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Ligand-Free RAR Can Interact with the RNA Polymerase II Subunit hsRPB7 and Repress Transcription

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Upon binding retinoic acid (RA), the retinoic acid receptors (RARs) are able to positively and negatively regulate transcription. It has been shown that the DNA-binding domain and carboxy terminus of RARs are necessary for the ligand-dependent ability of the receptor to repress AP-1 transcriptional activity. A fusion of these two regions, shown to constitutively inhibit AP-1 activity, was used in a yeast two-hybrid screen to identify a novel hRARα-interacting protein. This protein, hsRPB7, a subunit of RNA polymerase II, interacts with hRARα in the absence of RA and addition of RA disrupts the interaction. Truncation analysis indicates that hsRPB7 specifically interacts with the hRARα DNA-binding domain. This interaction appears to compromise transcription, since overexpressed hRARα, in the absence of RA, is able to repress the activity of several RNA polymerase II-dependent activators, including AP-1 and the glucocorticoid receptor. This repression is relieved by transfected hsRPB7, strongly suggesting that ligand-free hRARα can block AP-1 activity by sequestering hsRPB7. The repression is dependent on the integrity of the hRARα DBD, since a mutation within the DBD blocks both the hRARα-hsRPB7 interaction and ligand-free hRARα repression of AP-1. These results provide evidence that non-liganded hRARα can regulate transcription by directly interacting with RNA polymerase II, and thus suggest a novel pathway by which hRARα can cross-talk with AP-1 and perhaps other families of transcriptional activators.

Key words: RAR; RNA polymerase II; hsRPB7; transcription; AP-1; nuclear receptors.

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Introduction

Transcriptional repression is emerging as an important mechanism for regulation of gene expression (reviewed in ref. *1*). Nuclear receptors, which comprise a large superfamily of ligand-dependent transcriptional activators (reviewed in ref. *2*), can also act as transcriptional repressors in the absence of ligand. Among these receptors, repression was first described for the thyroid hormone receptors (TRs) and their oncogenic counterpart, v-erbA *(3,4)*. Subsequent work revealed that the retinoic acid receptors (RARs) were capable of similar ligand-independent repression of transcription *(5)*. These negative effects by TRs and RARs require receptor DNA binding and are mediated by the ligand-independent interaction between the receptor and a repressor complex, consisting of the corepressors N-CoR *(6)* and SMRT *(7)* together with mSin3 and histone deacetylases *(8,9)*.

In contrast to this ligand-independent activity, nuclear receptors also have ligand-dependent negative effects on transcription. The best-studied is the ligand-dependent transrepression of AP-1, a complex composed of the protein products of the nuclear protooncogenes *c-jun* and *c-fos (10)*. This negative activity on AP-1-responsive genes occurs in the absence of receptor DNA binding or interaction with corepressor proteins and is not restricted to RARs and TRs, but is found in several other nuclear receptors (reviewed in ref. *11*). This ligand-dependent anti-AP-1 activity by RARs and TRs has been proposed to be mediated by CREB-binding protein (CBP) and p300 *(12,13)*, two highly related proteins required for the transcriptional activity of both nuclear receptors and AP-1 *(12)*. While competition for limiting CBP/p300 is an appealing mechanism for AP-1 transrepression, it does not explain the cell- or promoter-specific nature of the RAR effect on AP-1. Indeed, retinoic acid (RA)-induced RAR inhibition of AP-1 occurs in only certain mammalian cells, *(14)* and, moreover, there are several examples of cooperative interactions between AP-1 and nuclear receptors *(15,16)*. Indeed, nuclear receptor-AP-1 interactions result in different transcriptional outcomes in different human cells *(11)*. Our recent results have shown that liganded RARs can

disrupt the *in vivo* dimerization between c-Jun and c-Fos in a cell-specific manner *(17)*. Additionally, it has been suggested that nuclear receptors antagonize AP-1 activity by inhibiting Jun amino-terminal kinase (JNK), a protein that phosphorylates, and thereby stimulates, c-Jun's transcriptional activity *(18)*. All these data suggest that ligand-bound nuclear receptors can repress AP-1 activity via multiple mechanisms.

Surprisingly, it has been demonstrated that the negative effect of the glucocorticoid receptor (GR) on AP-1 transcriptional activity can occur in the absence of its cognate ligand, dexamethasone *(19)*. In this paper, we provide evidence for a ligand-independent activity of RAR on AP-1. Importantly, however, this RAR effect represents a novel and distinct mechanism for AP-1 transrepression, mediated via a recently identified RNA polymerase II subunit, hsRPB7 *(20)*. Our data show that hRARα can interact with hsRPB7 in the absence of RA and that RA disrupts this interaction. This interaction appears to compromise the transcriptional activity of not only AP-1, but perhaps other RNA polymerase II-dependent transcriptional activators.

Results

The ligand-dependent inhibition of AP-1-responsive promoters by RARs has been previously shown to be cell-specific *(14)*. In agreement with these earlier results, we have found that transfected hRARα can block endogenous AP-1 activity in HeLa (3–4 fold) but not Cos cells (Fig. 1B and C); the same RAR effect has been seen with transfected c-Jun (data not shown). This difference in RAR transrepression activity has been attributed to the presence of cell-specific factors mediating the interaction between RAR and AP-1 *(11)*. To identify such factors, we have performed a yeast two-hybrid screen using as a bait regions within the human RARα (hRARα) previously demonstrated to be important for AP-1 transrepression *(14)*. These are the C region, encoding the DNA-binding domain (DBD), and the F region on the carboxy terminus, which has no known function. A fusion of these two regions, giving hRARα(CF) (see Fig. 1A), was made and tested by transient transfection. It was found that hRARα(CF) was able to constitutively repress both endogenous and exogenous (data not shown) AP-1 activity nearly as well as does the ligand-bound full-length hRARα (Fig. 1B). Interestingly, this negative effect with hRARα(CF) was seen not only in HeLa, but also Cos cells (Fig. 1C), strongly suggesting that hRARα(CF) is inhibiting AP-1 through a mechanism distinct from that responsible for the ligand-dependent repression by full-length hRARα that is observed in HeLa cells. As expected, hRARα(CF) had no positive transcriptional activity on RA-responsive promoters but was able to block RA-induced transcription (data not shown). This negative effect on RA-induced transcription required less hRARα(CF) protein than did the effect on AP-1 transactivation, and

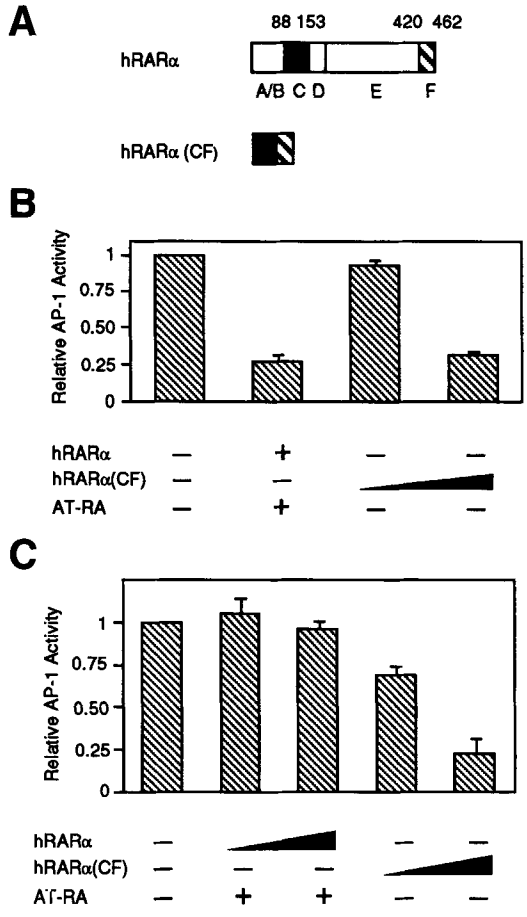

Fig. 1. hRARα(CF) is a constitutive repressor of AP-1 transcriptional activity. **(A)** A schematic representation showing full-length hRARα and the fusion protein hRARα(CF), which was used as a bait in the yeast two-hybrid screen. Numbers represent amino-acid residues of the C and F regions. hRARα(CF) inhibits transcription of induced by endogenous AP-1 in both HeLa **(B)** and Cos cells **(C)**. HeLa and Cos cells were transfected with 1 μg of TRE-tk-CAT reporter plasmid together with either (B) 1 μg of hRARα expression plasmid and 1 or 5 μg of hRARα(CF) expression plasmid or (C) 1 or 5 μg of expression plasmids for either full-length hRARα or hRARα(CF). Cells receiving full-length receptor were treated with 10^{-7} M AT-RA as indicated. Note that CAT activity is represented relative to activity of the first condition, which was set to 1.

thus, the effect on RAR most likely is due to this fusion protein's ability to compete with endogenous RARs for DNA binding. Hence, hRARα(CF) allows separation of hRARα-induced transactivation from that of AP-1 transrepression and represents a bait suitable for a yeast two-hybrid screen.

The yeast two-hybrid screen with hRARα(CF) as a bait resulted in the isolation of a complementary DNA (cDNA) clone derived from HeLa cells. This cDNA clone, which we call 3–59, encoded an amino-acid sequence with identity to hsRPB7 *(20)*, the human homologue of RPB7, which is the seventh largest subunit of yeast RNA polymerase II *(21)*. Clone 3–59 was found to encode the C-terminus (amino acids 51 to 172) of hsRPB7. This identity was con-

firmed by complete DNA sequencing of clone 3–59 (data not shown).

The yeast two-hybrid system was initially used to study the interaction between the protein encoded by 3–59, designated as hsRPB7(Δ1–50), fused to the B42 transcriptional activation domain, and hRARα, joined to the LexA DBD. Since our initial screen indicated that hsRPB7(Δ1–50) can interact with a fusion of the C and F regions of hRARα, we wanted to determine if either of these regions alone can undergo this interaction. B42-hsRPB7(Δ1–50) interacted significantly with LexA-hRARα(C) but not LexA-hRARα(F) (Fig. 2A), showing that hsRPB7(Δ1–50) targets the hRARα DBD. This interaction appears to be specific for the hRARα DBD, since the DBDs of the highly related mouse retinoid X receptor α (mRXRα), the human androgen receptor (hAR), or the Drosophila Bicoid protein all failed to interact with hsRPB7(Δ1–50) (Fig. 2A). Importantly, when full-length hRARα was tested, its interaction with hsRPB7(Δ1–50) was 5- to 10-fold stronger than that of either hRARα(CF) or hRARα(C) (Fig. 2A). Interestingly, full-length hRARα interacted with hsRPB7(Δ1–50) in the absence of all-trans retinoic acid (AT-RA), the cognate ligand for RARs.

To look for an *in vivo* association between full-length hRARα and hsRPB7, hRARα-containing complexes were immunoprecipitated from transiently transfected HeLa cells with an anti-hRARα antibody, and tested by Western blot analysis for the presence of co-precipitated hsRPB7 by Western blot analysis (Fig. 2B). Extracts derived from cells that were not treated with AT-RA exhibited a significant amount of hsRPB7 in the immunoprecipitates (lane 1). However and importantly, addition of AT-RA to the cells reduced the amount of precipitated hsRPB7 (lane 2) to the background level found in immunoprecipitates using a control anti-GAL4 antibody (lane 3). Note that the three nuclear extracts had approximately similar amounts of hsRPB7 protein (lanes 4–6). To confirm the efficiency of the immunoprecipitation, the same Western blot was probed with an anti-hRARα antibody. This showed that hRARα was very efficiently immunoprecipitated with the anti-hRARα antibody, either in the absence (lanes 7, 10, and 11) or presence of AT-RA (lanes 8, 12, and 13), while the anti-GAL4 antibody did not precipitate any detectable levels of hRARα (lanes 9, 14, and 15). These results strongly suggest that hRARα is able to physically associate with hsRPB7 *in vivo*, and that this interaction is disrupted by AT-RA.

To further analyze this *in vivo* interaction between hRARα and hsRPB7, GST pulldown experiments were carried out with GST fusion proteins expressed in Cos cells (Fig. 2C). Coexpression of GST-hRARα and hsRPB7 resulted in the copurification of hsRPB7 (lane 1), suggesting an association between GST-hRARα and hsRPB7. Again, treatment of cells with AT-RA disrupted this association (lane 2). Supporting the yeast two-hybrid data (see Fig. 2A), hsRPB7 specifically associates with the hRARα DBD, since it copurified with GST-hRARα(C) (lane 3), but not with either GST-hRARα(F) (lane 4) or GST (lane 5). Expression of these various GST fusion proteins did not alter the expression level of hsRPB7 (lanes 6–10) and all four GST proteins were expressed and bound to glutathione-sepharose (lanes 11–15). Thus, hsRPB7 represents a new target of the RAR DBD, suggesting the existence of novel activities of this hRARα domain. However, hsRPB7 does not appear to influence RAR/RXR DNA binding as measured by in vitro gel mobility shift (data not shown), suggesting that the RAR-hsRPB7 interaction does not take place when RAR is bound to DNA.

Our finding of an interaction between the hRARα C region and hsRPB7 provides a possible mechanistic model for how the truncated hRARα, hRARα(CF), inhibits AP-1 transcriptional activity. It is possible that hRARα(CF) is interfering with RNA polymerase II responsiveness to AP-1 by titrating away from endogenous RNA polymerase II endogenous hsRPB7, a protein that is known to be expressed in HeLa cells (data not shown, ref. *20*). If this is the case, then it is necessary to show that hsRPB7 should be involved in AP-1-mediated transactivation. This was examined by cotransfecting HeLa cells with the AP-1-responsive reporter TRE-tk-CAT and increasing amounts of hsRPB7 expression plasmid. Indeed, hsRPB7 stimulated AP-1 activity in a dose-dependent manner (Fig. 3A), suggesting that hsRPB7 is in a limiting concentration for AP-1 activity in HeLa cells. By contrast, exogenous expression of another RNA polymerase II subunit, hsRPB4 *(20)*, does not influence AP-1 activity (data not shown). The results above in mammalian cells show that hsRPB7 can interact with full-length, RA-free hRARα as well as with hRARα(C) (see Figs. 2B and 2C). If the hRARα(CF) interaction with hsRPB7 is responsible for its negative effect on AP-1 activity (see Fig. 1B), then the ligand-free full-length receptor should have the same effect on AP-1 as does hRARα(CF). This possibility was tested by transfecting HeLa cells with increasing amounts of hRARα expression plasmid and measuring AP-1 transcriptional activity. Interestingly, overexpression of hRARα did repress both endogenous and exogenous (data not shown) AP-1 activity in the absence of AT-RA (Fig. 3B), while it did not affect c-Jun/c-Fos dimerization (data not shown). However, this ligand-independent activity required markedly more hRARα protein than did the ligand-dependent repression of AP-1 (Fig. 3B). Western blot analysis allowed us to estimate that this ligand-independent effect requires about fivefold more hRARα than does the ligand-dependent effect (data not shown). Addition of RA in this experiment did not restore AP-1 activity, but in fact resulted in stronger anti-AP-1 activity exhibited by hRARα (data not shown). This is undoubtedly reflecting the well-documented ligand-dependent repression of AP-1 activity by RARs, which has been attributed to several activities of liganded RAR, including the titration of a common coactivator from AP-1 *(12,13)* [reviewed

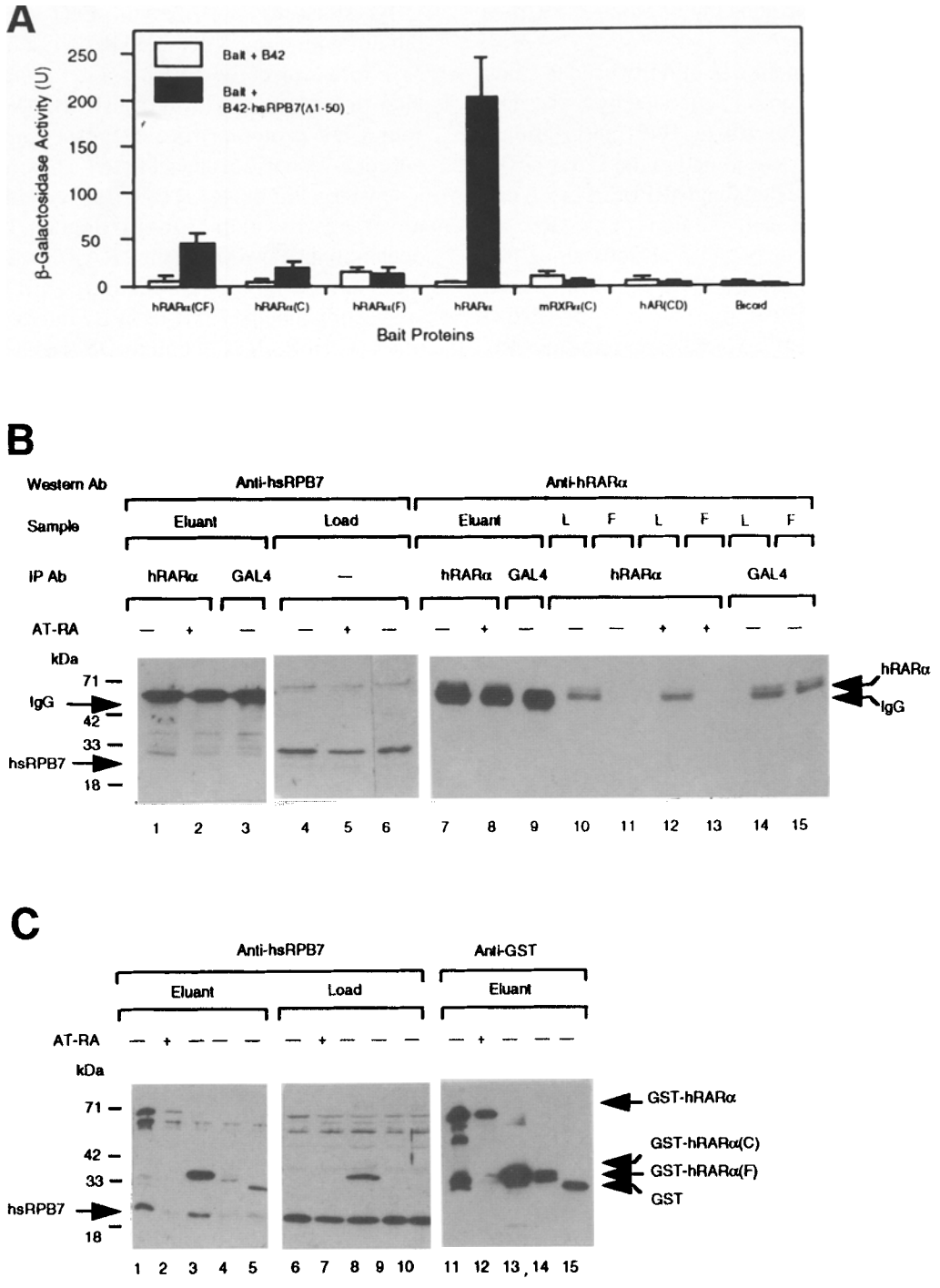

Fig. 2. hsRPB7 directly interacts with the DBD of hRARα in the absence of AT-RA. (**A**) hsRPB7(Δ1–50) specifically interacts with the DBD of hRARα in yeast. Bait proteins are LexA fusions with full-length hRARα, the DBD (C region) and/or the COOH-terminus (F region) of hRARα, the mRXRα DBD (C region), the hAR DBD and hinge region (C and D regions), or the Bicoid protein (residues 2–160). The target proteins are B42 and B42 fusion with hsRPB7(Δ1–50). Each bar represents the average of 3 to 4 independent experiments plus the standard deviation. (**B**) hRARα associates with hsRPB7 *in vivo* and AT-RA disrupts this association. Immunoprecipitations were performed with nuclear extracts from HeLa cells that had been transfected with 5 μg each of expression plasmids for hRARα and hsRPB7 using either an anti-hRARα or a control anti-GAL4 antibody. Eluant is the bound proteins and load is the nuclear extract. L represents load and F flowthrough from the glutathione-sepharose purification. Note that the intense band found in lanes 7–9 is the IgG heavy chain. Quantification by densitometric scanning showed the following values: lane 1, 0.12; lane 2, 0.03; lane 3, 0.03; lane 4, 1.20; lane 5, 0.71; lane 6, 0.92. (**C**) hsRPB7 specifically interacts with the DBD of hRARα *in vivo*. GST pulldowns were performed with nuclear extracts from Cos cells that had been transfected with 5 μg each of expression plasmids for GST-hRARα (lanes 1, 2, 6, 7, 11, 12), GST-hRARα(C) (lanes 3, 8, 13), GST-RARα(F) (lanes 4, 9, 14) and GST (lanes 5, 10, 15). Cells receiving full-length receptor were treated with 10^{-7}M AT-RA as indicated. Quantification by densitometric scanning showed the following values: lane 1, 1.34; lane 2, 0.17; lane 3, 1.01; lane 4, 0.12; lane 5, 0.25; lane 11, 2.09 ; lane 12, 1.58; lane 13, 3.12; lane 14, 1.04; lane 15, 1.69.

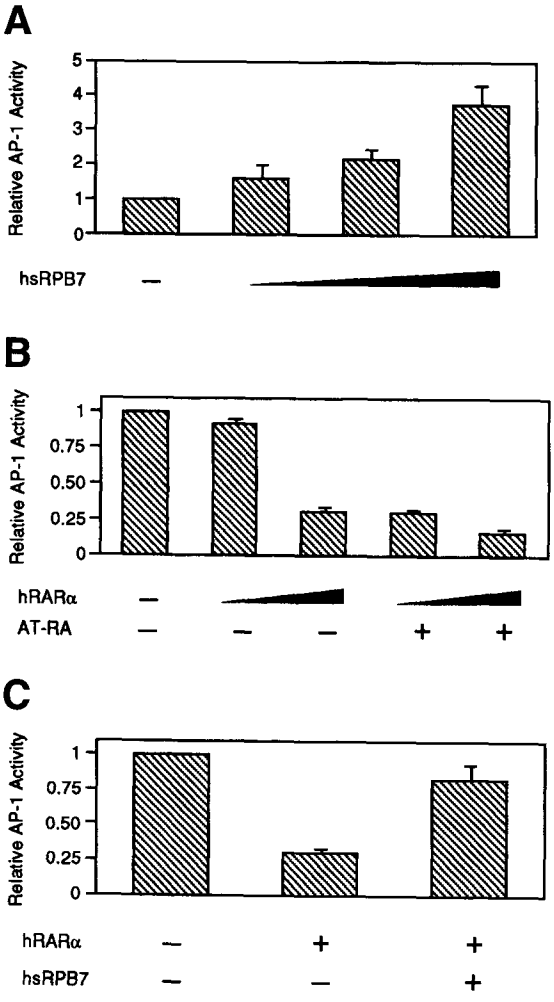

Fig. 3. hRARα inhibits AP-1 in the absence of RA. **(A)** Exogenous hsRPB7 supports AP-1-mediated transcription. HeLa cells were transfected with 1 μg of TRE-tk-CAT reporter plasmid and increasing amounts (1, 3, 5 μg) of hsRPB7 expression plasmid. **(B)** hRARα inhibits AP-1-induced transcription in the absence of RA. HeLa cells were transfected with 1 μg TRE-tk-CAT and 1 or 5 μg of hRARα expression plasmid in the presence or absence of AT-RA, as indicated. **(C)** hsRPB7 restores transcription inhibited by hRARα. HeLa cells were transfected with 1 μg of TRE-tk-CAT with or without 5 μg of expression plasmids for hRARα and/or hsRPB7. Note that, in all cases, CAT activity is represented relative to activity of the first condition, which was set to 1.

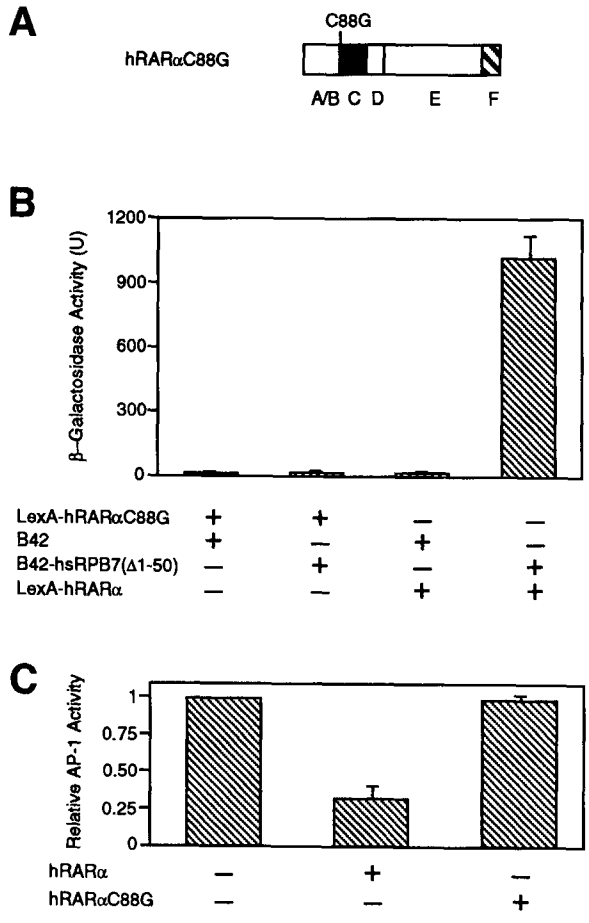

Fig. 4. A DBD mutant of hRARα is unable to interact with hsRPB7 or repress AP-1 activity. **(A)** A schematic representation showing hRARαC88G, which has cysteine 88 in the DBD mutated to a glycine. **(B)** hRARαC88G does not interact with hsRPB7(Δ1–50) in yeast. Bait protein is LexA fusions with full-length hRARα or hRARαC88G. The target proteins are B42 or B42 fusion with hsRPB7(Δ1–50). **(C)** hRARαC88G does not inhibit AP-1 activity. HeLa cells were transfected with 1 μg TRE-tk-CAT and 5 μg of expression plasmid for either wild-type hRARα or hRARαC88G in the absence of AT-RA. Note that CAT activity is represented relative to activity of the first condition, which was set to 1.

in ref. *11*], inhibition of JNK activity *(16)*, and disruption of AP-1 dimerization *(17)*. If titration of limiting quantities of hsRPB7 by hRARα accounts for the ligand-independent effect, then increased levels of hsRPB7 should restore AP-1-dependent transactivation. When this was tested, the inhibitory effect was almost completely relieved with transfected hsRPB7 (Fig. 3C). These results show for the first time that hRARα can inhibit AP-1 activity in a ligand-independent fashion and this may be mediated by the hRARα interaction with hsRPB7. Interestingly, exogenous hsRPB7 also restored, but only partially, AP-1 activity repressed by liganded hRARα (data not shown), an expected result in view of the limiting levels of endogenous hsRPB7 that are found in Cos cells as suggested by our data (see Fig. 3A).

If the hRARα DBD is the *in vivo* target of hsRPB7 interaction, as our data suggest, then it might be possible to disrupt the hRARα-hsRPB7 association by mutating the hRARα DBD. The hRARα mutant C88G, which has cysteine 88 in the first zinc finger of the DBD mutated to a glycine (Fig. 4A), has previously been shown to be deficient in *in vitro* DNA binding *(22,23)*. When this mutant was tested in our yeast two-hybrid system, it was incapable of interacting with hsRPB7(Δ1–50) (Fig. 4B), indicating that the integrity of the hRARα DBD is essential for hsRPB7 interaction. Importantly, this hRARα mutant deficient in interacting with hsRPB7 failed to exhibit a significant ligand-free negative effect on AP-1 transcriptional activity (Fig. 4C). These results strongly suggest that the

hRARα ability to interact with hsRPB7 is a prerequisite for the RA-independent effect of this receptor on AP-1.

Since hsRPB7 is a component of RNA polymerase II, it is possible that ligand-free hRARα has a general effect on RNA polymerase II-catalyzed transcription. This was examined by testing another transcriptional activator, the GR *(24)*. Overexpression of hRARα significantly repressed GR-induced transactivation, and this repression is partially relieved with coexpression of hsRPB7 (Fig. 5). This relief of repression is expected since exogenous hsRPB7 is able to stimulate GR-induced transcription (Fig. 5), suggesting hsRPB7 involvement in GR function. Interestingly, addition of AT-RA did not relieve the inhibition (Fig. 5), possibly due to sequestration by liganded RAR of a common coactivator, such as CBP/p300 *(12,13)*, from liganded GR. Therefore, we suggest that ligand-free hRARα may have a general inhibitory effect on RNA polymerase II-dependent transcription in HeLa cells by directly associating with hsRPB7.

Discussion

Interaction between transcriptional activators and the RNA polymerase II complex has been previously suggested to be important for transcriptional activation *(25)*. In contrast to this, we provide evidence here for such an interaction resulting in transcriptional repression. Our data show that nonliganded hRARα is able to physically associate, via its DBD, with the RNA polymerase II subunit hsRPB7 and, thereby, block the activity of RNA polymerase II-dependent transcriptional activators, such as AP-1. These data suggest a novel pathway in which RAR can repress AP-1 transcriptional activity, distinct from the well-documented RA-dependent effect on AP-1 *(12,13)*. Previous data have suggested that GR is also capable of ligand-independent repression of AP-1 activity *(19)*. However, in this case, the integrity of the receptor DBD was not essential for the inhibitory effect. Therefore, ligand-free GR may be inhibiting AP-1 via a mechanism that is distinct from the hsRPB7-mediated pathway that we propose here for RAR.

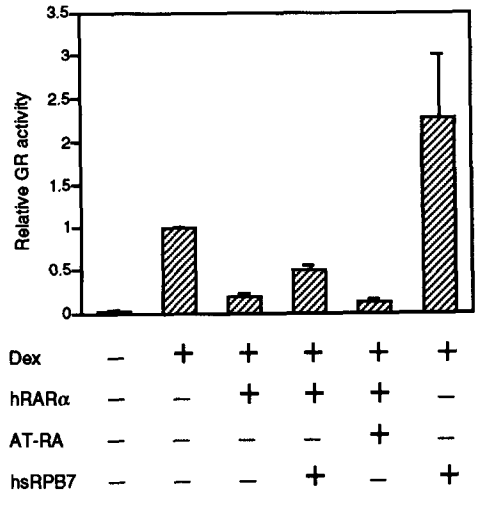

Fig. 5. hRARα can block GR-dependent transcription in the absence of RA. HeLa cells were transfected with 1 μg MMTV-CAT and 5 μg of expression plasmids for hsRPB7 or hRARα without and with 5 μg of hsRPB7 expression plasmid. Dex and AT-RA were added, as indicated, at a concentration of 10^{-7} M. Note that CAT activity is represented relative to activity of the second condition, which was set to 1.

The yeast homolog of hsRPB7, RPB7, has been shown to be essential for yeast viability and important for the yeast stress response and cell morphology *(9,10)*. This stress response appears to require RPB7 and RPB4, another subunit of RNA polymerase II with which RPB7 specifically and directly interacts *(20,21)*. Recently, the human homolog of RPB4, hsRPB4, has been cloned and shown to associate with the human hsRPB7 in a RNA polymerase II complex *(26)*. It has been suggested that RPB7 association with RPB4 is a prerequisite for RPB7 interaction with RNA polymerase II *(21)*. This raises the possibility that nonliganded RARα blocks the transcriptional activity of AP-1, and perhaps other activators, by disrupting the hsRPB7-hsRPB4 interaction and thereby attenuating RNA polymerase II activity. However, our preliminary results in yeast demonstrate that hRARα does not interfere with the hsRPB7–hsRPB4 interaction (data not shown). Thus, it is possible that hRARα affects either the ability of the hsRPB7–hsRPB4 subcomplex to become associated with the RNA polymerase II holoenzyme or hsRPB7 functions which may be independent of hsRPB4. In support of the latter, we have not detected hsRPB4 in our immunoprecipitates that have hRARα and hsRPB7 (data not shown), suggesting that there exists within the cell a hsRPB4-free hsRPB7 that is able to associate with hRARα. Recent results have suggested that the RPB7–RPB4 subcomplex may be essential for promoter recognition by RNA polymerase II *(27)*. In support of this, work with *rpb4*⁻ yeast suggests that the RPB4–RPB7 subcomplex may be involved in promoter selectivity *(28)*. If the same holds for the human counterpart, the hsRPB4–hsRPB7 subcomplex, then it is possible that RAR's negative effect on transcription via hsRPB7 is promoter-specific.

Based on earlier work, transcriptional repression by RARs in the absence of ligand appears to be mediated via interactions between RARs and the corepressors N-CoR and SMRT *(6,7)*. It has been proposed that these corepressors mediate ligand-independent transcriptional repression in a complex together with mSin3 and histone deacetylases *(6–9)*. We propose that a second mechanism by which ligand-free hRARα is able to inhibit transcription is through its association with hsRPB7. While the transcriptional outcome is the same, these two mechanisms are clearly distinct. Unlike the hRARα interaction with N-CoR and SMRT, which is mediated through a region within the ligand-binding domain of the receptor *(6,7)*, the interaction with hsRPB7 is mediated via the receptor DBD, which is in the C region. In addition, the repressive activities of ligand-

free hRARα through a N-CoR/SMRT complex are targeted to genes harboring retinoic acid-response elements, while the activity through hsRPB7 appears to be independent of receptor DNA binding. Our transfection data thus far suggest that hsRPB7 is involved transcriptional activation induced by various activators, including several nuclear receptors, p53, and the chimeric protein GAL-VP16 (data not shown). In support of the hsRPB7-dependent pathway, our results thus far demonstrate that hRARα can inhibit transcription via hsRPB7 without, as of yet, an apparent activator specificity (data not shown). However, based on the degree of complexity associated with hsRPB7 expression at the RNA and protein levels *(20)*, it has been suggested that RPB7 may be found in some, but not all, RNA polymerase II complexes. This makes it possible that there are RPB7-dependent and -independent promoters, as has been suggested by in vitro transcription studies *(28)*. This would provide specificity in the RAR regulation of transcription via hsRPB7. Therefore, it is attractive to speculate that hsRPB7 may represent a target of a divergent pathway by which non-liganded hRARα can modulate the transcriptional efficacy of RNA polymerase II and, thereby, affect transcription of a diverse, but limited, array of genes.

Recently, a novel SET (Su, E(z), Trx) protein, NSD1, has been demonstrated to interact with non-liganded RARs *(29)*. Like the interaction with hsRPB7 and corepressors, RAR's interaction with NSD1 is disrupted by ligand. However, unlike hsRPB7 but like the corepressors, NSD1 is able to interact with several nuclear receptors, in addition to RARs, and it targets the ligand-binding domain, not the DBD, of RARs *(29)*. It is noteworthy that NSD1 is tightly associated with chromatin *(29)* and that the corepressor complex harbors histone deacetylase activity *(8,9)*. Thus, it appears that non-liganded RARs, and perhaps other nuclear receptors, can negatively regulate transcription by, at minimum, two mechanisms, either by effecting chromatin remodeling via its LBD region or modulating the enzymatic activity of RNA polymerase II via its DBD. It is possible that one of these proposed mechanisms is the preferred choice in vivo or that these different modes of actions may cooperate to ensure the appropriate temporal and spatial expression of certain RNA polymerase II-dependent genes.

Interestingly, while the hRARα DBD can alone participate in a protein-protein interaction with hsRPB7, it is not able to repress AP-1 activity (data not shown). By contrast, both wild-type RAR, in the absence of RA, and a CF fusion of hRARα can very efficiently inhibit AP-1, presumably via the hsRPB7 interaction. These data suggest that interaction with hsRPB7 via the DBD is not sufficient for the negative effect on AP-1, but requires additional regions of the receptor, including the poorly understood F region.

What is the physiological relevance of the hRARα-hsRPB7 interaction? RARs, as well as several other nuclear receptors, are known to act as transcriptional repressors in the absence of ligand, by virtue of their associations with the corepressor complex *(6–9)*. Our data extend this RAR activity to what may be a general effect on RNA polymerase II transcription. Whether this is indeed a global effect of RAR on RNA polymerase II transcription is not known, and will likely remain as such until the role of hsRPB7 in transcription is better defined. However, in this vain, it is noteworthy that p53 has recently been demonstrated to act as a general repressor of RNA polymerase III transcription *(30)*. Thus, it is conceivable that RAR may have a general inhibitory effect on RNA polymerase II transcription, perhaps involved in keeping polymerase II-transcribed genes silent until the correct developmental signal for transcriptional activation appears. Yet, it is also possible that the RAR-hsRPB7 interaction may target a subset of genes that are dependent on hsRPB7 for their transcription. While in vivo evidence is lacking for the existence of both hsRPB7-dependent and -independent promoters, data from in vitro transcription studies have suggested that hsRPB7 may play a role in promoter recognition and selectivity by RNA polymerase II *(27,28)*. Finally, it has been suggested that the stress-response effects of yeast RPB7 are mediated by functions of this protein which may be independent of its association with RNA polymerase II *(21)*. In view of the homologous functions between the yeast and human RPB7 proteins, this opens up the intriguing possibility that non-liganded hRARα can influence mammalian cells through a mechanism that may not directly involve transcriptional regulation.

Materials and Methods

Plasmids

For yeast two-hybrid analysis, LexA-hsRPB7 *(20)*, LexA-Bicoid *(31)*, and B42-hsRPB4 *(26)* have been described. To construct LexA-hRARα, full-length hRARα was cloned into the EcoRI/XhoI sites of pEG202 *(31)*. LexA fusions with hRARαC88G, hRARα(C) (amino acids 88–153), hRARα(F) (amino acids 420–462), hRARα(CF), mRXRα(C) (amino acids 140–205), and hAR(CD) (amino acids 556–665) were all generated by polymerase chain reaction (PCR) amplification and cloning into pEG202. B42-hsRPB7 was constructed by cloning hsRPB7 into the EcoRI/XhoI sites of pJG4–5 *(31)*. hRARα was also expressed in yeast from the plasmid pYE10 *(31)*.

For mammalian expression, hRARα *(22)*, hRARαC88G *(22,23)*, GR *(22)*, and c-Jun *(23)* in pSG5 *(33)*, and hsRPB7 in pcDNA3 *(26)* have been described. hRARα(CF) in pSG5 was constructed by PCR amplification of regions encoding amino acids 88–153 (C region) and 420–462 (F region) with an artificial start codon initiating translation and a linker of Lys-Leu-Met between the two regions of hRARα. In vitro translated and mammalian expressed GST (glutathione S-transferase)-tagged proteins were expressed from pSG5-derived plasmids that contain a sequence

encoding GST, while in vitro translated hsRPB7 was expressed from pSG5, which was constructed by PCR amplification of hsRPB7 and insertion into the EcoRI/XhoI sites of pSG5. The reporter plasmids TRE-tk-CAT and MMTV-CAT have been previously described *(15)*.

Yeast Two-Hybrid Screen and Analysis

A yeast two-hybrid system was employed to screen a HeLa cDNA library *(32,34)*. The bait construct, LexA-hRARα(CF), contains LexA fused to the DNA-binding domain (DBD) (residues 88–153) and carboxy-terminus (residues 420–462) of hRARα with a linker of Lys-Leu-Met between the two regions of hRARα. This construct was confirmed by both DNA sequencing and Western blot analysis using an anti-hRARα(F) antibody (data not shown). The plasmid expressing LexA-hRARα(CF) was transformed into the *Saccharomyces cerevisiae* strain EGY48 together with a HeLa cell cDNA library inserted into the pJG4–5 plasmid *(31)*. Approximately 3×10^6 primary yeast transformants were screened and recovered on glucose/CM-Ura,-His,-Trp media. Of these, four produced colonies that showed galactose-dependent growth on Leu^- media and galactose-dependent blue color on X-Gal media. One of these clones was 3–59, that which expresses the truncated hsRPB7 (amino acids 51–172).

Yeast two-hybrid analysis was also used to study protein–protein interactions, which were quantified by measuring β-galactosidase activity with *o*-nitrophenyl-β-D-galactoside (ONPG) as described *(34)*.

Mammalian Cell Transfections and CAT Assays

HeLa and Cos cells were grown in DMEM (Sigma) and supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories). Cells were grown in 60-mm dishes and transiently transfected using the calcium phosphate precipitation method as previously described *(15)*. CAT (chloramphenicol acetyltransferase) assays were standardized according to β-galactosidase activity and performed as previously described *(15)*. hRARα and GR were activated by the addition of 10^{-7} M of *all-trans* retinoic acid (AT-RA) (Sigma) or dexamethasone (Dex) (Sigma), respectively. CAT assay results were quantified by densitometric scanning of autoradiograms (420 oe scanner, PDI, Inc.) of at least three repeats for each transfection, and, hence, each value represents the average of 3 to 4 repetitions plus standard deviation.

Nuclear Extracts

For immunoprecipitation and GST pulldown studies, cells were transfected with 5 μg each of expression plasmids for hRARα, hsRPB7, GST-hRARα, GST-hRARα(C), GST-hRARα (F), and GST, and 2 μg of pCH110 which harbors the Lac Z gene. Cells were harvested in ice-cold PBS and spun at 5000 rpm for 5 min; 10% of the cells were used to do a β-galactosidase assay for quantification of transfection efficiency. The remainder of the cells were resuspended in buffer I (10 m*M* Tris-HCl, pH 7.5; 10 m*M* NaCl; 5 m*M* $MgCl_2$) and incubated at 4°C for 5 min. 0.3 *M* sucrose was then added and cells were lysed with a dounce homogenizer. Nuclei were pelleted by centrifuging lysed cells at 2500 rpm (600*g*) for 10 min. The nuclear pellet was washed once with buffer II (buffer I containing 0.3 *M* sucrose). Then, the nuclear pellet was resuspended in buffer III (50 m*M* Tris-HCl, pH 8; 150 m*M* NaCl; 5 m*M* EDTA; 0.1% Nonidet P-40) with protease inhibitors and incubated with shaking at 4°C for 30 min. The lysed nuclei were centrifuged at 15,000 rpm for 15 min and the supernatant, constituting the nuclear extract, was saved. The amount of extract used was standardized according to β-galactosidase activity.

Protein–Protein Interaction

In the immunoprecipitation studies, nuclear extracts of equal β-galactosidase activity were precleared by incubating with protein A-sepharose beads at 4°C for 30 min. The precleared extracts were transferred to new Eppendorf tubes and 10 μL of either anti-hRARα antibody (sc-551, Santa Cruz Biotechnology) or anti-GAL4 antibody (sc-577, Santa Cruz Biotechnology) were added. After incubation at 4°C overnight, the mixture was added to protein A-sepharose beads and shaken at 4°C for 2 h. The sepharose beads were washed three times with Buffer III, and bound proteins were eluted by boiling beads in SDS sample buffer (63 m*M* Tris, pH 6.8; 20% glycerol; 2% SDS; 5% β-mercaptoethanol) and analyzed by Western blot.

In the GST pulldown experiments, proteins were derived either from mammalian cell nuclear extracts or *in vitro* translation. For the former, nuclear extracts of equal β-galactosidase activity were incubated with glutathione-sepharose beads and shaken at 4° C overnight. The sepharose beads were washed three times with buffer III, and bound proteins were eluted by boiling beads in SDS sample buffer and were analyzed by Western blot. For *in vitro* translated proteins, hsRPB7 radiolabeled with [^{35}S]methionine was incubated for 2 h at 4°C with GST-hRARα, GST-hRARα(C), or GST in 100 μL of $T_{20}N_{20}E_2$ buffer (20 m*M* Tris, pH 7.5, 20 m*M* NaCl, 2 m*M* EDTA). After a 2-h incubation at 4°C, the reaction mixture was added to 30 μL of glutathione-sepharose beads in 500 μL of $T_{20}N_{20}E_2$. After a 3-h incubation at 4°C, the beads were washed three times with $T_{20}N_{20}E_2$ and bound proteins were eluted by boiling beads in Laemmli Sample Buffer. The eluted proteins were analyzed in a 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to autoradiography. All proteins were in vitro translated in reticulocyte lysate.

SDS-PAGE and Western Blot

Proteins were separated by SDS-PAGE and were electrotransferred onto nitrocellulose (Micron Separations Inc., Westborough, MA). After blocking with nonfat dry milk, the nitrocellulose blots were probed with antibodies

against hRARα antibody (sc-551, Santa Cruz Biotechnology), GST antibody (sc-138, Santa Cruz Biotechnology), or hsRPB7 *(20)*. The blots were developed using the chemiluminescence detection kit from Amersham. Western blot results were quantified by densitometric scanning of autoradiograms (420 oe scanner, PDI, Inc.).

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