TIF2 Mediates the Synergy between RARα1 Activation Functions AF-1 and AF-2*

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Nuclear receptors recruit coregulator complexes through both their AF-1 and AF-2 activation domains. Here we demonstrate that TIF2, a p160 coactivator, is able to bridge the two activation domains of the retinoic acid (RA) receptor isotype RARα1, resulting in synergistic activation of transcription. Bridging requires the presence of motifs in region A of RARα1 and in the activation domain AD1 of TIF2. Notably, only RARα1 exerted this interaction, which requires additional unknown factors. This is the first observation of a RAR isotype-selective coactivator interaction. Because another p160 coactivator, SRC-1, has no effect, this is also the first demonstration of a difference between the members of this coactivator family.

There are three types of nuclear retinoic acid receptors, (RARα, RARβ, and RARγ), and for each isotype there are at least two main isoforms differing only in their N-terminal region (1). RARs contain two autonomous activation functions, AF-1 and AF-2. The AF-1 located at the N-terminal end (A/B region) is ligand-independent and contains conserved serine residues that are "consititutively" phosphorylated by the cdk7 subunit of the general transcription factor TFIIH (2, 3). In contrast, the AF-2 domain, located in the C-terminal E region is ligand-dependent (4). It contains the ligand-binding domain and requires the integrity of a highly conserved amphipathic α-helix, the AF-2 core that corresponds to helix 12. Retinoic acid (RA) binding induces a major structural change in the conformation of this helix (5) creating a new surface for the binding of coregulators such as RIP140 and the p160 family of nuclear receptor coactivators such as NcoA-1/SRC-1, NcoA-2/GRIP1/TIF2, and pCIP/ACTR/AIB1/RAR3 (6–8). The p160 co-activators, including TIF2, interact with nuclear receptor ligand-binding domain through a central conserved domain (NID) with three LXXLL motifs (see Fig. 1A) (9). Through another conserved C-terminal transcriptional activation domain (AD1), coactivators also mediate the recruitment of p300/CREB and large histone acetyltransferase complexes such as the p/CAF complex that lead to chromatin decondensation (10, 11). Finally, liganded receptors bind the TRAP/ DRIP/ARC complex, also termed the Srb- and mediator protein-containing complex, which establishes contacts with the RNA pol II holoenzyme and its associated general transcription factors (12, 13).

It has been established that the AF-2 of a given RAR isotype cooperates with the AF-1 of the same or different isoatypes in a response element and promoter context manner (14, 15). This observation led to the hypothesis that the cooperativity between AF-1 and AF-2 might be mediated through coactivators interacting simultaneously with the two AFs, as previously shown for PPARγ (16), ERα (17–19), ERβ (20), SF-1 (21), or AR (22). As the modulating functions present in the AF-1 of RARα and RARγ are different, we speculated that the AF-1 of each RAR isotype might interact with specific intermediary proteins participating to the bridge between the two AFs.

The present study was undertaken to determine whether known AF-2 coregulators mediate the synergism between the two AFs of RARs. We found that TIF2 is indeed involved in this synergism and bridges the AF-1 and AF-2 domains of the RARα1 isotype. Moreover our data suggest that this bridging requires additional factor(s), which may account for the distinct isotype-specific activities of RARα and RARγ AF1s.

MATERIALS AND METHODS

Plasmids—The (17mer-ERE)-TATA-CAT and (17mer)x5-TATA-CAT reporter constructs as well as the RARα, RARγ, and ERα chimeric constructs have been described elsewhere (15, 17). Gal4-RARα1(AB), Gal4-RARα1(AB), Gal4-RARα1(AB57A), Gal4-RARα1(A), and Gal4-RARα1(B) were constructed with PCR-amplified AB, A, or B fragments that were inserted into XhoI-BglII-digested pG4 M Poly II. Deletions in the A region of RARα1 were introduced by double PCR amplification according to standard protocols. GST-RARα1(DEF) was previously described (9, 23). GST-RARα1(AB) was constructed with PCR-amplified AB fragments that were inserted into BamHI-EcoRI-digested pGEX-2T.

The different TIF2 constructs (Fig. 1) were described previously (9, 17, 24). TIF2.1 deleted for the AD1 domain (amino acids 1007–1147) was constructed by double PCR amplification to generate a HpaI-XbaI fragment containing the appropriate mutation. The other deletions (amino acids 1012–1034, 1031–1073, and 1107–1047 in TIF2.1) were previously described (23, 25). The CDNA for SRC-1 (gift from M. Parker) was cloned in pSG5. The expression vector for RIP-140 was a gift from M. Parker and that for CBP was from C. Glass.

Cells, Transfections, and CAT Assays—HeLa cells were transiently transfected in six-well plates, using the DMRIE-C reagent (Invitrogen). All transfections contained the reporter plasmid, the different receptor chimeric constructs, the β-galactosidase expression vector pCH110, and BlueScript as a carrier. After a 16-h incubation with the DNA, the cells
were washed and maintained for a further 24 h in the appropriate medium with or without RA (10⁻⁷ M). CAT assays were performed using the ELISA method (CAT ELISA, Roche Molecular Biochemicals) after normalization to equal β-galactosidase activity.

Immunoblotting and Antibodies—Expression levels of recombinant proteins in transfected COS-1 cells were determined by standard SDS-PAGE and subsequent electrotransfer to nitrocellulose membranes. Proteins were revealed by immunoblotting and chemiluminescence. Rabbit polyclonal antibodies against TIF2 were raised against an epitope within residues 624 and 869. Monoclonal antibodies against the Gal4 DNA-binding domain (2GV3) were as described (26).

GST-based Interaction Assays—Glutathione-Sepharose beads (Amersham Biosciences) were incubated with bacterial extracts containing the GST proteins and then with rabbit reticulocyte lysates containing 3⁵-S-labeled translated protein (TnT T7 Quick Coupled Transcription/Translation System, Promega) as described (23). Bound proteins were recovered in SDS loading buffer, subjected to SDS-10% polyacrylamide gel electrophoresis, and analyzed by autoradiography of dried gels or by immunoblotting.

RESULTS AND DISCUSSION

TIF2 Mediates the Synergy between RARα1 AF-1 and AF-2—The observation that both AF-1 and AF-2 of RARs can synergize (14, 15), led us to evaluate whether this synergy might be mediated through members of the p160 transcriptional coactivators. As the coactivator TIF2 is able to interact independently with the two activation functions AF-1 and AF-2 of several steroid hormone receptors and can bridge their N- and C-terminal domains (17, 22), we investigated whether TIF2 could mediate the synergy between both AFs of RARα1. COS-1 cells were cotransfected with a hybrid reporter gene comprising an artificial minimal promoter containing a Gal4 binding site juxtaposed to an estrogen responsive element, (17mer-ERE)-TATA-CAT, and the Gal4-RARα1(DEF) and Gal4-RARα1(AB)-ER(C) hybrid constructs (see Fig. 2). Under these conditions, the AF-1 of RARα1 displays very weak if any activity (see Fig. 2B, lane 8), whereas AF-2 is significantly more active in the presence of RA (see Fig. 2B, lane 3). TIF2 WT enhanced the RA-stimulated transcriptional activity of the isolated AF-2 (Fig. 2B, lane 4) but not of AF-1 (Fig. 2B, lane 9). A TIF2 mutant lacking the three LXXLL motifs of the nuclear receptor interacting domain (NID), which are necessary and sufficient for interaction with the AF-2 domain of nuclear receptors (TIF2m123 in Fig. 1A) (9), did not exhibit any stimulatory activity (Fig. 2B, lane 5).

When coexpressed together, AF-1 and AF-2 cooperated (Fig. 2B, compare lanes 3 and 10). Overexpression of TIF2 markedly enhanced this effect (Fig. 2B, lane 11), while TIF2m123 failed to do so (Fig. 2B, lane 12). Interestingly, a mutant TIF2 protein encompassing only the NID (TIF2.5 in Fig. 1A) acted as a dominant-negative mutant and abrogated the synergy between the two AFs (Fig. 2B, lane 13).

Altogether, these results indicate that TIF2 can mediate the synergy between both AFs of RARα1. Moreover, the observation that TIF2.5 acts as a dominant negative, shows that the endogenous factor(s) mediating AF-1/AF-2 synergy of RARα1 are functionally similar to TIF2 and correspond most likely to members of the p160 coactivators family.

FIG. 1. Constructs. A, schematic representation of the different TIF2 mutants (note to scale) with their functional domains: NID, AD1 and AD2, two autonomous activation domains, bHLH (sequence similarity with basic helix-loop helix motifs), PAS (sequence similarity with the Per Arnt-Sim motif), and Q-rich (glutamine-rich sequence). B, schematic representation of the RARα1(AB) mutants. C–E, representative Western blots illustrating the expression levels of the TIF2 and Gal4-RARα1(AB) proteins in transfected COS-1 cells.

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TIF2 Bridges the AF-1 and AF-2 Activation Domains of RAR in an Isotype-selective Manner—To further study the molecular basis of the synergy mediated by TIF2, mammalian-bridged two-hybrid experiments were performed in COS-1 cells. The basis of this assay (see Fig. 3A) is that in COS-1 cells neither the AF-1 function present in Gal4-RAR(DEF) (Fig. 3B, lane 5) nor the non-ligated RAR-DEF-VP16 construct (Fig. 3B, lane 3), activate the (17mer)x5-TATA-CAT reporter gene because AF-1 alone is inactive (see above) and the VP16 activation domain is not recruited to this promoter. However, such a recruitment can occur if a factor binds concomitantly to AF-1 and AF-2. In the presence of RA, coactivators (e.g. p160 proteins) will bind to AF-2, and, provided the p160 proteins can also bind to AF-1, this will finally lead to recruitment of VP16 AD to the Gal4 reporter. Indeed, the activity of the CAT reporter was significantly enhanced (about 3-fold) when RAR(DEF)-VP16 was coexpressed with the A/B region of RAR1 in the presence of RA (Fig. 3B, lane 7). This suggests that the effect of TIF2 would be specific for the RAR1 isotype. To assess the selectivity of the RAR1 AF-1/AF-2 mediator effect, we tested whether other coactivators could mediate the observed synergy. Most surprisingly, SRC-1, which is a member of the same p160 coactivator family, was completely inefficient in this bridging assay (Fig. 3C, lane 6). In addition, neither RIP140, CBP, ADA2, nor SUG1 exhibited any effect (Fig. 3C, lanes 7–10). Note that none of these coregulators had any significant effect either with the RAR3 isotype (Fig. 3C, lanes 14–18).

TIF2 Bridges the RAR1 AF-1 and AF-2 Domains through Its NID and AD1 Domains—While TIF2 interaction with the AF-2 of nuclear receptors requires the LXXL boxes located in the NID, binding to AF-1 was reported previously to involve residues in the glutamine-rich domain (17, 18, 22). Indeed, in the present bridged two-hybrid experiments, TIF2 lacking this domain (TIF2m123 in Fig. 1A) was unable to bridge the AF-1 and AF-2 domains of RAR1 and therefore recruit liganded RAR(DEF)-VP16 to the promoter. This increase was markedly enhanced (5-fold) upon coexpression of TIF2WT (Fig. 3B, lane 8 and Fig. 3C, lane 5) but not of TIF2m123 (Fig. 3B, lane 9).

When the A/B region of RAR1 was replaced by that of RAR3 in the bridged two hybrid assay, overexpressed TIF2 did not significantly increase the activity of the CAT reporter (Fig. 3C, lanes 11–13), confirming that the RAR AF-1 functions are different. This suggests that the effect of TIF2 would be specific for the RAR1 isotype. To assess the selectivity of the RAR1 AF-1/AF-2 mediator effect, we tested whether other coactivators could mediate the observed synergy. Most surprisingly, SRC-1, which is a member of the same p160 coactivator family, was completely inefficient in this bridging assay (Fig. 3C, lane 6). In addition, neither RIP140, CBP, ADA2, nor SUG1 exhibited any effect (Fig. 3C, lanes 7–10). Note that none of these coregulators had any significant effect either with the RAR1 isotype (Fig. 3C, lanes 14–18).

TIF2 Bridges the RAR1 AF-1 and AF-2 Domains through Its NID and AD1 Domains—While TIF2 interaction with the AF-2 of nuclear receptors requires the LXXL boxes located in the NID, binding to AF-1 was reported previously to involve residues in the glutamine-rich domain (17, 18, 22). Indeed, in the present bridged two-hybrid experiments, TIF2 lacking this domain (TIF2m123 in Fig. 1A) was unable to bridge the ERα AF-1 and AF-2 domains (Fig. 3B, compare lanes 15–17). In striking contrast, the same mutant was still able to strongly potentiate the cooperation between the two ERα AFs (Fig. 3B, compare lanes 8 and 10), indicating that the glutamine-rich region is not required for mediating the effect of TIF2 on RAR1A functions.

To delineate the region responsible for the effect of TIF2, a series of deletion mutants (9) were tested (Fig. 1A). The TIF2.10 construct encoding the NID and the N-terminal part of
TIF2 (amino acids 1–869) was unable to increase the cooperation between the two AF domains of RARα1 (Fig. 3D, lane 6). In contrast, a TIF2 fragment from amino acids 624–1287 (TIF2.1) that also contains the NID, has maintained this capacity (Fig. 3D, lane 7). As expected from the above data, TIF2.1 lacking the Q region (TIF2.3) was still effective (Fig. 3D, lane 9). However, removal of the subsequent 169 C-terminal amino acids (TIF2.4) made the protein inactive (Fig. 3D, lane 10). As these amino acids encompass the AD1 (See Fig. 1A), TIF2.1 deleted for this domain (TIF2.1ΔAD1) was constructed. This mutant was also ineffective (Fig. 3D, lane 12) indicating that TIF2 recruits RARα1 AF-1 through its AD1 domain.

The AD1 activation domain harbors several exposed motifs that are candidates for protein-protein contacts. Among them, the conserved LLL motif (amino acids 1080, 1081, and 1084), which is involved in CBP binding (9), is not required for RARα1 AF-1 interaction since its mutation (in TIF2LLL) had no effect (Fig. 3D, lane 5). Interestingly, sequence alignment studies indicated that among the other motifs, three are not conserved between the different coregulators. Deletion of two of these
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Figure 4. TIF2 recruits RARα1 AF-1 domain through an adaptor. A, set-up of the experiments shown in B. COS-1 cells were transfected with the (17mer)x5-TATA-CAT reporter gene and the expression vectors for Gal4-RARα1(AB), Gal4-RARα1(AB), and TIF2.1-VP16. Results are expressed as fold induction relative to the CAT activity displayed in the absence of RA and are the mean ± S.E. of three individual experiments. C, interaction assay of in vitro translated TIF2 with GST, GST-RARα1(AB), or GST-RARα1(DEF) in the absence or presence of RA. Bound TIF2 was detected by immunoblotting. Equal loading of the GST fusion proteins was confirmed by Coomassie staining (not shown). D, 35S-labeled in vitro translated TIF2, ADA-2, and CBP proteins were incubated with GST or GST-RARα1(AB). Bound proteins were detected by autoradiography of dried gels. E, model for the synergy between the two AFs of the RARα1 isotype through TIF2. This synergy involves an adaptor protein interacting specifically with motifs located in the A region of the RARα1 isotype and in the AD1 domain of TIF2.

surfaces (amino acids 1011–1034 and amino acids 1031–1073 in TIF2.1Δ1 and TIF2.1Δ2, respectively, see Fig. 1A) did not affect TIF2 efficiency (Fig. 3D, lanes 13 and 14). However, deletion of the third one (amino acid residues 1107–1147 in TIF2.1Δ3) decreased significantly TIF2.1 action (Fig. 3D, lane 15).

The TIF2.1Δ3 mutant was also unable to increase the synergy between the two AFs of RARα1 when the Gal4-RARα1(DEF) and RARα1(1AF)-ER(C) hybrid proteins were cotransfected with the (17mer-ERE)-TATA-CAT reporter gene (see Fig. 2B, lane 15). Note also that in that context, TIF2ΔQ was as effective as TIF2WT in mediating the synergy between the two AFs (Fig. 2B, lane 14 and Fig. 2D, lane 6).

Collectively, these results indicate that TIF2 mediates selectively the cooperation between the AF-1 and AF-2 domains of the RARα1 isotype through a surface located in its AD1 domain. It is interesting to note that this sequence contains a proline-rich motif (KPPXXP) that is not conserved between p160 coactivators and could be recognized by proteins with SH3 or WW domains (27–29).

Phosphorylation at Serine 77 Is Not Required for TIF2-mediated RARα1 AF-1- AF-2 Cooperativity—As the AF-1 domain of RARα1 is constitutively phosphorylated at serine 77 (Ser77) (28) in COS-1 cells, we tested whether phosphorylation of this residue plays a role in the cooperation between the two RARα1 AFs as for other nuclear receptors (20, 21, 30). Mutation of serine 77 into alanine, in Gal4-RARα1(AB)S77A (Fig. 1C), did not affect the synergy between both AFs of RARα1 (Fig. 2B, compare lanes 18 and 19 to lanes 10 and 11). It did not affect either the activity of the CAT reporter nor TIF2 efficiency in the bridged two-hybrid assay (Fig. 3E, compare lanes 2–5), indicating that the ability of TIF2 to bridge the two AFs of RARα1 is AF-1 phosphorylation-independent.

TIF2-mediated RARα1 AF-1- AF-2 Cooperativity Involves the A Region of RARα1—To delineate which motif(s) in the A/B region of RARα1 might be responsible for recruiting TIF2, a series of mutants were tested (Fig. 1A). First, in agreement with the conservation of the B region between the different RAR isotypes (1), we found that deletion of this region did not affect the ability of the AF-1 domain to recruit RARα1(DEF)-VP16 to the promoter, nor TIF2 function (Fig. 3E, lanes 8 and 9). In contrast, deletion of the A region abrogated the activation of the reporter gene either in the absence or presence of TIF2 (Fig. 3E, lanes 6 and 7). Thus the A region of RARα1 would be involved in the recruitment of TIF2.

These results were corroborated by using a construct expressing TIF2 fused to the VP16 activation domain (TIF2.1-VP16) (Fig. 4A). Indeed, when coexpressed with Gal4-RARα1(AB), TIF2.1-VP16 activated the promoter (Fig. 4B, lane 4) confirming that it can be recruited by the AF-1 domain of
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In the present study, we show that the synergy between the AF-1 and AF-2 domains of the RARα1 isoform involves the coactivator TIF2. Because no direct interaction between TIF2 and the AF-1 domain of RARα1 could be detected, we propose that TIF2 recruits this domain through an adaptor molecule (see Fig. 4E) as previously described for ERα (30, 34). According to our results, such an adaptor would specifically interact on the one hand with the RARα1 AF-1 domain through a motif located at the N-terminal end of the A region and on the other hand with TIF2 via a motif within its AD1 domain. As both these RAR1 and TIF2 motifs depict proline-rich motifs, proteins with at least two WW or SH3 domains (27–29) might be good adaptor candidates. Yeast two-hybrid screening experiments with the A/B domain of RARα1 as a bait are presently under way to identify the putative RARα1 AF-1 domain interacting proteins. The same strategy will be used to identify the proteins involved in the synergy between the AF-1 and AF-2 domains of the other RAR isoforms. The goal of these studies is to determine RAR isotype- and/or isoform-specific functions, which may provide at the same time clues to the cell type specificity of AF-1.

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