RXRα on DNA. Although this expectation was confirmed for a palindromic or inverted palindromic TRE (19, 20), it was unexpectedly shown that such heterodimers do form on a DR TRE (20). We tested TRv2-RRX heterodimer formation on four DR TREs (Table I), which differ only in whether the half-sites contain the traditional hexamer AGGTCA (7) or the extended octamer TRE (20)). Heterodimer formation is barely detectable when just the 3’ half-site is an octamer (86DR; lane 24), but DNA binding is not detectable when both half-sites are hexamers (6DR; lane 24). Heterodimer formation is barely detectable when just the 5’ half-site is an octamer (86DR; Fig. 2, lane 12) and is somewhat more pronounced when just the 3’ half-site is an octamer (68DR; Fig. 2, lane 18). Similar results were obtained using proteins produced in E. coli, except that binding of TRv2-RRX heterodimers to 8DR was as strong as the binding of TRα1-RRX heterodimers, and TRv2 monomer binding to DNA was detectable (data not shown). The data indicate that TRv2-RRX heterodimers can form on DR TREs, but this requires at least one half-site to be an octamer (preferably the 3’ half-site), and strong binding requires both sites to be octamers.

Effect of the Ninth Heptad on TRv2-RRX Binding to DNA—The fact that TRv2-RRX heterodimers can form on some DR TREs but not on Pal or IP TREs suggests that the importance
4. As expected, TR both half-sites are octamers but the orientations of the half-sites were then used in a series of EMSAs with TREs in which the construct is denoted underlined.

LANE5 bands on all three TREs (Fig. 4, lane 5), showing as a nonspecific spacer to separate inhibitory sequences in the unique TRvo2 carboxyl terminus from other critical sequences of TRvo2. Most Ala heterodimerizes strongly on 8IP but not on 8Pal (Fig. 4C, lane 11).

The Ability (or Lack of Ability) of TRvo2 Proteins to Heterodimerize with RXR on 8IP Is Not Related to Their Phosphorylation State—It has been shown that TRvo2 is phosphorylated in its unique carboxyl terminus when translated in reticulocyte lysate, and that this phosphorylation underlies the deficient TRvo2 monomer-TRE binding (29). A series of EMSAs was performed to determine whether phosphorylation influences the ability of the TRvo2 proteins to heterodimerize. Specifically, we wished to know whether the unexpected ability of reticulocyte lysate-translated o2+Ala to heterodimerize with RXR on 8IP was due to moving the phosphorylated residues away from other critical regions of the TRvo2 protein. The strategy, modeled after that of Katz et al. (29), was to produce TRvo2 proteins in E. coli, in which case they are not phosphorylated, or to treat the reticulocyte lysate-translated proteins with alkaline phosphatase. If o2+Ala promotes heterodimerization on 8IP by sterically separating the phosphorylated res-
idues from other critical residues, then one would expect that heterodimerization also would be observed with wild type, non-phosphorylated TRvα2 produced in E. coli or phosphatase-treated TRvα2 from reticulocyte lysate. Fig. 6 shows, however, that E. coli-produced TRvα2 does not form detectable heterodimers with RXR on 8IP (lane 4), although heterodimers are seen with α2+9H (lane 12) and α2+Ala (lane 20). Furthermore, heterodimerization with RXR is not induced by dephosphorylation of reticulocyte lysate-translated TRvα2 (lane 10). As expected, however, dephosphorylation does promote binding of monomers of TRvα2 (Fig. 6, lane 9 versus lanes 7 and 5), α2+9H (Fig. 6, lane 17 versus lanes 15 and 13), and α2+Ala (Fig. 6, lane 25 versus lanes 23 and 21), as well as a small amount of homodimer binding (Fig. 6, lanes 9, 17, and 25). The overall conclusion is that the ability of α2+Ala to restore heterodimerization on 8IP is not related to the TRvα2 phosphorylation state.

The Role of TRvα2-RXR Heterodimerization and DNA Binding in the Dominant Negative Activity of TRvα2—To assess the role of TRvα2-RXR heterodimerization in the dominant negative activity of TRvα2, we first compared this activity on 8DR, 86DR, 68DR, and 6DR by transient transfection (Fig. 7). The percentages of repression of T3 induction by TRvα2 on these response elements were: 8DR, 38.6%; 86DR, 30.7%; 68DR, 21.6%; and 6DR, 29.6%. These data roughly parallel the relative binding of TRvα2-zRXR to these TREs (Fig. 2) in the sense that binding to 8DR was the strongest. However, the difference in dominant negative activity between 8DR and the other elements was not statistically significant (Dunnett’s test). Thus, the lack of a strict parallel between the EMSA and transfection data might suggest that heterodimerization and DNA binding is not critical for the dominant negative activity or that the EMSAs do not reflect the DNA binding within the cell.

To help address this question, a TRvα2 protein was produced with a cysteine to alanine mutation within the first zinc finger (C56A). This mutation prevents formation of the zinc finger and abolishes DNA binding of TRvα2-RXR heterodimers on 8DR (Fig. 8). In a transfection system, TRvα2 C56A no longer possesses dominant negative activity on 8DR, 8Pal, or 8IP (Fig. 9). To confirm that this mutant protein is expressed, transfected cells were metabolically labeled with [35S]methionine, and immunoprecipitation was performed using a TRvα2-specific antibody. The results, quantified by PhosphorImager analysis, indicate that C56A is expressed at 65% of the level of TRvα2 (Fig. 10). It is unlikely that this modest difference in expression could account for the complete loss of dominant
Cells were cultured for 2 days after transfection with or without T3.

Type TRv has potent dominant negative activity. However, by EMSA wild (as analyzed by EMSA); hence, this restoration could explain a role of ical regulators in addition to DNA binding.

The dominant negative activity, or perhaps by there being critical regulators within the cell, by DNA binding not being a critical regulator of the dominant negative activity, or perhaps by there being critical regulators in addition to DNA binding.

To address this, the C56A mutation was placed in the context of a2+9H and analyzed by transfection. Immunoprecipitation from metabolically labeled cells demonstrated that a2+9H and its C56A mutant were expressed at equivalent levels (Fig. 10). The transfection results (Fig. 11) indicate that a2+9H C56A loses ~50% of its dominant negative activity on 8DR and 8IP, but it remains a powerful inhibitor on 8Pal. This suggests two independent mechanisms for the dominant negative activity, one requiring DNA binding and one not. The simplest explanation for the dominant negative activity that requires DNA binding is competition between TRα1-RXR and a2+9H-RXR heterodimers for DNA. A straightforward explanation for the dominant negative activity that does not require DNA binding would be sequestration of RXR in solution. If this were the case, one would predict that cotransfection of RXR would partially alleviate the dominant negative activity of a2+9H. This was tested and, surprisingly, found not to be the case (Fig. 11).

This study sought to evaluate several questions regarding thyroid hormone receptor heterodimerization with RXR and the dominant negative activity of TRv2. It is clear that TR-RXR heterodimers bind to many TREs with much higher affinity than TR monomers or homodimers (13, 14), and this has led to the belief that the heterodimer is the biological mediator of T3 action. A substantial body of mutagenesis data supports the notion that the TR ninth heptad is a critical contact site in the heterodimer with RXR (16, 17), and this is further supported by the observation that the ninth heptad and RXR are lacking. Although this region was originally named as one of nine possible leucine zipper motifs that might form an interface for interaction with RXR (35), these heptad repeats are unlikely to form a traditional leucine
The data indicate that, in contrast to wild type TRv2, the C56A mutant lacks dominant negative activity on all three TREs.

Heterodimerization of TRv2

gesting that the 5′-Tthd is more critical than the following Ado in the octamer, and this has been confirmed.2 Still, even with octamer half sites, TRv2-RXR heterodimers are not detectable on Pal or IP TREs. For DR TREs, it appears that having an octamer half-site is more important than as the 5′-half-site. This fits with the polarity preference of TRs occupying the 3′-half-site when they heterodimerize with RXR on DR TREs (37, 38).

To test whether restoration of the full ninth heptad would permit TRv2 to heterodimerize on 8Pal and 8IP, we made the construct α2+9H. As anticipated, α2+9H heterodimerizes with RXR on both of these TREs, suggesting that the full ninth heptad is critical for this process. However, restoring the full ninth heptad into TRv2 also adds separation between the first 370 amino acids of this protein and its unique 122-amino acid carboxyl terminus. To control for this, we made the construct α2+Ala. As expected, α2+Ala had only minimal ability to heterodimerize with RXR on 8Pal. Surprisingly, however, α2+Ala and RXR heterodimerize strongly on 8IP. This suggests that the role of the ninth heptad in TR-RXR heterodimerization is different on each of the orientations of TREs: (a) on 8DR the full ninth heptad is not critical for heterodimerization; (b) on 8Pal the full ninth heptad sequence is critical; this situation most closely mimics heterodimerization in the absence of DNA; and (c) on 8IP the full ninth heptad also is critical, but only as a nonspecific spacer, since a series of alanines functions equally well. Presumably heterodimerization of wild type TRv2 on 8IP is sterically impaired by the unique TRv2 carboxyl terminus, and the extra alanines or ninth heptad serves to move the inhibitory domain out of the way. Although the exact location of this inhibitory domain is unknown, it is not simply the TRv2

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phosphorylation sites, since the phosphorylation state of TRv2 and its mutants did not regulate heterodimerization on 8IP. However, TRv2 phosphorylation may have some influence on TRv2-RXR binding to 8DR, since (phosphorylated) reticulocyte lysate-translated TRv2 heterodimerized somewhat less well on this element than did (nonphosphorylated) E. coli-derived TRv2. This probably would represent an extension of the process whereby phosphorylation impairs TRv2 monomer binding to DNA (29).

The above conclusions suggest that the steric interactions between TR and RXR are not identical on DR, Pal, and IP TREs. Previous models of heterodimerization (1) postulated that a flexible hinge separates the DNA and ligand binding (heterodimerization) domains, such that the TR-RXR ligand binding domain contacts can be the same, even though the relative orientations of the DNA binding domains differ. Our data, however, are not consistent with this model. Even if the putative hinge does exist, it apparently is not fully flexible, since it is clear that the stereochemistry of heterodimerization differs on DR, Pal, and IP TREs. This implies that details of the TRE sequence, specifically the half-site orientations, actively influence the TR-RXR interaction. Thus, the TRE is not simply a passive recipient of a preformed protein heterodimer, but rather, it actively influences the heterodimer structure.

This suggests that certain TR mutations could impair heterodimerization selectively depending on the orientation of the TRE half-sites. This is of possible relevance to the syndrome of RTH, which is caused by mutations in the TRβ ligand binding domain (see Ref. 39 for a recent review). The syndrome is generally inherited in an autosomal dominant manner, and the mutant TR is not neutral but, rather, has dominant negative activity on the wild type TR. It has been shown that mutant TRβ in this syndrome must retain DNA binding and RXR binding activity for them to possess dominant negative activity (17, 40). The clinical phenotype in this syndrome is variable and cannot be predicted simply by the decrease in T₃ binding affinity. If a mutation impaired heterodimerization on a subset of TREs, this could result in greater dominant negative activity on some TREs than on others and could help explain variations in phenotype.

In addition to its role in heterodimerization, the ninth heptad also appears to play a role in TR monomer binding to DNA. For example, when the ninth heptad of TRα1 is mutated to become the partial ninth heptad of TRv2 (α1–9H), there is a decrease in monomer binding to 8DR, 8Pal, and 8IP, even though heterodimer binding is significantly impaired only on 8Pal. Similarly, restoration of the full ninth heptad into TRv2 tends to enhance monomer binding to DNA, especially with E. coli-derived protein. In this regard, it is interesting to note that, due to their nonphosphorylated state, E. coli-derived TRv2 monomers bind DNA much better than do (phosphorylated) reticulocyte lysate-derived TRv2 monomers (29). This suggests a functional interaction between the ninth heptad and the phosphorylation state of TRv2. In any case, since the ninth heptad is not thought to contact DNA directly, it is presumed that this effect of the ninth heptad on TR monomer DNA binding is indirect.

The mechanism of the dominant negative activity of TRv2 has been contentious. A simple model, direct competition for DNA binding, is supported by some studies (20, 21). However, other data suggest that the effect is independent of DNA binding (22). Related to this is the question of why TRv2 has such weak dominant negative activity relative to mutant TRs from patients with RTH. Using essentially all TREs except 8DR, TRv2 shows a very minimal ability to heterodimerize with RXR and, hence, a similarly minimal ability to bind to the DNA. This would help explain the weak dominant negative activity of TRv2 if the mechanism is direct competition for DNA binding (presumably heterodimerization and DNA binding do occur on TREs such as 8Pal, 8IP, and 6DR, but too weakly to detect by traditional EMSA).

The DNA binding-deficient mutant of TRv2 (C56A) sheds light on this issue, since this protein is devoid of dominant negative activity. This supports the simple model of direct competition for DNA binding. However, the dominant negative activity on 8DR is only marginally greater than that on other TREs, even though by EMSA the DNA binding of TRv2-RXR heterodimers appears to be substantially greater on 8DR. This quantitative discrepancy could be explained in a number of ways. It is plausible that DNA binding is necessary but not sufficient for the dominant negative activity of TRv2. Recently nuclear receptor corepressors have been identified, which can bind to unliganded TRs and cause them to repress basal transcription (41, 42). A similar molecule might need to interact with DNA-bound TRv2 to permit dominant negative activity, and if this were the case, there may only be a weak correlation between the magnitude of TRv2-RXR binding to TREs by EMSA and the dominant negative activity in a transfection assay.

An alternative explanation for the above discrepancy between DNA binding and dominant negative activity on 8DR might be that the EMSA conditions do not accurately mimic DNA binding within the cell. Thus perhaps in vivo, binding of TRv2-RXR heterodimers to 8DR may be much weaker than binding of TRα1-RXR, even though our standard EMSA conditions show similar degrees of DNA binding. To assess this, we examined several properties of TRv2-RXR and TRα1-RXR binding to 8DR by EMSA. First, we performed the EMSA incubations with increasing concentrations of KCl (50–500 mM). Increasing salt had a parallel inhibitory effect on TRv2-RXR and TRα1-RXR binding to 8DR and thus did not yield conditions that could substantially weaken TRv2 binding relative to TRα1 (data not shown). Second, we examined the stability of TRv2-RXR and TRα1-RXR complexes on 8DR by adding a 1000-fold excess of nonradiolabeled 8DR after the EMSA binding reactions reached equilibrium and then loading aliquots onto the EMSA gel at various time points. The TRα1 and TRv2 heterodimer complexes were remarkably stable, with both showing only minimal loss of signal after 80 min of incubation with the competitor DNA (data not shown). In short, we were unable to find EMSA conditions that could demonstrate TRv2-RXR heterodimers to be substantially less stable or less likely to form on 8DR than TRα1-RXR heterodimers.

The above discussion presumes that the lack of dominant negative activity of TRv2 C56A indicates that DNA binding is essential for dominant negative activity. However, we cannot totally exclude the possibility that the TRv2 C56A mutation disrupts some unknown protein-protein interaction, which accounts for the loss of dominant negative activity, rather than the disruption of TR-DNA binding.

The reason why the dominant negative activity of TRv2 is substantially weaker than that of RTH mutant TRs is complex. Restoring the full ninth heptad into TRv2 (α2+9H) restores highly potent dominant negative activity, indicating that the loss of the full ninth heptad accounts for the weak TRv2 dominant negative activity (all known RTH mutations occur outside the ninth heptad). It would be expected that the role of the ninth heptad in this situation is either to enhance DNA binding (by enhancing heterodimerization with RXR) and/or to sequester RXR in solution. The DNA binding mutant α2+9H C56A retains ~50% of its dominant negative activity on 8DR.
and 8IP, indicating that enhanced DNA binding is only one-half of the explanation on these TREs. We expected sequestration of RXR in solution to be the other half, in which case cotransfection of RXR should at least partially overcome the dominant negative activity of a2+9H. Unexpectedly, this did not occur, suggesting that the full ninth heptad plays a role in sequestering a currently unknown RXR factor, which is important for full T3 induction of gene expression on 8DR and 8IP. The results with 8Pal are somewhat different in that a2+9H C56A retains near complete dominant negative activity of TRv2. As with 8DR and 8IP, cotransfection of RXR did not relieve the dominant negative activity of a2+9H on 8Pal, again suggesting that the mechanism involves sequestration of some unknown non-RXR factor. Clarification of this mechanism and identification of the postulated factor would be of importance in understanding T3 regulation of gene expression, the dominant negative activity of TRv2, and the syndrome of RTH.

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