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

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Martin Bommer \ddagger , Arndt Benecke \S , Hinrich Gronemeyer, and Cécile Rochette-Egly \parallel

From the Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/Université Louis Pasteur, BP 10142, 67404 Illkirch Cedex, France

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 RAR α 1, resulting in synergistic activation of transcription. Bridging requires the presence of motifs in region A of RAR α 1 and in the activation domain AD1 of TIF2. Notably, only RAR α 1 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, (RAR α , RAR β , and RAR γ),¹ 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 “constitutively” phosphorylated by the cdk7 subunit of the general transcription factor TFIID (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 coactivators, including TIF2, interact with nuclear receptor li-

gand-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/CBP 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 isotypes 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 PPAR γ (16), ER α (17–19), ER β (20), SF-1 (21), or AR (22). As the modulating functions present in the AF-1 of RAR α and RAR γ 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 RAR α 1 isotype. Moreover our data suggest that this bridging requires additional factor(s), which may account for the distinct isotype-specific activities of RAR α and RAR γ AF1s.

MATERIALS AND METHODS

Plasmids—The (17mer-ERE)-TATA-CAT and (17mer) \times 5-TATA-CAT reporter constructs as well as the RAR α , RAR γ , and ER α chimeric constructs have been described elsewhere (15, 17). Gal4-RAR α 1(AB), Gal4-RAR γ 1(AB), Gal4-RAR α 1(ABS77A), Gal4-RAR α 1(A), and Gal4-RAR α (B) were constructed with PCR-amplified AB, A, or B fragments that were inserted into *Xho*I-*Bgl*II-digested pG4 M poly II. Deletions in the A region of RAR α 1 were introduced by double PCR amplification according to standard protocols. GST-RAR α (DEF) was previously described (9, 23). GST-RAR α -AB was constructed with PCR-amplified AB fragments that were inserted into *Bam*H1-*Eco*R1-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 *Hpa*I-*Xba*I fragment containing the appropriate mutation. The other deletions (amino acids 1012–1034, 1031–1073, and 1107–1047 in TIF2.1A1, TIF2.1A2, and TIF2.1A3, respectively) were introduced into TIF2 according to the same protocol. The ADA-2 and SUG-1 expression vectors were previously described (23, 25). The cDNA for SRC-1 (a gift from B. O'Malley) 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—COS-1 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

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\ddagger Present address: Dept. of Biological Sciences, Imperial College of Science, Technology, and Medicine, London SW7 2AY, UK.

\S Recipient of Marie Curie Long Term Fellowship ERBFMBICT961269 from the European Commission. Present address: INSERM U147, Hospital Saint Antoine, 75571 Paris Cedex 12, France.

\parallel To whom correspondence should be addressed: Institut de Génétique et de Biologie Moléculaire et Cellulaire, BP 163, 67 404 Illkirch Cedex, CU de Strasbourg, France. Tel.: 33-3-88-65-34-59; Fax: 33-3-88-65-32-01; E-mail: cegly@igbmc.u-strasbg.fr.

¹ The abbreviations used are: RAR, retinoic acid receptors; RA, retinoic acid; AF, activation functions; CAT, chloramphenicol acetyltransferase; ELISA, enzyme-linked immunosorbent assay; GST, glutathione S-transferase; NID, nuclear receptor interacting domain; SH3, src homology 3; CBP, cAMP-response element-binding protein.

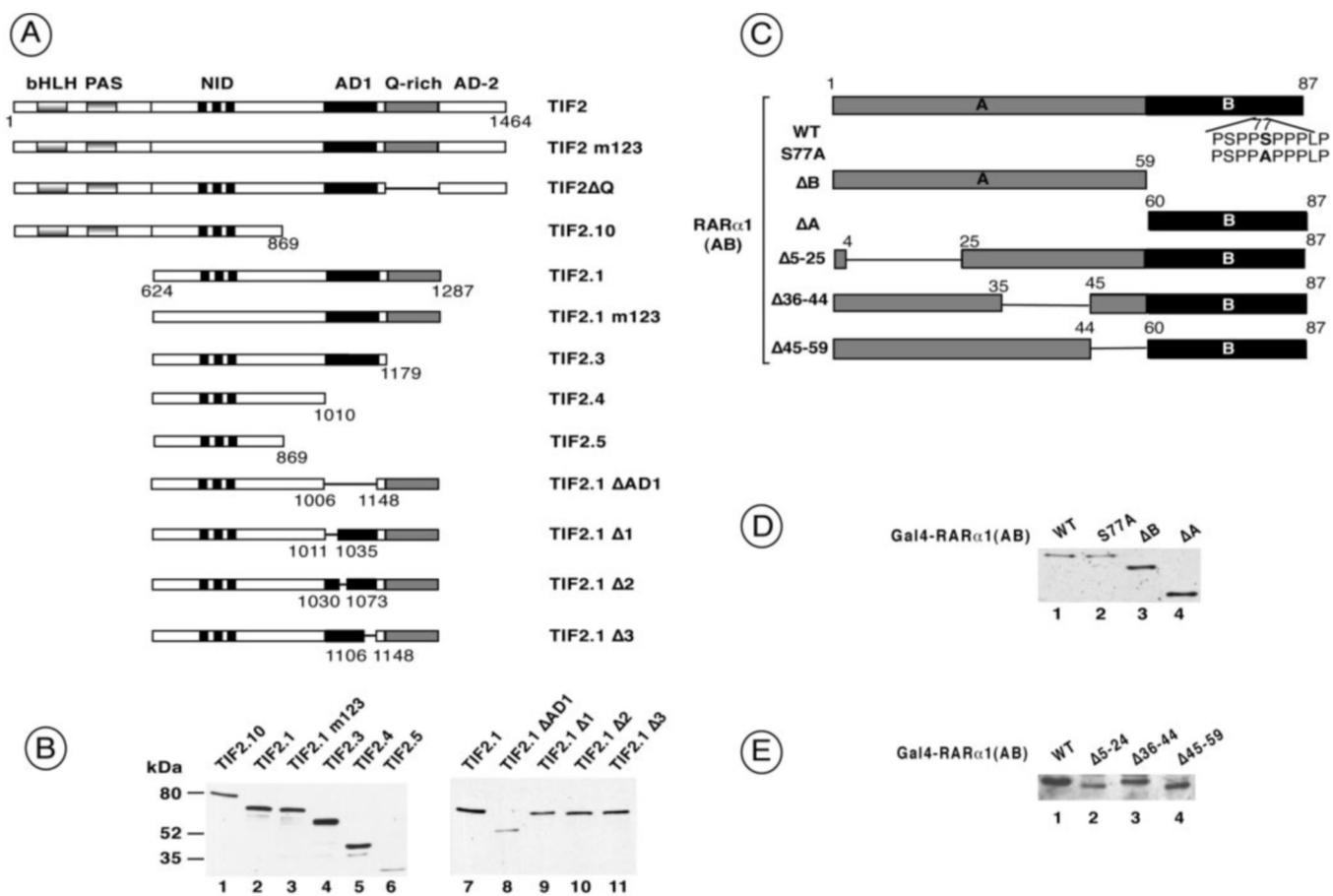


FIG. 1. Constructs. *A*, schematic representation of the different TIF2 mutants (note to scale) with their functional domains: NID, AD1 and AD2, two autonomous activation domains, bHLH (sequence similarity with basic helix-loop helix motifs), PAS (sequence similarity with the Per Arndt-Sim motif), and Q-rich (glutamine-rich sequence). *B*, schematic representation of the RAR α 1(AB) mutants. *C–E*, representative Western blots illustrating the expression levels of the TIF2 and Gal4-RAR α 1(AB) proteins in transfected COS-1 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 RAR α 1 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 RAR α 1. 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-RAR α (DEF) and RAR α 1(AB)-ER(C) hybrid constructs (see Fig. 2). Under these conditions, the AF-1 of RAR α 1 displays very weak if any activity (see Fig. 2*B*, lane 8), whereas AF-2 is significantly more active in the presence of RA (see Fig. 2*B*, lane 3). TIF2 WT enhanced the RA-stimulated transcriptional activity of the isolated AF-2 (Fig. 2*B*, lane 4) but not of AF-1 (Fig. 2*B*, 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. 1*A*) (9), did not exhibit any stimulatory activity (Fig. 2*B*, lane 5).

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

Similar results were obtained with full-length RAR α 1 in which region C was replaced by the core of the estrogen receptor DNA binding domain, termed ER.Cas (Fig. 2*C*). Cotransfection of TIF2WT potentiated the RA-dependent activation of the reporter gene by RAR α 1-ER.Cas (Fig. 2*D*, lane 4), whereas TIF2m123 had no effect (Fig. 2*D*, lane 5).

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

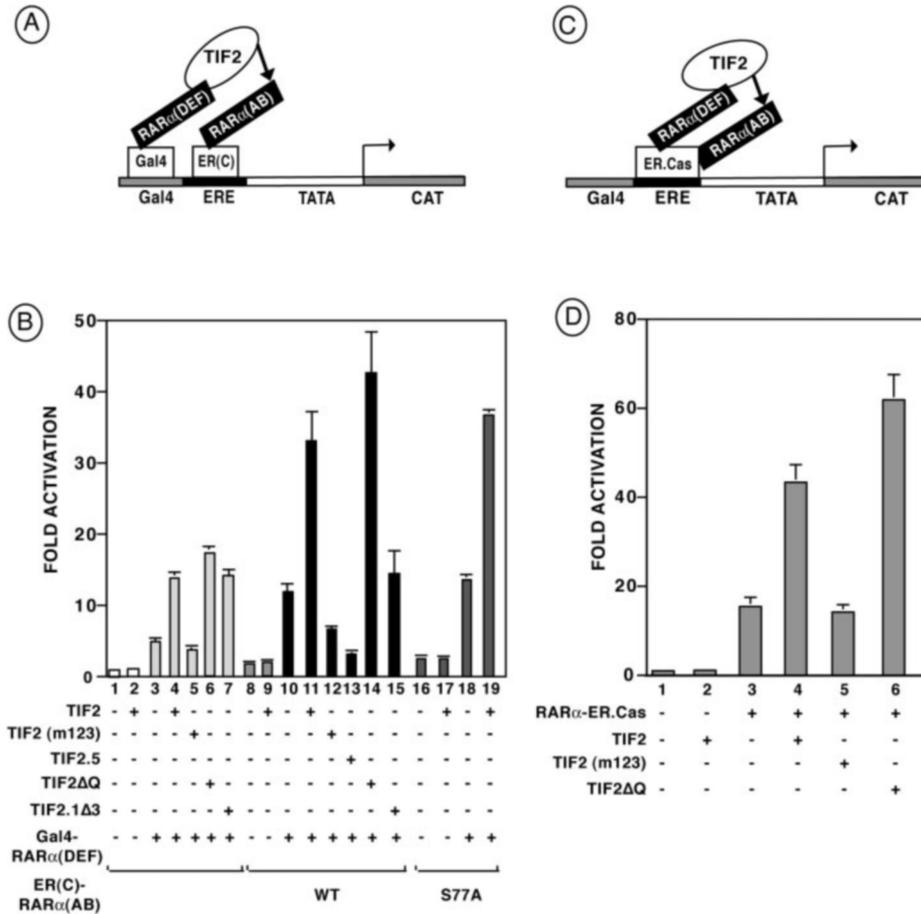


FIG. 2. TIF2 mediates synergy between the AF-1 and AF-2 domains of RAR α 1. *A*, schematic set-up of the experiments shown in *B*: COS-1 cells were cotransfected with the (17MER-ERE)-TATA-CAT reporter gene and the expression vectors for Gal4-RAR α (DEF), RAR α (AB)-ER(C), (either wild type (WT) or S77A) and the various TIF2 mutants in absence or presence of RA. Extracts were subjected to CAT ELISA. Results are expressed as fold-induction relative to the CAT activity displayed in the absence of RA and are the mean \pm S.E. of three experiments. *C*, set-up for the experiment shown in *D*: COS-1 cells were cotransfected with the reporter gene and the expression vectors for RAR α -ER.Cas and the various TIF2 mutants.

TIF2 Bridges the AF-1 and AF-2 Activation Domains of RAR α 1 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 α 1(AB) (Fig. 3B, lane 5) nor the non-liganded RAR-DEF-VP16 construct (Fig. 3B, lane 3), activate the (17mer) \times 5-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 RAR α 1 in the presence of RA (Fig. 3B, lane 7 and Fig. 3C, lane 4), indicating that endogenous factor(s) present in COS-1 cells can bridge the AF-1 and AF-2 domains of RAR α 1 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 RAR α 1 was replaced by that of RAR γ 1 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 RAR α 1 isotype. To assess the selectivity of the RAR α 1 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 RAR γ 1 isotype (Fig. 3C, lanes 14–18).

TIF2 Bridges the RAR α 1 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 (TIF2 Δ Q in Fig. 1A) was unable to bridge the ER α 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 RAR α 1 AFs (Fig. 3B, compare lanes 8 and 10), indicating that the glutamine-rich region is not required for mediating the effect of TIF2 on RAR α 1AF 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

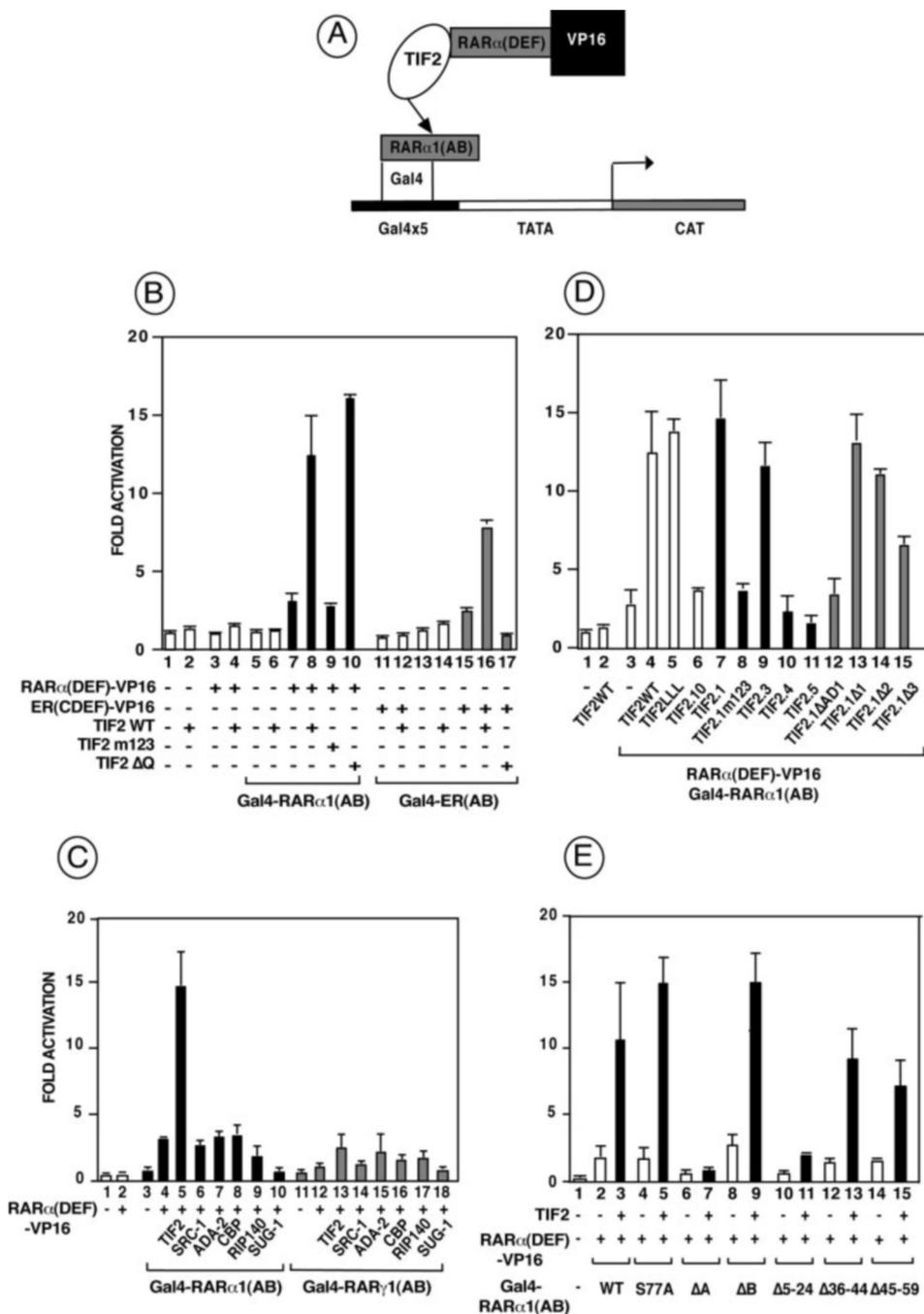


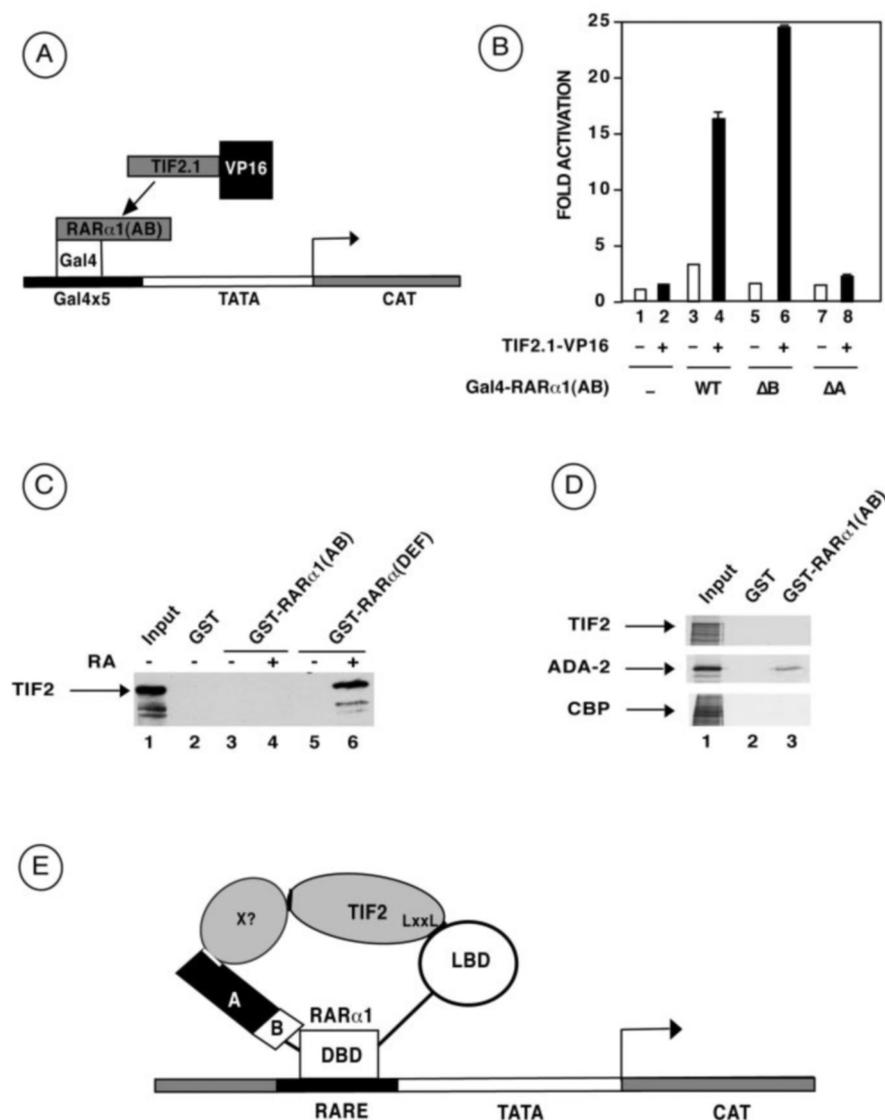
FIG. 3. TIF2 bridges the AF-1 and AF-2 domains of RAR α 1. *A*, schematic set-up of the mammalian bridged two-hybrid experiments. *B*, COS-1 cells were transfected with the (17Mer)x5-TATA-CAT reporter gene and the expression vectors for RAR α (DEF)-VP16, Gal4-RAR α 1(AB), TIF2WT, TIF2m123, or TIF2 Δ Q in the presence or absence of RA. In lanes 11–17, the same experiments were performed with the ER α (DEF)-VP16 and the Gal4-ER α (AB) expression vectors in the absence or presence of estrogen. Results are expressed as fold induction relative to the CAT activity displayed in the absence of ligand and are the mean \pm S.E. of three individual experiments. *C*, COS-1 cells were cotransfected with the reporter gene and the expression vectors for RAR α (DEF)-VP16 and Gal4-RAR α 1(AB) in the absence or presence of TIF2, SRC-1, RIP-140, p300/CBP, ADA-2, and SUG-1. Lanes 11–18 correspond to the same experiments performed with Gal4-RAR γ 1(AB). *D*, COS-1 cells were cotransfected as in *B* in the absence or presence of the various TIF2 mutants. *E*, COS-1 cells were cotransfected with the reporter gene and the expression vectors for RAR α (DEF)-VP16, TIF2, and the various Gal4-RAR α (AB) mutants.

TIF2 (amino acids 1–869) was unable to increase the cooperation between the two AF domains of RAR α 1 (Fig. 3*D*, lane 6). In contrast, a TIF2 fragment from amino acids 624–1287 (TIF2.1) that also contains the NID, has maintained this capacity (Fig. 3*D*, lane 7). As expected from the above data, TIF2.1 lacking the Q region (TIF2.3) was still effective (Fig. 3*D*, lane 9). However, removal of the subsequent 169 C-terminal amino acids (TIF2.4) made the protein inactive (Fig. 3*D*, lane 10). As these amino acids encompass the AD1 (See Fig. 1*A*), TIF2.1 deleted for this domain (TIF2.1 Δ AD1) was constructed. This

mutant was also ineffective (Fig. 3*D*, lane 12) indicating that TIF2 recruits RAR α 1 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 RAR α 1 AF-1 interaction since its mutation (in TIF2LLL) had no effect (Fig. 3*D*, 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

FIG. 4. TIF2 recruits RAR α 1 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-RAR α 1(AB), Gal4-RAR α 1(A), Gal4-RAR α 1(B), 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 \pm S.E. of three individual experiments. *C*, interaction assay of *in vitro* translated TIF2 with GST, GST-RAR α 1(AB), or GST-RAR α (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*, 35 S-labeled *in vitro* translated TIF2, ADA-2, and CBP proteins were incubated with GST or GST-RAR α 1(AB). Bound proteins were detected by autoradiography of dried gels. *E*, model for the synergy between the two AFs of the RAR α 1 isotype through TIF2. This synergy involves an adaptor protein interacting specifically with motifs located in the A region of the RAR α 1 isotype and in the AD1 domain of TIF2.



surfaces (amino acids 1011–1034 and amino acids 1031–1073 in TIF2.1 Δ 1 and TIF2.1 Δ 2, 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 TIF2.1 Δ 3) decreased significantly TIF2.1 action (Fig. 3D, lane 15).

The TIF2.1 Δ 3 mutant was also unable to increase the synergy between the two AFs of RAR α 1 when the Gal4RAR α (DEF) and RAR α 1(AB)-ER(C) hybrid proteins were cotransfected with the (17mer-ERE)-TAT-CAT reporter gene (see Fig. 2B, lane 15). Note also that in that context, TIF2 Δ Q 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 RAR α 1 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 RAR α 1 AF-1/AF-2 Cooperativity—As the AF-1 domain of RAR α 1 is constitutively phosphorylated at serine 77 (See Fig. 1C) (2) in COS-1 cells, we tested whether phosphorylation of this residue plays a role in the cooperation between the

two RAR α 1 AFs as for other nuclear receptors (20, 21, 30). Mutation of serine 77 into alanine, in Gal4-RAR α 1(ABS77A) (Fig. 1C), did not affect the synergy between both AFs of RAR α 1 (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 RAR α 1 is AF-1 phosphorylation-independent.

TIF2-mediated RAR α 1 AF-1-AF-2 Cooperativity Involves the A Region of RAR α 1—To delineate which motif(s) in the A/B region of RAR α 1 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 RAR α (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 RAR α 1 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-RAR α 1(AB), TIF2.1-VP16 activated the promoter (Fig. 4B, lane 4) confirming that it can be recruited by the AF-1 domain of

RAR α 1. Moreover, it was still efficient when coexpressed with Gal4-RAR α 1(AB) deleted for the B region but not of the A region (Fig. 4B, compare lanes 6 and 8).

According to sequence alignment studies, the A region of RAR α 1 depicts three exposed polar surfaces that are not conserved in the RAR γ 1 isotype. To delineate which part of the RAR α 1 A region is responsible for recruiting TIF2, the corresponding sequences were deleted from Gal4-RAR α 1(AB) (Fig. 1C) and tested in the bridged two-hybrid assay. The central region encompassing amino acids 25–35 was not considered, due to its highly hydrophobicity and to the prediction that its deletion would impair the whole structure of the A region, thus leading to false results. Deletion of amino acids 36–44 or 45–59 did not affect significantly the activation of the reporter gene both in the absence and presence of TIF2 (Fig. 3E, lanes 12–15). However, deletion of the N-terminal part (amino acid residues 5–24) caused complete loss of this activation (Fig. 3E, lanes 10 and 11). The lack of any significant sequence homology between this region and any region in the other RAR isotypes is in agreement with the selectivity for RAR α 1 in the bridged two-hybrid assay. It is interesting to note that this region harbors a proline-rich motif (PXPPY).

TIF2 Does Not Interact Directly with the AB Domain of RAR α 1 in Vitro—The above results suggest that TIF2 may be able to bind simultaneously the AF-1 and AF-2 domains of RAR α 1 as previously described for other nuclear receptors. However, it is also possible that TIF2 bound to liganded RAR α AF-2 recruits the AF-1 domain through an intermediary factor.

To assess whether TIF2 interacts directly with the AB domain of RAR α 1 we used *in vitro* GST-pulldown assays with recombinant TIF2 protein produced by *in vitro* translation in rabbit reticulocytes. Surprisingly, TIF2 did not interact with GST-RAR α 1(AB) (Fig. 4C, lane 4 and Fig. 4D, lane 3), while it was fully able to interact with GST-RAR α DEF in the presence of RA (Fig. 4C, lane 6). CBP did not interact either with GST-RAR α 1(AB) (Fig. 4D), while under our experimental conditions we could detect interaction with ADA-2 (Fig. 4D), which was previously described to interact also with GR (31) thus confirming that GST-RAR α 1(AB) is functional in this pulldown assay. It is important to point out that the absence of TIF2 interaction with the AF-1 domain of RAR α 1 does not reflect a partially folded state of this domain. Indeed, no interaction could be detected even when the GST-pulldown assays were performed in buffers containing the natural osmolyte trimethylamine N-oxide, which has been shown to fold the AF-1 of the glucocorticoid receptor into a more compact structure (32, 33) favoring interaction with certain coactivators (data not shown).

CONCLUSION

In the present study, we show that the synergy between the AF-1 and AF-2 domains of the RAR α 1 isotype involves the coactivator TIF2. Because no direct interaction between TIF2 and the AF-1 domain of RAR α 1 could be detected, we propose that TIF2 recruits this domain through an adaptor molecule (see Fig. 4E) as previously described for ER α (30, 34). According to our results, such an adaptor would specifically interact on the one hand with the RAR α 1 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 RAR α 1 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 experi-

ments with the A/B domain of RAR α 1 as a bait are presently under way to identify the putative RAR α 1 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 isotypes. 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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