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The AF-1 and AF-2 Domains of RAR γ 2 and RXR α Cooperate for Triggering the Transactivation and the Degradation of RAR γ 2/RXR α Heterodimers*

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In eukaryotic cells, liganded RAR γ 2/RXR α heterodimers activate the transcription of retinoic acid (RA) target genes and then are degraded through the ubiquitin-proteasome pathway. In this study, we dissected the role of the RAR γ 2 and RXR α partners as well as of their respective AF-1 and AF-2 domains in the processes of transactivation and degradation. RAR γ 2 is the “engine” initiating transcription and its own degradation subsequent to ligand binding. Integrity of its AF-2 domain and phosphorylation of its AF-1 domain are required for both the degradation and the transactivation of the receptor. Deletion of the whole AF-1 domain does not impair these processes but shifts the receptor toward other proteolytic pathways through RXR α . In contrast, RXR α plays only a modulatory role, cooperating with RAR γ 2 through its AF-2 domain and its phosphorylated AF-1 domain in both the transcription activity and the degradation of the RAR γ 2/RXR α heterodimers. Our results underline that the AF-1 and AF-2 domains of each heterodimer partner cooperate with one other and that this cooperation is relevant for both the transcription and degradation processes.

In response to retinoic acid (RA),¹ target genes are regulated by two families of nuclear receptors, the RARs (α , β , and γ) and

the RXRs (α , β , and γ) that bind as RAR/RXR heterodimers to response elements located in their promoters (1–3). RARs and RXRs are modular proteins (Fig. 1) with a highly conserved central DNA-binding domain and a less conserved ligand-binding domain that is composed of 11 α -helices (H1 and H3–H12), loops, and two short β -strands (Ref. 4 and references therein) with a dimerization interface formed mainly by helices H9 and H10 (5). The recent comparison of the crystal structures of the ligand-binding domain of unliganded and liganded RARs and RXRs (1, 6, 7) shed light on the molecular mechanism underlying the structural reorganization that accompanies ligand binding. The ligand-induced conformational changes in the ligand-binding domain result in the release of corepressors and in conformational rearrangements that affect mostly the N-terminal part of H3, H11, and the highly conserved amphipathic helix 12, which carries the autonomous activation function AF-2 (8). The new conformation generates an interaction surface for coactivators (9), which then recruit multiprotein complexes and lead to the activation of responsive genes (10, 11). Through this surface, RARs also interact with SUG-1 (12), which belongs to the 19 S regulatory complex of the 26 S proteasome (13).

In RAR/RXR heterodimers, RXR is subordinated to the nonliganded RAR and therefore cannot autonomously induce transcription upon binding of a cognate agonist (14). However, in the presence of both RAR and RXR ligands, RXR synergizes with RAR for the recruitment of coactivators and thus for the transcription of RA target genes.

The N-terminal region of RARs and RXRs contains another transcription activation domain called AF-1, which acts autonomously and ligand-independently (15). The interesting feature of this AF-1 domain is that it contains consensus phosphorylation sites for proline-dependent kinases (for a review, see Ref. 16). The AF-1 domain of unliganded RAR γ 2 (Fig. 1) is phosphorylated at serine 68 (17) by cdk7/cyclin H-associated to transcription factor IIH, a general transcription factor also involved in DNA repair (18). Phosphorylation of this serine is required for RA-induced transcription initiation (17). In response to RA, however, RAR γ 2 can also be phosphorylated at the nearby serine 66 by p38MAPK (12, 19). Phosphorylation of RXR α by MAPKs (Fig. 1) at three residues located in the AF-1 domain (Ser-61, Ser-75, and Thr-87) has been also reported (20).

We have recently shown that liganded RAR γ 2 is degraded by the ubiquitin-proteasome pathway when heterodimerized with RXR α and engaged in transcription of RA target genes (12) according to the following model. The fraction of RAR γ 2 that is bound to cognate response elements as heterodimers with

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¹ The abbreviations used are: RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoic X receptor; AF, activation function domain; CAT, chloramphenicol acetyltransferase; MAPK, mitogen-activated protein kinase; WCE, whole cell extract; WT, wild type; Z, benzyloxy-carbonyl; fmk, fluoromethyl ketone; m, mouse.

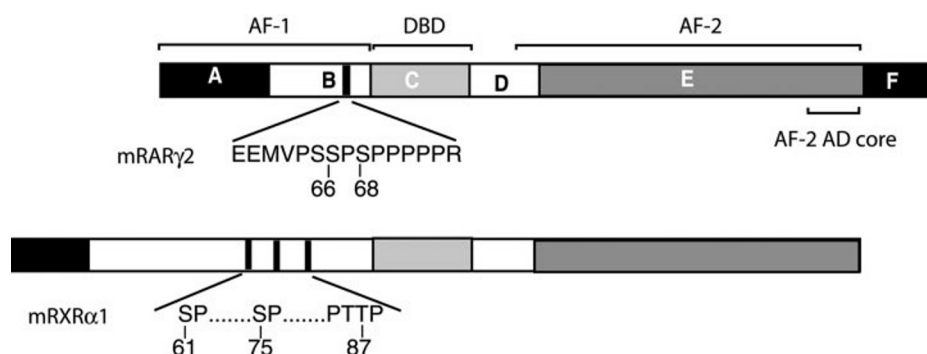


FIG. 1. Schematic representation of the functional domains and the major phosphorylation sites of RAR γ 2 and RXR α 1. The functional AF-1 and AF-2 domains that lie in the A/B and E regions respectively are schematically represented (not to scale). The DNA-binding domain (DBD) and the ligand-binding domain are also depicted. The target sequences for phosphorylation by proline-directed kinases are also shown.

RXR α is phosphorylated by the cdk7 subunit of transcription factor IIH and activates transcription, which increases up to 24–48 h and then reaches a plateau. This restriction in transcription is concomitant to the RA-induced activity of p38MAPK, which leads to further phosphorylation of the AF-1 domain of RAR γ 2. This marked increase in phosphorylation acts as a permissive signal, paving the way to RAR γ 2 degradation through an increase in its ubiquitylation and subsequent recognition by the proteasomal SUG-1 subunit bound at helix 12. Liganded RXR α is also degraded by the proteasome pathway (21–23), but whether this process is controlled by its AF-1 domain and/or its phosphorylation remains unknown. Most interestingly, both phosphorylation and the ubiquitin-proteasome machineries also play a role in RAR γ -mediated transcription. Therefore, we postulated that phosphorylation of the AF-1 domain would play a dual role, programming on the one hand the transcriptional pattern and on the other hand the receptor degradation, to prevent a single RAR γ /RXR α heterodimer from performing endless rounds of transcription of the cognate RA target genes and therefore controlling the magnitude of transcription.

In the present study, we have investigated how, within RAR γ 2/RXR α heterodimers, the AF-1 and AF-2 domains of each receptor regulate, in addition to their transcriptional properties, their own degradation and that of the partner. We show that the AF-2 domains of each receptor are crucial for their autonomous degradation. The phosphorylated AF-1 domain of RAR γ 2 is a fundamental determinant of the degradation and the transactivation of both partners. Unexpectedly, deletion of the whole AF-1 domain does not impair these processes but targets RAR γ 2 and its dimerization partner RXR α toward different mechanisms of regulation. We also show that the AF-1 of RXR α can be phosphorylated in response to RA. This phosphorylation has only a modulatory effect on the degradation of RXR α and its cooperation with RAR γ 2 for transcription.

EXPERIMENTAL PROCEDURES

Plasmids, Antibodies, and Chemicals—The pSG5-based expression vectors for mouse (m) RAR γ 2WT, mRAR γ 2 Δ AF-1, mRAR γ 2 Δ AF-2, mRXR α WT, mRXR α 2 Δ AF-1, mRXR α 2 Δ AF-2, mRXR α 2 Δ Het expression vectors and the DR5-tk-CAT reporter gene have been described elsewhere (21, 24, 25). The expression vectors for RAR γ 2S66/68A and for RXR α S61/S75/T87A were as described (17, 20).

Rabbit polyclonal antibodies raised against the F region of RAR γ , RP γ (F), have been described previously (26). The polyclonal and monoclonal antibodies raised against the A region of RXR α , RPRX α (A), and the E region of RXR α , Ab4RX3A2, respectively, were also described previously (27).

MG-132 and z-VAD-fmk were from Calbiochem. The synthetic retinoids BMS961 and BMS649 were a gift from Bristol-Myers Squibb.

Cell Lines, Transfections, and CAT Assays—F9 cells were grown in

Dulbecco's modified Eagle's medium under 7% CO $_2$ with a change of medium every 48 h, as described (28). F9 cells ablated for RXR α (RXR α ^{-/-} cells) and F9 cells reexpressing RAR γ 2 Δ AF-1 in a RAR γ -null background (RAR γ 2 Δ AF-1 rescue line) were described previously (29, 30). Rescue lines expressing RAR γ 2 Δ AF-1 in a RAR γ /RXR α -null background were established as described (30).

COS-1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, plated in 6-well plates, and transiently transfected using the DMRIE-C reagent, according to the manufacturer's protocol (Invitrogen). All transient transfections were carried out in OPTIMEM (Invitrogen) and contained the DR5-tk-CAT reporter gene (1 μ g/well), the pSG5-based expression vectors for mRAR γ 2 and mRXR α (0.05 μ g of each/well), Bluescript as a carrier, and the β -galactosidase expression vector pCH110 (0.5 μ g/well) to correct for variations in transfection efficiency. After a 16-h incubation with the DNA, the cells were washed and incubated for a further 48 h in medium in the absence or presence of RA (10⁻⁶ M), the RAR γ agonist (BMS961, 10⁻⁷ M), the pan-RXR agonist (BMS649, 10⁻⁶ M), or the combination of both agonists. CAT assays were performed using the enzyme-linked immunosorbent assay method (CAT enzyme-linked immunosorbent assay, Roche Applied Science). All assays were normalized to equal β -galactosidase activity, and the results were expressed as pg of CAT/unit of β -galactosidase.

Extracts and Immunoblotting—Whole cell extracts (WCEs) were prepared from F9 cells or transfected COS-1 cells as described previously (31). Proteins were resolved by SDS-10% PAGE, transferred onto nitrocellulose membranes by semidry blotting, and immunoprobed. The protein-antibody complexes were detected by chemiluminescence according to the manufacturer's protocol (Amersham Biosciences).

RNA Isolation and Real-time Reverse Transcriptase-PCR—Total RNAs were isolated using the guanidinium thiocyanate method, and aliquots (500 ng) were subjected to real-time quantitative reverse transcriptase-PCR by using the SYBR Green Light cycler detection system (Roche Applied Science and Idaho Technologies). Transcript levels were normalized according to 36B4 transcripts, which are unresponsive to retinoids. The oligonucleotides for 36B4, HNF3 α , and HNF1 β were as described (12, 30).

RESULTS

The AF-1 and AF-2 Domains of RAR γ 2 Control the Degradation of RAR γ 2/RXR α Heterodimers—To investigate how the activation functions of RAR γ 2 influence the degradation of both partners within RAR γ 2/RXR α heterodimers, COS-1 cells were cotransfected with a RAR γ 2 expression vector and a CAT reporter gene controlled by a DR5 RA-responsive element (DR5-tk-CAT) in the presence or absence of an RXR α expression vector and treated with RA. When concomitantly overexpressed, RAR γ 2 and RXR α bound as RAR γ 2/RXR α heterodimers to the DNA-response element, and RA treatment resulted in the degradation of RAR γ 2 that was maximal at 48 h (12) (Fig. 2A, lane 2). However, in the absence of overexpressed RXR α , RAR γ 2WT was degraded less efficiently (Fig. 2A, lane 4), confirming that RXR α modulates RAR γ 2 degradation and indicating that endogenous RXR α is present in limited amounts in COS-1 cells. The degradation of RAR γ 2WT was

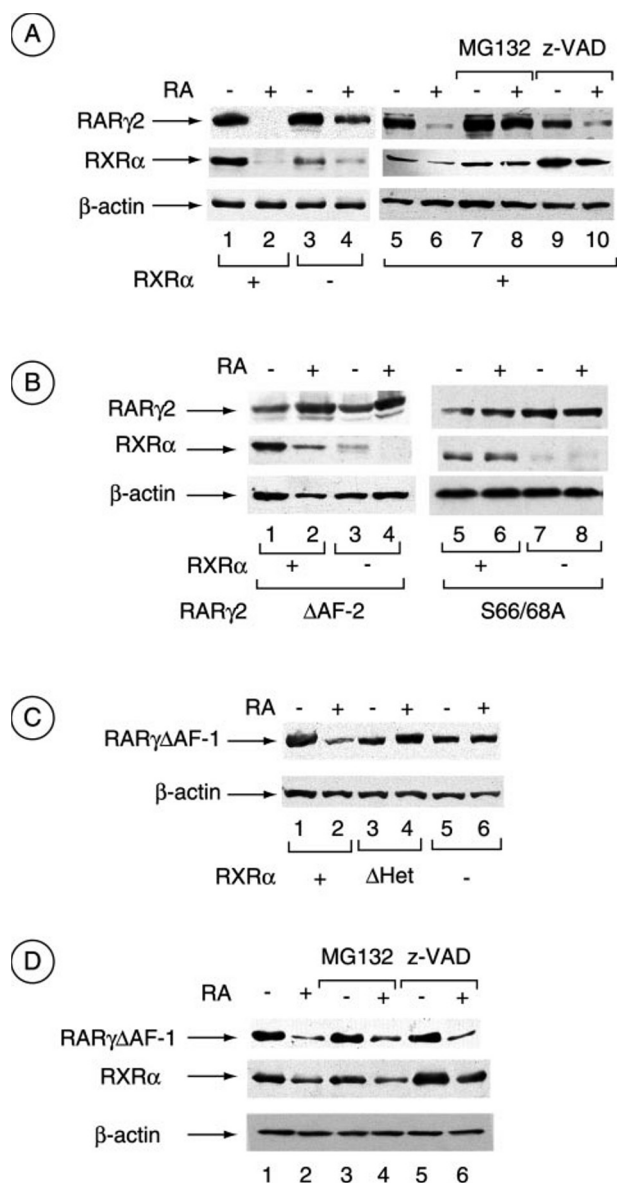


FIG. 2. Role of the AF-1 and AF-2 domains of RAR γ 2 in the degradation of both partners within RAR γ 2/RXR α heterodimers and influence of RXR α . As shown in A, COS-1 cells were cotransfected with the DR5-tk-CAT reporter construct and the expression vector for RAR γ 2 WT, in the absence or presence of the RXR α expression vector as indicated, and treated with vehicle or with RA (10^{-6} M) for 48 h. As indicated, MG132 ($8 \mu\text{M}$) or z-VAD-fmk ($50 \mu\text{g/ml}$) was added 16 h before harvesting. WCEs were immunoblotted with antibodies against RAR γ , RXR α , or β -actin. As shown in B, COS-1 cells were cotransfected with the DR5-tk-CAT reporter construct and with the RAR γ Δ AF-2 (lanes 1–4) or S66/68A (lanes 5–8) expression vectors in the absence or presence of the RXR α vector and processed as in A. C, as in A with the RAR γ Δ AF-1 expression vector in the absence (lanes 5 and 6) or presence (lanes 1 and 2) of a vector expressing RXR α WT or mutated at the heterodimerization residues (RXR α Δ Het) (lanes 3 and 4). As shown in D, COS-1 cells were cotransfected with the DR5-tk-CAT reporter construct, the RXR α and RAR γ Δ AF-1 expression vectors, treated with RA for 48 h, and processed as in A. As indicated, MG132 ($8 \mu\text{M}$) or z-VAD-fmk ($50 \mu\text{g/ml}$) were added 16 h before harvesting.

reversed by the proteasome inhibitor MG132 (Fig. 2A, lane 8) but less by the caspase inhibitor z-VAD-fmk (Fig. 2A, lane 10).

Consistent with our previous results (12, 21), RAR γ 2 deleted for helix 12 (RAR γ 2 Δ H12) and RAR γ 2 mutated at the phosphorylation sites located in the AF-1 domain (RAR γ 2S66/68A) were refractory to RA-induced degradation, regardless of the presence of overexpressed RXR α (Fig. 2B). Unexpectedly, we found that RAR γ 2 deleted for its N-terminal AF-1 domain

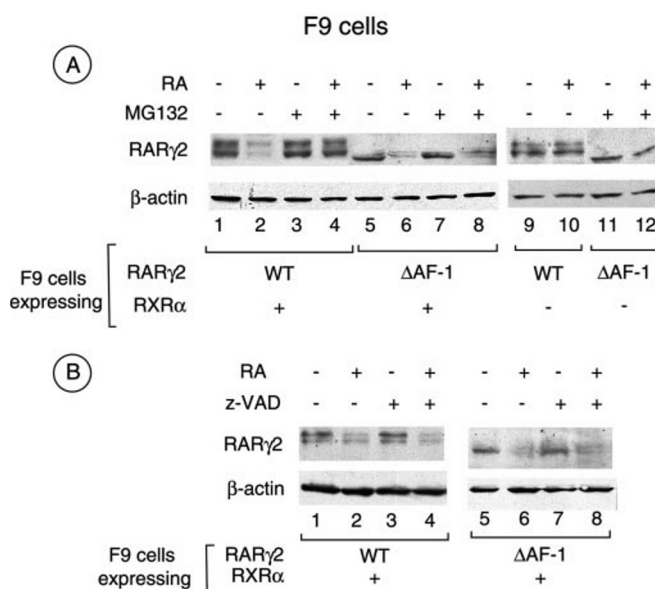


FIG. 3. RA-induced degradation of RAR γ Δ AF-1 in F9 cells. As shown in A, F9 cells, WT (lanes 1–4), RXR $\alpha^{-/-}$ (lanes 9 and 10), or expressing RAR γ Δ AF-1 in a RAR γ -null (lanes 5–8) or in a double RAR γ /RXR α -null background (lanes 11 and 12), were treated for 48 h with vehicle or RA (10^{-7} M). As indicated, MG132 ($40 \mu\text{M}$) was added 16 h before harvesting. WCEs were immunoblotted with RAR γ and β -actin antibodies. As shown in B, F9 cells, either WT (lanes 1–4) or expressing RAR γ Δ AF-1 in a RAR γ -null background (lanes 5–8), were RA-treated for 48 h and processed as in A. As indicated, z-VAD-fmk ($50 \mu\text{M}$) was added 16 h before harvesting.

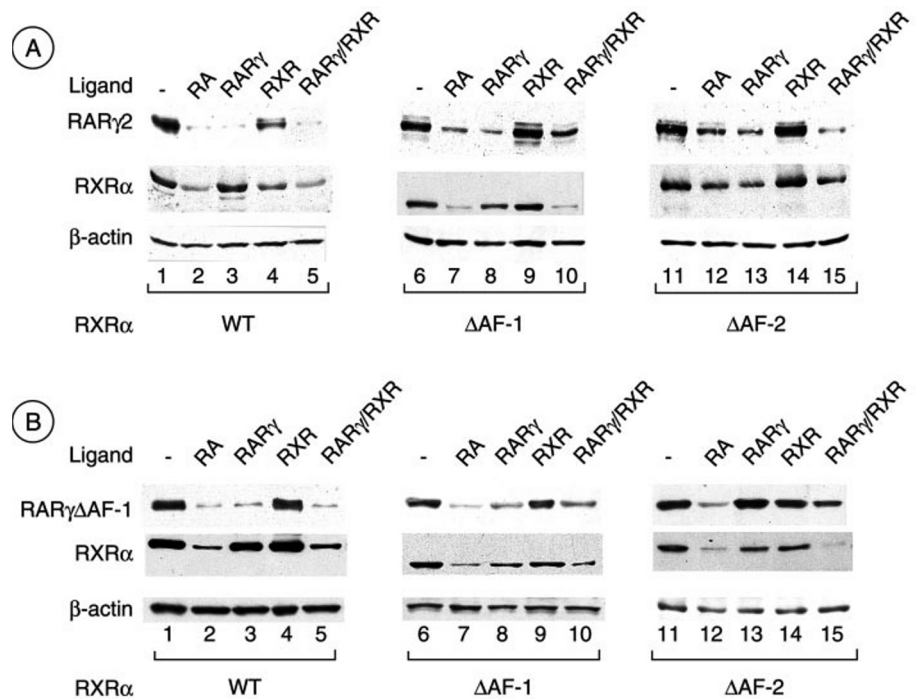
(RAR γ Δ AF-1) was significantly degraded when cotransfected with RXR α (Fig. 2C, lane 2). However, RAR γ Δ AF-1 was resistant to RA-induced degradation when overexpressed in the absence of RXR α (Fig. 2C, lane 6) or when cotransfected with RXR α mutated at its dimerization surface (mRXR α D364A/E384A/E399A/Y402A/E406A/R426A/E439A) (RXR α Δ Het) (21) (Fig. 2C, lane 4). This is different from what was observed with the other RAR isotype, RAR α , which does not require the phosphorylation sites located in the AF-1 domain for degradation (21). Indeed, RAR α Δ AF-1 and RAR α mutated at the phosphorylation sites (RAR α S77A) were degraded as efficiently as RAR α WT, regardless of RXR α overexpression (data not shown). The degradation of RAR γ Δ AF-1 was neither reversed by MG132 (Fig. 2D, compare lanes 2 and 4) nor reversed by z-VAD-fmk (Fig. 2D, lane 6), suggesting that deletion of the AF-1 domain targets RAR γ 2 toward a proteolytic pathway that is distinct from those controlled by the proteasome or by caspases.

In transfected COS cells, RXR α was also degraded at 48 h of RA treatment, whether its heterodimerization partner, RAR γ 2, was WT (Fig. 2A, lanes 1–4), Δ AF-2 (Fig. 2B, lanes 1–4), or Δ AF-1 (Fig. 2D, lanes 1 and 2). The only exception was when RXR α heterodimerized with RAR γ 2S66/68A (Fig. 2B, lanes 5–8). The degradation of RXR α was reversed by MG132 in the context of heterodimers with RAR γ 2WT (Fig. 2A, compare lanes 6 and 8) but not with RAR γ Δ AF-1 (Fig. 2D, compare lanes 2 and 4). However, in both cases, z-VAD-fmk promoted the accumulation of RXR α , indicating that the turnover of RXR α is sensitive to caspases (Fig. 2, A (lanes 9 and 10) and D (lanes 5 and 6)).

Similar results were obtained in F9 embryocarcinoma cells with the endogenous RAR γ 2 and RXR α receptors. Indeed, in these cells, the degradation of RAR γ 2 that occurs at 48 h of RA treatment (12, 21) (Fig. 3A, lanes 1 and 2) required the presence of RXR α as RXR $\alpha^{-/-}$ F9 cells did not show any evidence of RAR γ 2 degradation (Fig. 3A, lanes 9 and 10). In agreement

FIG. 4. Role of the AF-1 and AF-2 domains of RXR α in the degradation of both partners within RAR γ 2/RXR α and RAR γ Δ AF-1/RXR α heterodimers.

As shown in A, COS-1 cells cotransfected with the DR5-tk-CAT construct, the RAR γ 2 expression vector, and the vector for RXR α WT, Δ AF-1, or Δ AF-2, were treated for 48 h with RA (10^{-6} M), the RAR γ agonist (BMS961, 10^{-7} M), the pan-RXR agonist (BMS649, 10^{-6} M), or the combination of both agonists. WCEs were immunoblotted with RAR γ , RXR α , and β -actin antibodies. In lanes 6–10, RXR α Δ AF-1 was detected with the monoclonal antibodies 4RX1D2, raised against the E region of RXR α . Note that under our experimental conditions, deletion of the AF-2 AD core in RXR α (amino acids 455–467) does not change significantly the migration of the receptor (lanes 11–15), as compared with RXR α WT (lanes 1–5). B, as in A with RAR γ Δ AF-1.



with the results obtained with transfected COS-1 cells, RAR γ Δ AF-1 expressed in RAR γ -null cells (*i.e.* in the presence of RXR α) was degraded in response to RA (Fig. 3A, lanes 5 and 6). This degradation was neither reversed by MG132 (Fig. 3A, compare lanes 6 and 8) nor reversed by z-VAD-fmk (Fig. 3B, lanes 6 and 8). However, degradation did not occur when RAR γ Δ AF-1 was expressed in the double knock-out RAR $\gamma^{-/-}$ /RXR $\alpha^{-/-}$ cells (Fig. 3A, lanes 11 and 12).

The Degradation of RAR γ Δ AF-1 Involves the AF-2 Domain of RXR α —To gain insight into the mechanisms through which RXR α participates in the degradation of RAR γ 2, either WT or deleted of its AF-1 domain, synthetic agonistic ligands specific for RAR γ (BMS961) or RXRs (BMS649) were tested. The consequences of deleting the AF-1 and AF-2 domains of RXR α (in the RXR α Δ AF-1 and RXR α Δ H12 mutants respectively) were also tested.

When dimerized with RXR α WT, RAR γ 2WT was degraded in response to its cognate ligand (Fig. 4A, lane 3) but not in response to the pan-RXR agonist (Fig. 4A, lane 4). The combination of the two ligands was as efficient as the RAR γ agonist alone in inducing RAR γ 2 degradation (Fig. 4A, lane 5). Deletion of the AF-1 or AF-2 domains of RXR α , in RXR α Δ AF-1 and RXR α Δ AF-2, respectively, did not affect this process (Fig. 4A, lanes 6–15).

Similarly, RAR γ Δ AF-1 underwent degradation upon binding of its cognate ligand (Fig. 4B, lane 3) but not in response to the pan-RXR agonist (Fig. 4B, lane 4). Deletion of the AF-1 domain of RXR α had no influence (Fig. 4B, lanes 6–10). However, deleting the AF-2 domain of RXR α made the RAR γ ligand unable to signal degradation (Fig. 4B, lane 13), indicating that the helix 12 of RXR α may be of functional importance for the proteolysis of liganded RAR γ Δ AF-1. However, the absence of this domain could be compensated either partially or totally upon addition of the two agonists (Fig. 4B, lane 15) or of RA (Fig. 4B, lane 12), respectively. Thus, one can hypothesize that the two agonists, as well as RA and its 9-cis metabolite, cooperate for inducing allosteric transconformation changes (7) that would overcome the absence of the AF-2 domain of RXR α .

The Autonomous Degradation of RXR α Requires, in Addition to Its AF-2 Domain, the AF-1 Domains of Both Partners—The

degradation of RXR α heterodimerized with RAR γ 2, either WT or Δ AF-1, was also considered. When paired with RAR γ 2WT, RXR α WT was also degraded in response to its specific ligand, a pan-RXR agonist (Fig. 4A, lane 4), and not in response to the RAR γ agonist (Fig. 4A, lane 3). The combination of the two agonists did not influence the degrading activity of the RXR agonist (Fig. 4A, lane 5). Thus, RXR α WT can be autonomously degraded. Accordingly, RXR α degradation still occurred upon deletion of helix 12 in the RAR γ 2 partner (Fig. 2B, lanes 1 and 2) or in the absence of RAR γ 2 overexpression (21).

The pan-RXR agonist-induced degradation of RXR α was abrogated upon deletion of the AF-2 domain (Fig. 4A, lane 14), confirming that helix 12 is as crucial for the autonomous degradation of RXR α as for that of RAR γ . However, in contrast to the same RAR γ mutant, RXR α Δ H2 was not totally resistant since its degradation could be triggered by the liganded RAR γ partner (Fig. 4A, lanes 13 and 15).

Deletion of the AF-1 domain also made RXR α refractory to its agonist-induced degradation (Fig. 4A, lane 9). Importantly, deletion of the AF-1 domain of the RAR γ 2 partner led to the same effect (Fig. 4B, lane 4), indicating that the autonomous degradation of RXR α is regulated through mechanisms involving the AF-1 domains of both partners. However, in both cases, the resistance was overcome upon addition of the two agonists (Fig. 4A (lane 10) and B (lane 5)) or of RA (Fig. 4A (lane 7) and B (lane 2)).

Phosphorylation of the AF-1 Domain Also Accounts for the Autonomous Degradation of RXR α —Because the AF-1 domain plays a crucial role in the degradation of RXR α and phosphorylation serving as a positive signal for the degradation of several proteins (32–35), including RAR γ 2 (12, 21), we investigated whether it was the same for RXR α . The AF-1 domain of RXR α can be phosphorylated by MAPKs at three residues (Ser-61, Thr-75, and Ser-87) located in the AF-1 domain (20). Interestingly, we found that either in transfected COS-1 cells (Fig. 5A, lanes 1–4) or in F9 WT cells (Fig. 5B), RA induces an upward shift in the electrophoretic mobility of the receptor. This shift, which appears within 2 h of RA treatment and disappears at 24 h, reflects the phosphorylation of the three residues located in the AF-1 domain (20) as it is abrogated upon

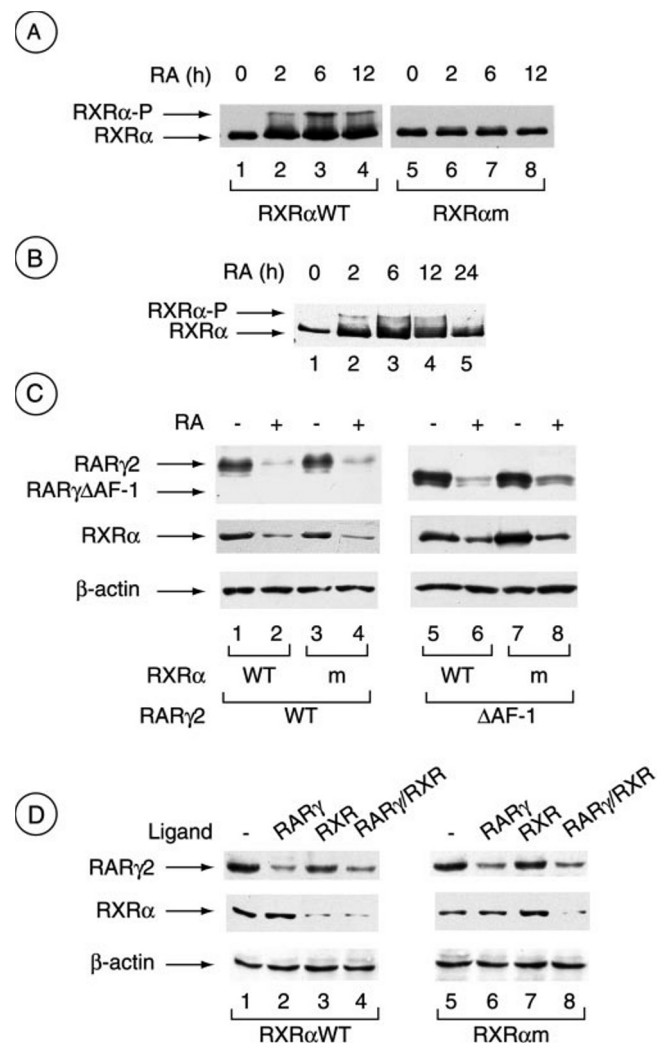


FIG. 5. RXR α is phosphorylated in its AF-1 domain in response to RA. This phosphorylation accounts for the autonomous degradation of RXR α . As shown in *A*, COS-1 cells were transfected with the expression vector for RXR α , either WT or S61/S75/T87A (RXR α m), and treated with RA for 2, 6, or 12 h. Equal amounts of nuclear extracts were immunoblotted with RXR α antibodies. As shown in *B*, F9 cells were treated with RA for different times, and nuclear extracts were immunoblotted as in *A*. As shown in *C*, COS-1 cells were cotransfected with the DR5-tk-CAT expression vector, the expression vector for RAR γ 2, either WT (lanes 1–4) or Δ AF-1 (lanes 5–8), and the vector for RXR α WT or mutated at the phosphorylation sites. After a 48-h treatment with RA, equal amounts of WCEs were immunoblotted with RAR γ , RXR α , and β -actin antibodies. As shown in *D*, COS-1 cells cotransfected with the DR5-tk-CAT expression vector, the expression vector for RAR γ 2WT and the vector for RXR α WT (lanes 1–4) or mutated at the phosphorylation sites (lanes 5–8) were treated with the RAR γ agonist (BMS961, 10^{-7} M), the pan-RXR agonist (BMS649, 10^{-6} M), or the combination of both agonists.

their mutation into alanine in the RXR α S61A/T75A/S87A mutant (RXR α m) (Fig. 5*A*, lanes 5–8).

RXR α mutated at the phosphorylation sites was degraded after 48 h of RA treatment, as efficiently as RXR α WT (Fig. 5*C*, compare lanes 2 and 4 and lanes 6 and 8), indicating that phosphorylation is not required for signaling the degradation of RXR α within RAR γ 2/RXR α heterodimers. Mutation of the RXR α phosphorylation sites did not affect the degradation of the RAR γ 2 partner, either WT or Δ AF-1 (Fig. 5*C*, lanes 3, 4, 7, and 8).

However, mutation of the phosphorylation sites made RXR α less efficiently degraded in response to its agonist (Fig. 5*D*, compare lanes 3 and 7), as did the deletion of the AF-1 domain.

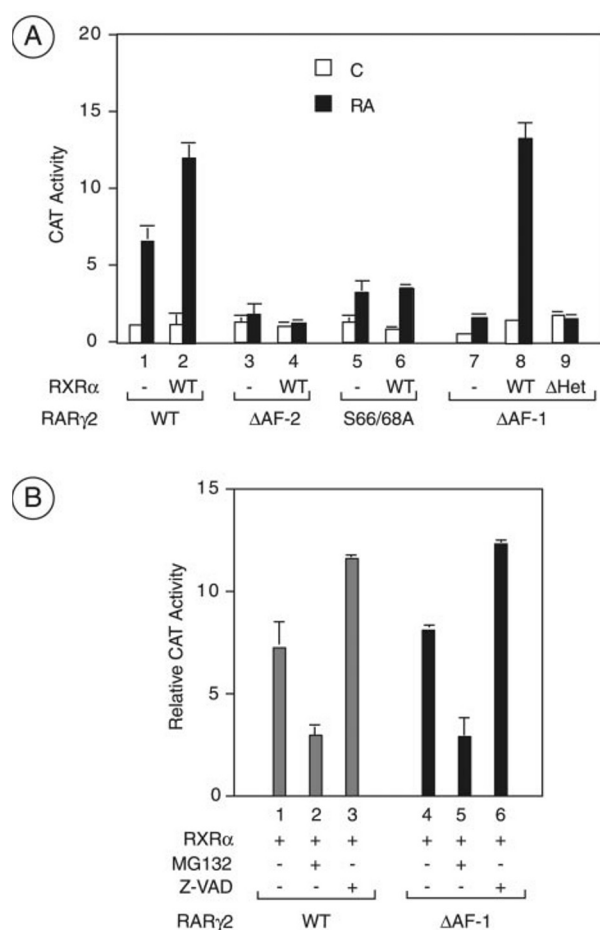


FIG. 6. The AF-1 and AF-2 domains of RAR γ 2 control the transcriptional activity of RAR γ 2/RXR α heterodimers. As shown in *A*, COS-1 cells were cotransfected with the DR5-tk-CAT construct and the expression vector for RAR γ 2 WT (lanes 1–2), Δ AF-2 (lanes 3–4), S66/68A (lanes 5–6), or Δ AF-1 (lanes 7–9) in the absence or presence of an expression vector for RXR α WT or mutated at the heterodimerization sites (Δ Het). After a 48-h treatment with RA, the cells were analyzed for CAT activity. The results are the mean \pm S.D. of at least three experiments. As shown in *B*, COS-1 cells cotransfected with the DR5-tk-CAT construct, the RXR α vector and the expression vector for RAR γ 2 WT (lanes 1–3), or Δ AF-1 (lanes 4–6) were RA-treated and analyzed as in *A*. As indicated, MG132 (8 μ M) or z-VAD-fmk (50 μ g/ml) were added 16 h before harvesting.

This could be overcome upon addition of the two agonists (Fig. 5*D*, lane 8).

RAR γ Δ AF-1 Heterodimerized with RXR α Is Transcriptionally Active—Because RAR γ 2 degradation and transactivation are intimately linked (12, 19), we compared the transcriptional activity of RAR γ 2WT and RAR γ Δ AF-1. RAR γ 2/RXR α heterodimers bind to response elements located in the promoters of RA target genes, and upon ligand binding, they induce transcription. Accordingly, in COS-1 cells coexpressing RAR γ 2WT, RXR α , and a CAT reporter gene under the control of a DR5-RA-response element (DR5-tk-CAT), RA induces a 10-fold increase in CAT activity (Fig. 6*A*, lane 2). This induction was less efficient in the absence of overexpressed RXR α , in agreement with the known heterodimer requirement for activation of transcription (Fig. 6*A*, lane 1). In contrast, RAR γ 2S66/68A and RAR γ 2 Δ H12 were inactive regardless of the presence of overexpressed RXR α (Fig. 6*A*, lanes 3–6), confirming that the transcriptional properties of RAR γ 2/RXR α heterodimers are strictly dependent on the integrity of the AF-1 and AF-2 domains of RAR γ 2.

Importantly, RAR γ Δ AF-1 cotransfected with RXR α was

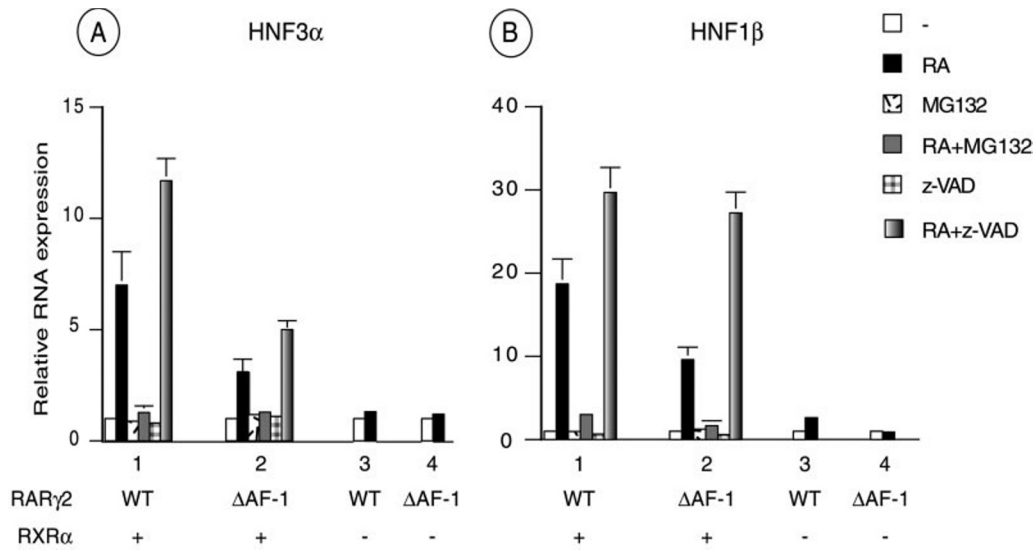


FIG. 7. Induction of RA target genes in F9 cells expressing RAR γ 2WT or RAR γ 2 Δ AF-1. F9 cells, WT (lane 1), RXR α ^{-/-} (lane 3), or expressing RAR γ 2 Δ AF-1 in a RAR γ -null background (lane 2) or in double RAR γ ^{-/-}/RXR α ^{-/-} cells (lane 4), were treated for 48 h with RA. As indicated, MG132 (40 μ M) or z-VAD (50 μ g/ml) were added 16 h before harvesting. Transcripts for HNF3 α (A) and HNF1 β (B) were analyzed by quantitative reverse transcriptase-PCR. The presented results are the mean \pm S.D. of two individual experiments and correspond to the -fold induction relative to the amount of transcripts present in vehicle-treated cells, which was given an arbitrary value of 1.

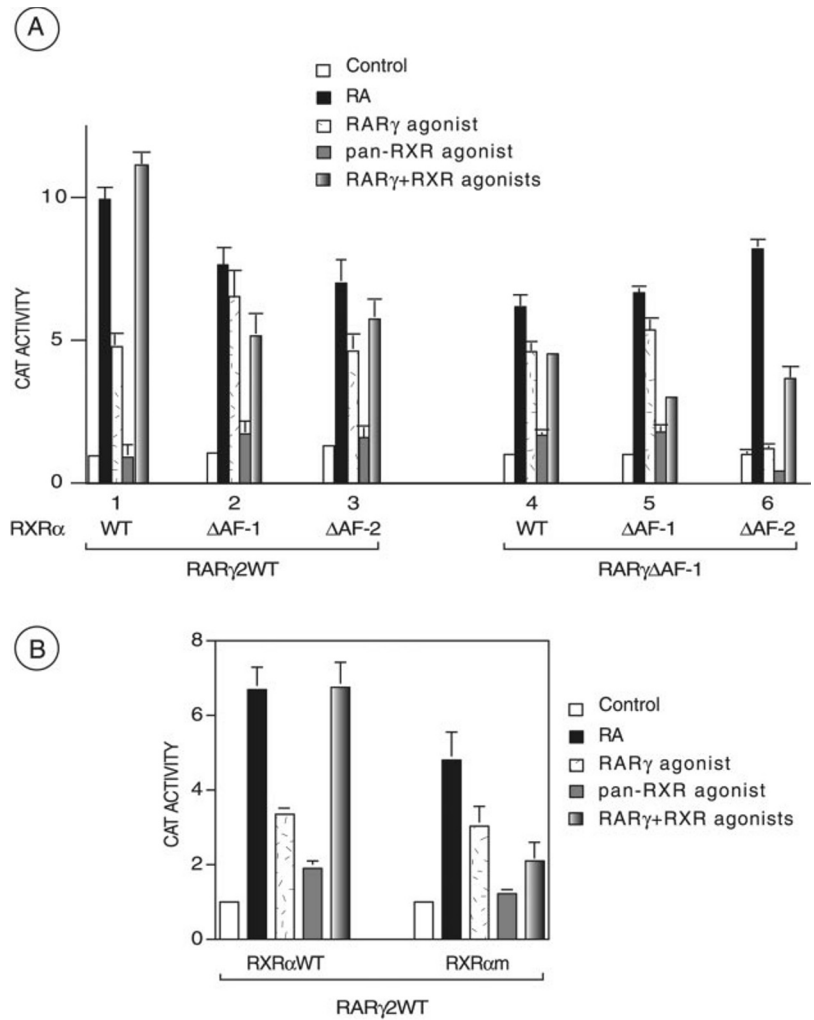


FIG. 8. RAR γ 2 and RXR α synergize for transcription through their phosphorylated AF-1 domains and the AF-2 domain of RXR α . As shown in A, COS-1 cells were cotransfected with the DR5-tk-CAT construct, the RAR γ 2 expression vector, and the vector for RXR α WT, Δ AF-1, or Δ AF-2. The cells were treated for 48 h with RA (10⁻⁶ M), the RAR γ agonist (BMS961, 10⁻⁷ M), the pan-RXR agonist (BMS649, 10⁻⁶ M), or the combination of the RAR γ and RXR agonists and then analyzed for CAT activity. The results are the mean \pm S.D. of at least three experiments. As shown in B, COS-1 cells were cotransfected with the DR5-tk-CAT construct, the RAR γ 2 expression vector, and the vector for RXR α , WT or mutated at the phosphorylation sites. The cells were treated and analyzed for CAT activity as in A. The results are the mean \pm S.D. of three experiments.

transcriptionally active (Fig. 6A, lane 8). However, RAR γ 2 Δ AF-1 was unable to activate transcription when cotransfected with RXR α Δ Het (Fig. 6A, lane 9) or when overexpressed in the absence of RXR α (Fig. 6A, lane 7). All together, these results

highlight the importance of the heterodimerization partner RXR α , not only for the degradation but also for the transactivation of RAR γ 2 Δ AF-1.

Transactivation by RAR γ 2, either WT or Δ AF-1, was abro-

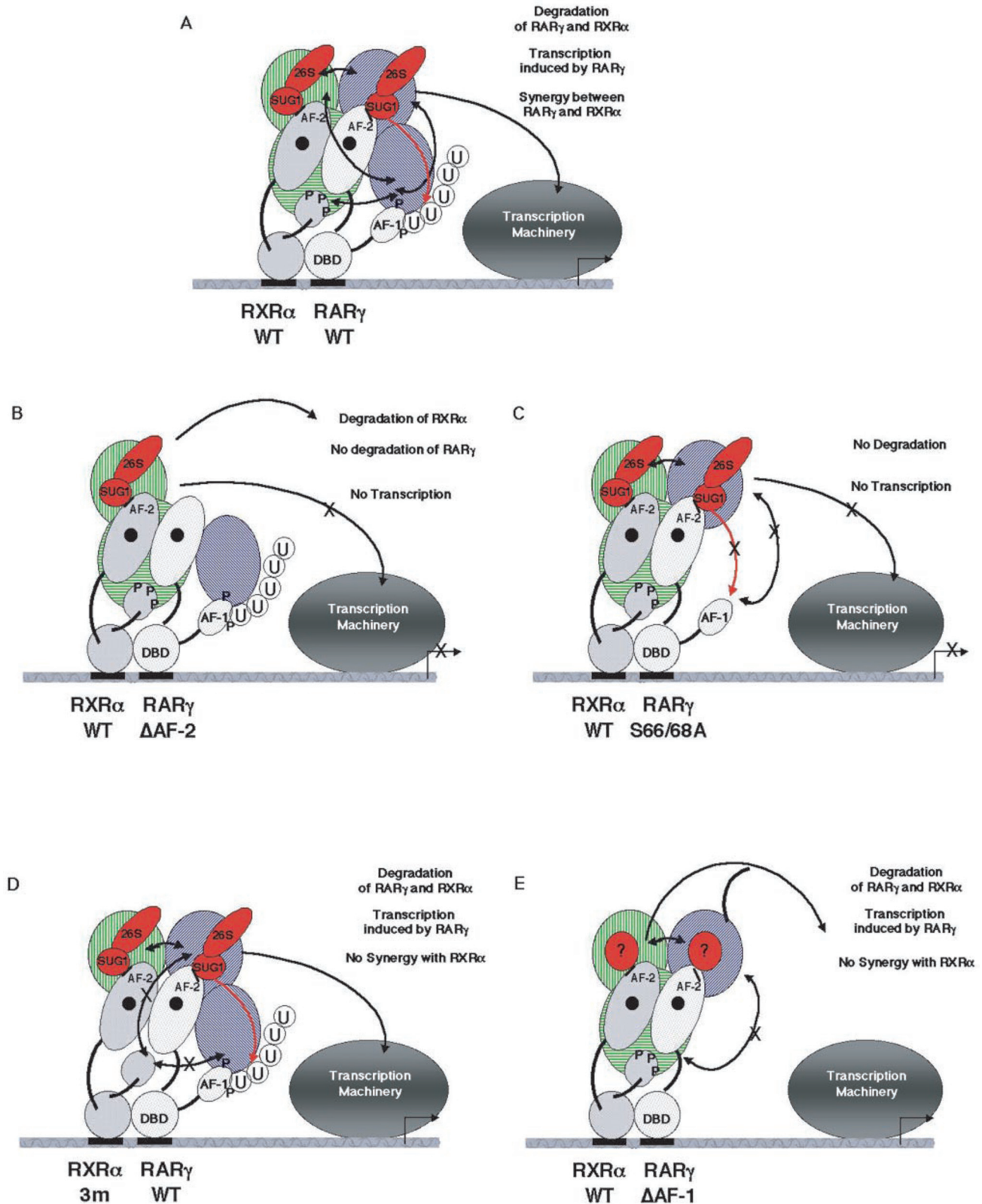


FIG. 9. Model recapitulating the role of the AF-1 and AF-2 domains of both partners in the transactivation and the degradation of RAR γ 2/RXR α heterodimers. As shown in A, liganded RAR γ 2WT/RXR α WT heterodimers bound at a response element recruit coregulator complexes through their AF-2 domains (green-stained for RXR α and blue-stained for RAR γ). These complexes cooperate to decompact chromatin and allow the recruitment of RNA polymerase II and the general transcription factors at the promoter. In that context, the RXR α partner is subordinated to liganded RAR γ 2 for the recruitment of the regulatory complexes. The AF-2 domains are also involved in the recruitment of the 26 S proteasome (red-stained) through SUG-1. The AF-1 domains of RAR γ 2 and RXR α are phosphorylated and also recruit coregulator complexes that cooperate with the AF-2 and with the two AFs of the partner for transcription. Phosphorylation of the AF-1 domain of RAR γ 2 is also a signal for ubiquitylation and subsequent recognition and degradation of both partners by proteasomal SUG-1 bound to the AF-2 domain. The double arrowheads indicate the cooperation between the complexes recruited by the different AF domains. DBD, DNA-binding domain. B, same as A with RAR γ 2 deleted for the AF-2 domain. The resultant heterodimer is transcriptionally inefficient due to the absence of recruitment of regulatory complexes. In addition, it is not degraded due to the absence of recruitment of the 26 S proteasome through the SUG-1 subunit. The AF-2 domain

gated by the proteasome inhibitor MG132 (Fig. 6B, lanes 2 and 5), supporting the concept that the proteasome can also carry out non-proteolytic tasks and modulate transcription (36). In contrast, z-VAD-fmk increased transcription mediated by RAR γ 2, either WT or Δ AF-1 (Fig. 6B, lanes 3 and 6), in agreement with the accumulation of RXR α (Fig. 2, A and C).

The above observations were recapitulated in F9 embryocarcinoma cells. In these cells, the transactivation of RA target genes such as HNF1 β and HNF3 α was maximal and reached a plateau at 48 h (Fig. 7, A and B, lane 1). In agreement with the results obtained with transfected COS-1 cells, the RA-induced expression of these genes was completely impaired in RXR α ^{-/-} cells (Fig. 7, A and B, lane 3) and in F9 cells expressing RAR γ Δ AF1 in a double RXR α /RAR γ -null background (Fig. 7, A and B, lane 4). However, it was only decreased in F9 cells expressing RAR γ Δ AF-1 in a RAR γ -null background (*i.e.* in the presence of endogenous RXR α) (Fig. 7, A and B, lane 2). As in transfected COS cells, MG132 abrogated the RA-induced expression of these RA target genes in F9 cells expressing either RAR γ 2WT or RAR γ Δ AF1 (Fig. 7, A and B, lanes 1 and 2), whereas z-VAD-fmk increased transcription in both cell lines (Fig. 7, A and B, lanes 1 and 2).

RAR γ 2 and RXR α Synergize for Transcription through the Phosphorylation of the AF-1 Domain of RXR α —The mechanisms through which the activation domains of each partner participate in the transactivation of RAR γ 2/RXR α heterodimers were further investigated by using the same strategy as that described for degradation. Within RAR γ 2WT/RXR α WT heterodimers, liganded RAR γ 2 is able to induce transcription, whereas RXR α is subordinated to RAR γ (14). Accordingly, RXR α cannot autonomously induce transcription in response to its cognate ligand (Fig. 8A, lane 1) but synergizes with liganded RAR γ (Fig. 8A, lane 1). Therefore, deleting helix 12 impaired the ability of RXR α to synergize with RAR γ 2 (Fig. 8A, lane 3). Deletion of the AF-1 domain of RXR α also abrogated the synergy between RAR γ and RXR ligands (Fig. 8A, lane 2). Interestingly, mutation of the three phosphorylation sites located in the AF-1 domain of RXR α had the same effect (Fig. 8B).

In the context of RAR γ Δ AF-1/RXR α heterodimers, transcription was still efficiently activated by the RAR γ agonist. However, synergy with liganded RXR α was abrogated, indicating that the AF-1 domain of RAR γ 2 also accounts for this process (Fig. 8A, lane 4). It is noteworthy that the autonomous activity of RAR γ Δ AF-1 was not affected upon deletion of the AF-1 domain of RXR α (Fig. 8A, lane 5) but was impaired subsequently to the deletion of the AF-2 domain of RXR α (Fig. 8A, lane 6), indicating that the helix 12 of the partner accounts for the transactivation of liganded RAR γ Δ AF-1. However, some transcriptional activity of the RAR γ Δ AF-1/RXR α Δ H12 heterodimers could be detected in the presence of the two agonists or of RA (Fig. 8A, lane 6).

DISCUSSION

Within RAR/RXR heterodimers (Fig. 9A), the AF-2 domain of each partner cooperates for the recruitment of multiprotein

complexes that act in coordinated and/or combinatorial manner to decompact chromatin and direct RNA polymerase II and the general transcription factors to the promoter (10, 11). According to recent studies, the AF-1 domains would also recruit intermediary proteins (37). Here we demonstrate that the AF-1 and AF-2 domains of each partner are key elements that cooperate with one other for controlling not only their own transcriptional activity and degradation but also that of the partner. We also demonstrate that deletion of one AF within one partner renders each one dependent on the other, highlighting the complex cooperation mechanisms between the AF-1 and AF-2 domains of each receptor.

The AF-2 Domain of RAR γ 2 but Not That of RXR α Is Strictly Required for the Degradation and Transactivation of RAR γ 2/RXR α Heterodimers—Within transcriptionally active RAR γ 2/RXR α heterodimers, both RAR γ 2 and RXR α are autonomously degraded in response to their cognate ligand, through their AF-2 domains. However, the role played by the AF-2 domain of each partner is different. Considering RAR γ 2 (Fig. 9B), the integrity of its AF-2 domain is strictly required for the degradation of the receptor, in accordance with our previous observation that the 26 S proteasome is recruited through this domain (12). In addition, RAR γ 2 degradation is not influenced by the AF-2 domain of RXR α , excluding a cooperative role for the liganded partner in the recruitment of the degradation machinery. Thus, one can suggest that RXR α might rather influence RAR γ 2 degradation through promoting its binding to the cognate response elements (38). In contrast, RXR α deleted for this same domain was not refractory to degradation as it could be degraded in response to the ligand of its partner.

The contribution of the AF-2 domains of RAR γ 2 and RXR α in the transcriptional properties of the heterodimers is also somehow different. Indeed, upon deletion of the AF-2 domain of RAR γ 2, transcription cannot be induced by RA or any agonist, either alone or in combination (Fig. 9B). In contrast, deletion of the AF-2 domain of the RXR α partner did not affect the autonomous activity of RAR γ 2 but impaired the synergy between the two receptors. These results confirm the model according to which, within RAR/RXR heterodimers, liganded RXR is subordinated to the liganded RAR partner for the dissociation of co-repressors and the recruitment of coactivators (14).

In conclusion, the AF-2 domain of RAR γ 2 plays an “indispensable” role as it promotes both transactivation and degradation, and its absence cannot be compensated by the AF-2 of the RXR α partner. In contrast, the AF-2 domain of RXR α instead appears to play a “permissive” role as it only cooperates with RAR γ 2 during transcription and can be substituted by the AF-2 of RAR γ 2 in the degradation process.

Phosphorylation of the AF-1 Domain of RAR γ 2 Is Strictly Required for the Degradation and Transactivation of RAR γ 2/RXR α Heterodimers, whereas That of RXR α Plays Only a Cooperative Role—The AF-1 domain of RAR γ 2 is also crucial to the degradation process through its phosphorylation and ubiquitylation (12, 21). Accordingly, RAR γ 2S66/68A is completely refractory to RA-induced degradation (Fig. 9C). Interestingly,

of RXR α is unable to compensate the absence of RAR γ 2 AF-2. As shown in C, upon mutation of the phosphorylation sites located in the AF-1 domain of RAR γ 2, the resulting heterodimers are also unable to activate transcription and to be degraded. Indeed, in the absence of phosphorylation, the AF-1 domain cannot be ubiquitylated, and therefore, there is no signal for degradation by the proteasome. In addition, the AF-1 domain becomes unable to recruit some coregulatory complexes and consequently makes the AF-2 domain inefficient at recruiting the transcription machinery. As shown in D, mutation of the phosphorylation sites in the AF-1 domain of RXR α does not impede the degradation of both partners nor does it impede transcription. It only abrogates the synergy between both partners, maybe due to the absence of recruitment of some coregulatory complexes involved in the cooperation with RAR γ 2. Similar results were obtained upon deletion of the whole AF-1 domain or of the AF-2 domain, corroborating our conclusion that the complexes recruited at the two AFs of RXR α do not play active roles but only help the action of the complexes recruited by the RAR γ 2 partner. As shown in E, deletion of the whole AF-1 domain of RAR γ 2 does not abrogate the degradation and the transcriptional properties of the heterodimers. However, the action of liganded RAR γ 2 becomes dependent on complexes recruited by the RXR α partner. In addition, the heterodimers are shifted toward other proteolytic pathways through RXR α .

when heterodimerized to this mutant, RXR α was also refractory to degradation, suggesting that the phosphorylated AF-1 of RAR γ 2 signals the degradation of both partners. The phosphorylated AF-1 domain also plays a crucial role in transcription (15, 17), probably through helping the recruitment of co-activators and/or intermediary proteins, in cooperation with the AF-2 domain, as already demonstrated for RAR α (37) and other nuclear receptors (39–41) and/or with the RXR α partner (compare Fig. 9, A and C).

Our results also illuminate a role for the AF-1 domain of RXR α , through its phosphorylation, in the degradation and transactivation of the heterodimers. We observed that RXR α responds to RA through an increase in the phosphorylation of its AF-1 domain. However, the role of this phosphorylated AF-1 domain (Fig. 9D) differs from that of RAR γ 2 as it is not critical but rather influences its own degradation as well as its cooperation with its partner for transcription.

First, mutation of the phosphorylation sites (similarly to the deletion of the AF-1 domain) did not influence the degradation of the RAR γ 2 partner. Further, it did not make RXR α refractory to degradation. However, RXR α degradation was no more autonomous and required the influence of its liganded partner. Thus, one can postulate that the phosphorylation sites located in the AF-1 domain of RXR α cooperate with RAR γ for the recruitment and/or the action of the degradation machinery and that this action can be substituted by liganded RAR γ 2.

Second, mutation of the RXR α phosphorylation sites did not affect the ability of RAR γ 2/RXR α heterodimers to transactivate RA target genes. However, it made RXR α unable to synergize with liganded RAR γ 2 for transcription, indicating that not only the AF-2 domain but also the phosphorylation sites located in the AF-1 domain of RXR α cooperate with RAR γ for the recruitment of cofactors and maximal transcriptional activity.

Deletion of the AF-1 Domain Does Not Make RAR γ 2 Refractory to RA-induced Degradation Transactivation but Renders It Dependent on RXR α —The novelty of this study is that unexpectedly, upon deletion of the whole AF-1 domain of RAR γ 2, the degradation and the transcriptional properties of the heterodimers were not abrogated but were regulated through different molecular mechanisms (Fig. 9E). First, RAR γ Δ AF-1 required the AF-2 domain of RXR α to activate transcription and to be degraded in response to its cognate ligand. However, in the absence of this domain, both processes could occur in response to the combination of both the RAR γ and RXR agonists, probably due to allosteric conformational changes (7). In that context, the degradation of RXR α WT, Δ AF-1, or Δ AF-2 also required allosteric conformational changes induced by the two liganded partners. Thus, deletion of the AF-1 domain renders RAR γ 2 dependent on its partner. Second, RAR γ Δ AF-1 degradation did not involve the proteasome but involved other proteases that remain to be characterized. Thus, in contrast to the mutation of the RAR γ 2 phosphorylation sites that makes the heterodimers completely refractory to proteasomal degradation, deletion of the whole AF-1 domain shifts the receptors toward other proteolytic pathways through RXR α . Finally, RAR γ Δ AF-1 was unable to synergize with liganded RXR α for transcription, indicating that not only the AF-2 domain but also the AF-1 domain of RAR γ 2 cooperates with RXR α for the recruitment of cofactors and maximal transcriptional activity.

In conclusion, our results highlight the crucial role played by the phosphorylated AF-1 domain of RAR γ 2 in the autonomous degradation of each partner and their cooperation for transcription. As many cancers characterized by aberrant kinase activities (42) are resistant to retinoids (43), it is tempting to speculate that the phosphorylation of RARs and/or RXRs is affected in these cells, leading to an aberrant degradation of the

receptors and a deficient transcription of the RA target genes. To gain insight in the mechanisms regulating these processes, we are currently investigating which proteins are interacting with the AF-1 domains of RAR γ 2 and RXR α .

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