On the Role of the Carboxyl-Terminal Helix of RXR in the Interactions of the Receptor with Ligand[†]

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ABSTRACT: The retinoid X receptor (RXR), a ligand-inducible transcription factor that is activated by 9-*cis*-retinoic acid, is a member of the superfamily of nuclear hormone receptors. The ligand-induced transcriptional activity of nuclear receptors is coordinated by their C-terminal region termed the ligand-binding domain. Structural analyses of several nuclear receptors showed that the most dramatic ligand-induced conformational change in these proteins involves a positional shift in the receptors' C-terminal helix, termed helix 12. Consequently, in the liganded state, helix 12 is folded over the entrance to the ligand-binding pocket where it serves as a lid, and it has been proposed that this region functions to stabilize ligand binding by at least some nuclear receptors. Here, to examine the possible role of helix 12 in contributing to the association of RXR with its ligand, the equilibrium and kinetic parameters of the interactions of 9-*cis*-retinoic acid with RXR and with a deletion mutant lacking helix 12 were measured. Deletion of the region did not significantly alter the ligand-binding affinity of RXR at equilibrium. However, both the rate of dissociation and the rate of association of the RXR−9-*cis*-retinoic acid complex were significantly slower in the absence of helix 12. Taken together, these observations suggest that helix 12 of RXR facilitates both the entry and the exit of the ligand from the binding pocket without affecting the equilibrium ligand-binding affinity. The results thus point at a previously unsuspected function for this region.

Retinoic acid (RA)[1] is a potent modulator of rates of transcription of multiple target genes, and, consequently, plays pleiotropic roles in regulating cell proliferation and differentiation in a variety of tissues both in the embryo and in the adult. The biological activities of RA are mediated by two types of ligand-inducible transcription factors that belong to the superfamily of nuclear hormone receptors. These proteins, the retinoid X receptors (RXRs) and the retinoic acid receptors (RARs), are activated by stereoisomers of RA. RARs are activated both by the 9-cis and by the all-trans isomers of RA (9cRA and tRA, respectively), whereas RXRs respond to 9cRA exclusively (*1*). Nuclear receptors share similar structural organization and functional motifs. They consist of an amino-terminal domain (A/B) which contains an autonomous constitutive activation function (activation function 1, AF-1), a DNA-binding region (domain C), a hinge region D, and a C-terminal ligand-binding domain, termed E. Some nuclear receptors contain an additional F extension at their C-terminal, the function of which is unknown (reviewed in ref *2*).

Nuclear receptors function as dimers. Some, such as steroid receptors and RXR, interact with DNA as homodimers (*3*−*5*). However, with the exception of RXR, receptors that are members of the nonsteroid branch of the superfamily, e.g., RAR, the vitamin D receptor (VDR), peroxisome proliferator-activated receptor (PPAR), and the thyroid hormone receptor (TR), require heterodimerization with RXR for tight binding to DNA (*4*−*6*). Hence, RXR serves as a common binding partner in multiple heterodimeric complexes, and is therefore a pivotal integrator of various nuclear hormone receptor signaling pathways.

The ligand-binding domain (LBD) of nuclear receptors coordinates their ligand-dependent transcriptional activity. This domain contains the ligand-binding pocket as well as regions that mediate multiple ligand-dependent protein−protein interactions, including association with transcriptional coregulators (*7*−*10*), formation of dimers (*11*), and, in the case of RXR, formation of tetramers (*12*). The LBDs of nuclear receptors are far less conserved than their DNA-binding domains. For example, there exists only 30% sequence identity between the ligand-binding domains of RXR and RAR. Nevertheless, all of the LBDs for which crystal structures have been described (*8*, *11*, *13*−*15*) share a common fold. They consist of 12 α-helices (numbered H1−H12 from the amino to the carboxyl termini), and a β-turn arranged as an antiparallel 'sandwich' in a three-layer structure (see ref *16* for details). Comparison between the structures of LBDs of nonliganded and liganded receptors led to the suggestion of a general mechanism, termed the 'mouse trap' model, which describes how a series of ligand-induced conformational changes may lead to receptor activation (*16*). According to this model, ligand binding by nuclear

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[1] Abbreviations: DOPC, dioleoylphosphatidycholine; ER, estrogen receptor; H12, helix 12; PPAR, peroxisome proliferator-activated receptor; RA, retinoic acid; 9cRA, 9-*cis*-retinoic acid; tRA, *all-trans*-retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; TR, thyroid hormone receptor; VDR, vitamin D receptor.

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receptors induces the C-terminal helices, H11 and H12, to undergo a large conformational change whereby H12 shifts from its extended position in the absence of ligand, to a position where it folds over the entrance to the ligand-binding pocket where it serves as a 'lid'. These conformational changes lead to the formation of a surface involving H12, H3, and H4 which mediates interactions of the liganded receptor with transcriptional coactivators. These coactivators, in turn, mediate between the receptor and the general transcription machinery (*7, 8, 16*).

The critical role of H12 in mediating the transcriptional activity of nuclear receptors has been established by numerous studies that showed that this amphipathic region comprises the core of the receptors' ligand-dependent activation function (AF-2), and that it acts via ligand-dependent assembly of a multicomponent coactivator complex. Other suggested functions for this region include mediation of the release of corepressors that are associated with some receptors in the absence of ligand (*17, 18*), and, in the case of RXR, communication with heterodimerization partners (*19*). The 'mouse trap' model also proposes that, in its closed position, H12 may interact with the ligand, thereby stabilizing ligand binding to the receptor. Indeed, in the holo-LBD structures of RAR and TR, certain amino acid residues within H12 appear to interact with the bound ligand (*13, 14*). The suggestion that H12 plays a role in regulating ligand binding by nuclear receptors is further supported by the observations that certain residues within H12 of RAR are important for determining the ligand selectivity of the receptor (*20*), that deletion of this region results in a 6-fold reduction of the ligand-binding affinity of RAR (*13*), and that mutations in specific H12 residues of TRα diminish the receptor's ligand-binding affinity by 3−13-fold (*21*).

The following question thus arises: Is interaction with bound ligand and thus stabilization of ligand−receptor complexes a general function of the C-terminal helix of nuclear receptors? Clear answers to this question await the solution of additional structures of liganded nuclear receptors. The proposed 'mouse trap' model was largely based on comparison of the crystal structures of holo-RARγ (*13*) and apo-RXRα (*11*), while the structure of holo-RXR has not been described yet. The study presented here was thus undertaken in order to examine whether H12 of RXR plays a role in stabilizing the association of this receptor with its ligand. To this end, the effects of truncation of H12 on the equilibrium and kinetic parameters characterizing the interactions of RXRα with 9cRA were studied. The data show that deletion of H12 has little effect on the equilibrium dissociation constant of the RXR−9cRA complex. Surprisingly, the observations indicate further that H12 facilitates both the entry and the exit of 9cRA into the ligand-binding pocket of RXR, suggesting an unexpected role for this region.

EXPERIMENTAL PROCEDURES

Ligand. 9-*cis*-Retinoic acid (9cRA) was purchased from ICN Pharmaceuticals Inc. (Costa Mesa, CA). Stock solutions in ethanol were stored in amber vials at −20 °C.

Proteins. mRXRα lacking the A/B domain (RXRαΔAB) was obtained by overexpression in *Escherichia coli* as previously described (*22*). This protein displays identical DNA-binding, ligand-binding, and oligomerization properties as the full-length protein (*12, 22−24*). RXRαΔAB lacking helix 12 (RXRαΔABΔH12, comprised of amino acid residues 140−448) was prepared by PCR amplification of the respective regions of full-length mRXRα. The purified PCR fragment was ligated into the PCR2.1 vector, amplified, and subcloned into the *Nde*I−*Xho*I sites of the bacterial expression vector pET16b. Plasmids were amplified in the *E. coli* strain DH5α and transfected into *E. coli* BL21 for protein expression. *E. coli* harboring the vector were grown at 37 °C to an $OD_{600\ nm}$ of 0.6, protein expression was induced with 0.5 mM IPTG, and cells were grown for an additional 3 h period, and pelleted by centrifugation. Following lysis, the protein was purified by metal-chelating affinity chromatography as previously described (*22, 24*). RXRαΔABΔH12 displayed wild-type ability to self-associate into dimers and tetramers (*12*) and to properly associate with cognate DNA (data not shown), verifying that the overall fold of the protein was not significantly altered. The purity of the isolated protein was analyzed by SDS−PAGE, and protein concentration was determined by the Bradford assay (Bio-Rad) using bovine serum albumin as a standard.

Fluorescence Titrations. The equilibrium dissociation constants of complexes of 9cRA with the appropriate receptors were studied by fluorescence titrations using a SPEX Industries Fluorolog-2 spectrofluorometer (Metachen, NJ). Protein (1 μM) was placed in a cuvette and titrated with 9cRA from a concentrated solution in ethanol. Ligand binding was monitored by following the ligand-induced decrease in the intrinsic fluorescence of the protein (λ_{ex} 280 nm, λ_{em} 340 nm). Ethanol concentration never exceeded 2%. Titration curves were analyzed by fitting to a binding equation (*25*) using the software Origin 4.1 (MicroCal).

Rates of Transfer of 9cRA from RXR to Unilamellar Vesicles. Unilamellar vesicles of dioleoylphosphatidylcholine (DOPC, Avanti Polar Lipids) were prepared by sonication as previously described (*26*). Following sonication, vesicle suspensions were centrifuged to remove multilamellar structures, and the lipid concentration of the suspension was determined by measuring its phosphorus content (*27*). The appropriate receptor was complexed with 9cRA at an equimolar ratio and mixed with vesicles using a rapid mixing apparatus (HiTech, Salisbury, U.K.). Rates of transfer of 9cRA from RXR to the vesicles were monitored by following the time-dependent increase of the fluorescence of the protein following mixing of holo-RXR with vesicles. Alternatively, to monitor movement of 9cRA to the vesicles, the fluorescent lipid probe 1-hexadecanoyl-2-(1-pyrenehexanoyl-*sn*-glycero)-3-phosphocholine (PY-PC) was incorporated into DOPC vesicles by cosonication (PY-PC/DOPC = 0.003 mol/mol). Transfer of 9cRA from RXR to the vesicles was followed by monitoring the time-dependent quenching of the fluorescence of the probe which was observed upon arrival of the ligand at the vesicles. Data were fitted to a single first-order reaction equation to yield the rate constant of the transfer reaction.

RESULTS

Deletion of H12 Does Not Affect the Equilibrium Dissociation Constant of the RXR−9cRA Complex. To examine whether H12 contributes to the ligand-binding affinity of RXR, the effect of deleting this region on the equilibrium

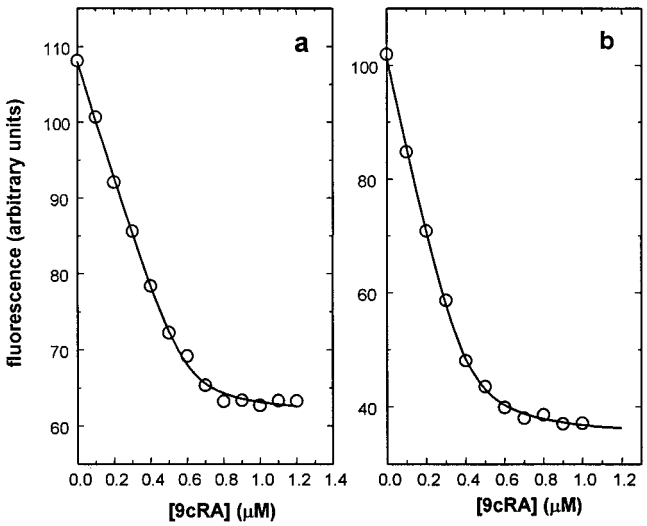

FIGURE 1: Fluorescence titrations of RXR and its ΔH12 mutant. RXRαΔAB or RXRαΔABΔH12 (1 μM) was titrated with 9cRA from a concentrated solution in ethanol. Binding of the ligand to RXRαΔAB (a) or RXRαΔABΔH12 (b) was monitored by following the ligand-induced decrease in the intrinsic fluorescence of the protein (λ_{ex} = 280 nm, λ_{em} = 340 nm). Titration curves were fitted to an equation derived from simple binding theory (solid lines through data points) to obtain the equilibrium dissociation constants of the proteins.

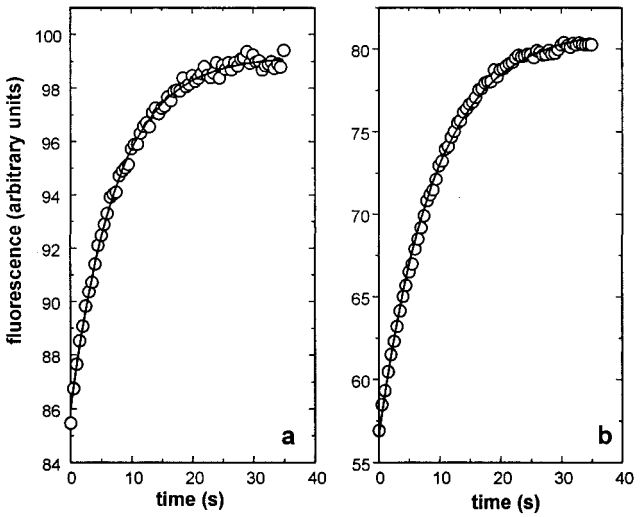

FIGURE 2: Rates of transfer of 9cRA from RXR and its ΔH12 mutant to lipid vesicles, monitored by following changes in the intrinsic fluorescence of the receptors. RXRαΔAB or RXRαΔAB-ΔH12 was complexed with 9cRA at an equimolar ratio and mixed with unilamellar vesicles of DOPC using a rapid mixing apparatus. Final concentrations of protein and vesicles were 1 μM and 20 mM, respectively. Movement of ligand from the proteins was monitored by following the time-dependent release of ligand-induced quenching of the intrinsic fluorescence of the receptors. Representative traces of transfer of ligand from RXRαΔAB (a) or RXRαΔABΔH12 (b) are shown. Data were analyzed by fitting to a single first-order reaction equation (solid lines through data points) to obtain the reaction rate constants.

dissociation constant characterizing the interaction of RXR with 9cRA was examined. K_ds for RXRαΔAB and for RXRαΔABΔH12 were measured by fluorescence titrations. Due to the extensive overlap of the fluorescence emission spectra of tryptophans and tyrosines and the absorption spectra of retinoids, the intrinsic fluorescence of many retinoid-binding proteins is quenched upon ligand binding. This phenomenon has been widely used to study the interactions of a variety of retinoid-binding proteins, including RXR, with their ligands (e.g., see refs *25*, *28*−*31*). Ligand binding by the receptors was monitored by following the saturable decrease in the fluorescence of the protein (λ_{ex} 280 nm, λ_{em} 340 nm) upon titration with 9cRA (Figure 1). Titration curves were carried out in duplicate for three separate preparations of RXRαΔAB or RXRαΔABΔH12 and analyzed by fitting the data to an equation derived from simple binding theory (see Experimental Procedures). The values of the K_ds thus extracted were found to be 41 ± 12 and 31 ± 10 nM for RXRαΔAB and RXRαΔABΔH12, respectively. These data indicate that truncation of H12 does not significantly affect the ligand-binding affinity of RXR.

Effect of Deletion of H12 on the Rates of Dissociation and Association of the RXR−*9cRA Complex*. The observations that the K_ds characterizing the association of 9cRA with RXRαΔAB and with RXRαΔABΔH12 are similar may be taken to suggest that H12 of RXR is not involved in receptor−ligand interactions. However, because of sensitivity limitations of the method, the K_d measurements described above were carried out using protein concentrations in the 0.5−1 μM range, i.e., at concentrations that are significantly higher than the K_d. Hence, although the derived values are within the range previously reported for the interactions of RXR with 9cRA (*32*−*35*), they should be considered upper limits for the actual values (see ref *25* for discussion). In addition, it should be noted that K_d values depend on the rate constants of both the dissociation and the association of the protein−ligand complex (k_{off} and k_{on}, respectively) by the relationship $K_d = k_{off}/k_{on}$. It is thus possible that truncation of H12 affected both of these rate constants to similar extents, resulting in a small overall change in the apparent K_d. To further examine possible involvement of H12 in the interactions of RXR with its ligand, the rate constant of dissociation of 9cRA from the receptor was measured directly. If H12 contributes to the stability of the RXR−9cRA complex, it can be expected that deletion of this region will result in a faster rate of dissociation of the ligand−receptor complex.

To induce dissociation of 9cRA from RXR, the holoprotein was mixed with unilamellar vesicles of DOPC. Lipid vesicles possess a high affinity for retinoids and thus can serve as an efficient hydrophobic sink for 9cRA (*26*, *36*). As binding of 9cRA to RXR results in quenching of the intrinsic fluorescence of the protein (Figure 1), movement of the ligand away from the receptor, following mixing of holo-RXR with vesicles, could be followed by monitoring the time-dependent release of protein fluorescence. In this setting, if transfer of ligand to vesicles is allowed to proceed to completion, i.e., if all the ligand is transferred at equilibrium, the rate of the reverse reaction is negligible, and the rate constant of the observed reaction directly reflects the apparent rate for dissociation of the ligand−protein complex ($_{app}k_{off}$) (*37*). RXRαΔAB or RXRαΔABΔH12 was precomplexed with 9cRA at an equimolar ratio and mixed with a large excess of DOPC vesicles using a rapid-mixing apparatus. Movement of 9cRA from the protein to the lipid vesicles was monitored by following the time-dependent increase in intrinsic fluorescence of the protein until equilibration was reached (Figure 2). The data were analyzed by fitting to a single first-order reaction (solid lines in Figure 2) to yield the rate constants of the transfer reactions. Under

Table 1: Effect of Concentrations of Acceptor Vesicles on the Rate Constant of Transfer of 9cRA from RXR[a]

receptor	[DOPC] (mM lipid)	$_{app}k_{off}$[b] (s^{-1})
RXRαΔAB	15	5.7 ± 0.4
	20	5.6 ± 0.4
	30	6.1 ± 0.3
RXRαΔABΔH12	15	8.5 ± 0.7
	20	8.8 ± 0.7
	30	8.3 ± 0.6

[a] Rate constants for the transfer reaction ($_{app}k_{off}$) were measured as described in the legend to Figure 2. Multiple measurements using three separate preparations of each receptor were carried out to obtain the mean values shown. [b] Mean ± SEM, $n = 15-23$.

the conditions used in these experiments, the observed rate constants were independent of the concentration of vesicles (Table 1), verifying that the observed rate constant directly reflected the rate constant of dissociation of 9cRA−RXR complexes. It may be noted that these measurements do not allow for identification of the exact nature of the rate-limiting step of the dissociation reaction. Such a step could be the actual movement of the ligand from the protein into the bulk solution, or it could reflect a reorganization of the ligand−protein complex which precedes the actual solvation of the ligand. However, the apparent values of k_{off} thus obtained characterize the overall reaction by which 9cRA dissociates from RXR and its ΔH12 mutant.

The studies depicted in Figure 2 utilized the response of the fluorescence of the protein to the presence of bound 9cRA as a 'read-out' for the reaction by which 9cRA moves off RXR. To further verify that the measured rate constants indeed reflected the rate of the overall dissociation reaction, transfer of 9cRA from RXR to lipid vesicles was also studied by measuring the rate of arrival of the ligand at the lipid bilayers. To this end, vesicles of DOPC containing the fluorescent lipid probe PY-PC were made. The absorption spectrum of 9cRA ($\lambda_{max} = 345$ nm) extensively overlaps with the fluorescence emission spectrum of pyrene ($\lambda_{max} = 377$ nm). Consequently, the presence of 9cRA in probe-containing vesicles results in quenching of the fluorescence of the probe (Figure 3a, inset). Hence, the rates of arrival of 9cRA at the vesicles could be monitored by the time-dependent decrease of the fluorescence of the probe following mixing of probe-containing vesicles with holo-RXRαΔAB or holo-RXRαΔABΔH12 (Figure 3).

As shown in Table 2, the mean apparent rate constant for dissociation of 9cRA from RXRαΔAB measured both by monitoring the change in the fluorescence of the protein and by following the rate of arrival of the ligand at the vesicles was found to be 0.12 s^{-1}, corresponding to a $t_{1/2}$ of 5.8 s. The similarity of the values obtained by the two types of assays confirms that the measurements indeed report on the rate-limiting step of the dissociation of the 9cRA−RXR complex. This value is also in excellent agreement with a previously reported value [$t_{1/2} = 5.1$ s, (*36*)]. The mean apparent rate constant for dissociation of 9cRA from RXRαΔABΔH12 was found to be 0.08 s^{-1} ($t_{1/2} = 8.5$ s). Based on the observed values of $_{app}k_{off}$ and K_ds obtained from the fluorescence titrations and by using the relationship $K_d = {}_{app}k_{off}/{}_{app}k_{on}$, the rate constants for association of 9cRA with RXR and with its ΔH12 counterpart were calculated. The extracted $_{app}k_{on}$s showed that removal of H12 resulted in a 2-fold decrease in the rate of association of 9cRA with RXR (Table 2). Overall, these observations reveal that the rates of both the association and the dissociation of the RXR−9cRA complex are significantly slower in a receptor lacking its C-terminal helix as compared to full-length RXR.

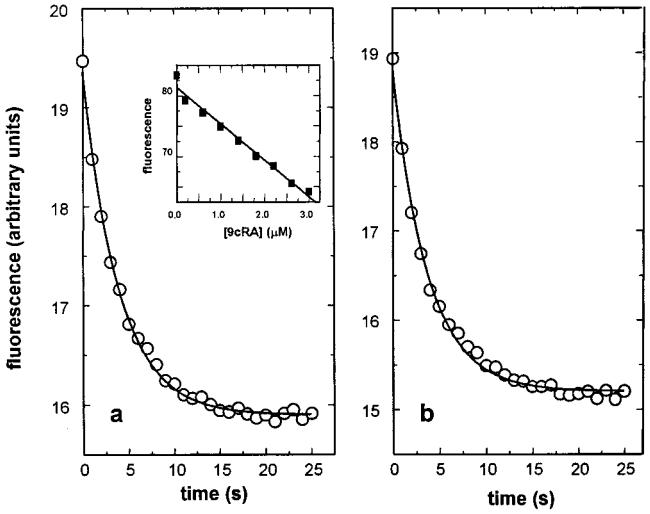

FIGURE 3: Rates of transfer of 9cRA from RXR and its ΔH12 mutant to lipid vesicles, monitored by following the rates of arrival of the ligand at the vesicles. Vesicles of DOPC (1 mM) containing the lipid fluorescent probe PY-PC (3 μM) were placed in a cuvette and titrated with 9cRA, demonstrating that the presence of 9cRA in the vesicles results in quenching of the fluorescence of the probe (λ_{ex} 329 nm, λ_{em} 377 nm; Figure 3a, inset). To measure the rates of transfer of 9cRA from RXRαΔAB or the ΔH12 mutant to PY-PC-containing vesicles, the appropriate receptors were complexed with 9cRA at an equimolar ratio and mixed with unilamellar vesicles of DOPC containing the probe PY-PC (final concentrations of protein and lipids 4 and 100 μM, respectively). Movement of 9cRA was monitored by following the time-dependent ligand-induced quenching of the fluorescence of the lipid-embedded probe. Representative traces of transfer of ligand from RXRαΔAB (a) or RXRαΔABΔH12 (b) are shown. Data were analyzed by fitting to a single first-order reaction equation (solid lines through data points) to obtain the reaction rate constants.

Table 2: Effect of Deletion of H12 on the Parameters Characterizing the Interactions of RXR with 9cRA[a]

receptor	K_d* (nM)	$_{app}k_{off}$** (s^{-1})	$_{app}t_{1/2\ off}$ (s)	$_{app}k_{on}$ ($M^{-1}\ s^{-1}$)
RXRαΔAB	41 ± 12	0.119 ± 0.004 (0.117 ± 0.003)***	5.8 ± 0.2	3.9×10^6
RXRαΔABΔH12	31 ± 10	0.082 ± 0.004 (0.077 ± 0.009)*	8.5 ± 0.4	2.0×10^6

[a] Equilibrium dissociation constants (K_d) were measured by fluorescence titrations as described in the legend to Figure 1. Apparent rate constants for dissociation ($_{app}k_{off}$) were obtained by using the fluorescence of RXR as a 'read-out' for dissociation of the RXR−9cRA complexes as described in the legend to Figure 2. Shown in parentheses are the values of $_{app}k_{off}$ obtained by following the rate of arrival of 9cRA at the vesicles, which were measured as described in the legend to Figure 3. Apparent rate constants for association ($_{app}k_{on}$) were calculated from the respective K_ds and k_{off}s by using the relationship: $K_d = {}_{app}k_{off}/{}_{app}k_{on}$. Values are mean ± SEM: $*n = 6$; $**n = 18-22$, $***n = 5$.

DISCUSSION

The details of the interactions of nuclear receptors with their cognate hormones are important for understanding how these transcription factors attain the activated state that allows

them to modulate gene expression. Structural and functional analyses of nuclear receptors showed that ligand-induced activation of these proteins is coordinated by their ligand-binding domain, and that the most dramatic conformational changes that occur in this region upon interactions with a cognate hormone involve a positional shift of the two most C-terminal helices, termed H11 and H12 (*16*). These changes result in the formation of a surface that mediates the association of the receptor with a multicomponent coactivator complex which, in turn, allows for transcriptional activation of the target gene (*1*). An additional function for H12 was suggested by the three-dimensional crystal structures of holo-hRARγ and holo-TRα which indicated that, in the liganded-position, certain residues within this region interact with the bound ligand (*13, 14*). It was thus proposed that while surface residues of H12 mediate receptor−coactivator association, residues with side chains that are directed toward the ligand-binding pocket serve to stabilize the interactions of these receptors with their ligands. Indeed, mutations or deletion of H12 of TR and of RAR significantly reduce their ligand-binding affinities (*13, 14*).

Despite their manyfold similarities, nuclear receptors display distinct functional and structural properties. Notably, RXR possesses several features that distinguish it among nuclear receptors. This protein is unique in that it can heterodimerize with numerous other receptors and is thus involved in multiple signaling pathways (*1*). Also unlike other receptors, RXR self-associates into transcriptionally inactive homo-tetramers, and thus acts as an auto-silencer in the absence of its cognate ligand (*38*). Tetramer formation by RXR was reported to be mediated through H11 of the receptor's ligand-binding domain, indicating that at least one region at the C-terminal of RXR plays a role that appears to be specific to this receptor. The present work focused on the most C-terminal helix of RXR, H12, and was undertaken in order to examine whether, similarly to its role in RAR and TR, this region stabilizes ligand binding by RXR. To this end, the equilibrium and kinetic parameters of the interactions of 9cRA with RXR and with a deletion mutant lacking H12 were measured.

The equilibrium dissociation constants of RXR and its ΔH12 mutant were found to be very similar (Table 2), suggesting that, unlike H12 of RAR and TR, H12 in RXR does not function to stabilize the ligand−receptor complex. The crystal structure of holo-RXR has not been reported, but a situation in which H12 does not directly contribute to ligand binding has been described for two nuclear receptors: the estrogen receptor α (*15*) and PPARγ (*8*). In both of these cases, none of the H12 residues were seen to directly coordinate with the bound ligands, although the region was found to be folded over the entrance to the ligand-binding pocket in agreement with the 'mouse trap' model (*16*). The uniformity of the position of H12 in all liganded nuclear receptors for which crystal structures have been described suggests that it will be similarly arranged in liganded RXR. The observations that truncation of this helix does not change the ligand-binding affinity of RXR may thus imply that, even in its folded position, movement of H12 is sufficiently dynamic so that it does not obstruct the exit of the ligand from the pocket to a measurable extent.

To further examine a potential contribution of H12 of RXR to the mode by which the receptor interacts with its ligand, the apparent rate constants of dissociation ($_{app}k_{off}$s) of 9cRA from RXR and from its ΔH12 mutant were measured directly, and $_{app}k_{on}$s were calculated based on the measured values of the K_ds and the $_{app}k_{off}$s. The equilibrium dissociation constant of the RXR−9cRA complex is in the 10−50 nM range [(*32*−*35*) and Table 2]. Hence, the ligand-binding affinity of RXR is markedly weaker than that displayed by RAR toward tRA, which was reported to be in the subnanomolar range (*32, 39*). The kinetic parameters by which RXR and RAR interact with their respective ligands are also notably different. The rate of dissociation of the RXR−9cRA complex ($t_{1/2}$ = 5.8 s, Table 2) is about 4-fold faster than that of the RARα−tRA complex ($t_{1/2}$ = 25 s, data not shown). The rate of association of RXR with 9cRA (k_{on} = 4×10^6 M^{-1} s^{-1}, Table 2) is over an order of magnitude slower than that of RARα−tRA (k_{on} = 5.6×10^7 M^{-1} s^{-1}, based on K_d = 0.5 nM and the measured k_{off}). Hence, the weak ligand-binding affinity of RXR vs RAR originates from both a slower rate of association and a faster rate of dissociation of the former complex, with a sluggish rate of association being the major contributor to the overall difference.

If H12 of RXR significantly contributes to the interactions of the receptor with 9cRA, it can be expected that deletion of this region will result in a faster rate of dissociation of the ligand−receptor complex. Surprisingly, $_{app}k_{off}$ for the dissociation of 9cRA from RXRΔH12 was found to be significantly smaller than the rate constant for dissociation from the full-length receptor (Table 2). Similarly, the rate constant for association of RXRΔH12 with 9cRA was 2-fold smaller than that of the full-length receptor. Hence, truncation of H12 hampered the kinetics of both the association and the dissociation of the RXR−9cRA complex, indicating that this region facilitates both the binding and the release of the ligand by RXR. The mechanism by which H12 exerts this effect is not clear, but a possible explanation is suggested by the amino acid composition of this region. H12 possesses several hydrophobic amino acid residues and is amphipathic in nature. It is thus reasonable to suggest that it will have an affinity toward the lipophillic RXR ligand. Consequently, in its extended position in the unliganded receptor, H12 may attract the ligand from the bulk solution and help to guide it toward the pocket, thereby facilitating ligand−protein interactions. In the same vein, in the liganded state, when H12 is believed to lie over the entrance of the pocket, it may have a similar effect; i.e., when movement of bound ligand within the pocket brings it toward the surface, it will be attracted by H12 which will help guide its exit. The results of the present study thus point at a previously unsuspected role for the C-terminal helix of RXR. The amphipathic nature and the extended position of H12 in other nuclear receptors raise the possibility that facilitation of ligand binding may be a general function of this region.

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