

The coactivator TIF2 contains three nuclear receptor-binding motifs and mediates transactivation through CBP binding-dependent and -independent pathways

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The nuclear receptor (NR) coactivator TIF2 possesses a single NR interaction domain (NID) and two autonomous activation domains, AD1 and AD2. The TIF2 NID is composed of three NR-interacting modules each containing the NR box motif LxxLL. Mutation of boxes I, II and III abrogates TIF2–NR interaction and stimulation, in transfected cells, of the ligand-induced activation function-2 (AF-2) present in the ligand-binding domains (LBDs) of several NRs. The presence of an intact NR interaction module II in the NID is sufficient for both efficient interaction with NR holo-LBDs and stimulation of AF-2 activity. Modules I and III are poorly efficient on their own, but synergistically can promote interaction with NR holo-LBDs and AF-2 stimulation. TIF2 AD1 activity appears to be mediated through CBP, as AD1 could not be separated mutationally from the CBP interaction domain. In contrast, TIF2 AD2 activity apparently does not involve interaction with CBP. TIF2 exhibited the characteristics expected for a *bona fide* NR coactivator, in both mammalian and yeast cells. Moreover, in mammalian cells, a peptide encompassing the TIF2 NID inhibited the ligand-induced AF-2 activity of several NRs, indicating that NR AF-2 activity is either mediated by endogenous TIF2 or by coactivators recognizing a similar surface on NR holo-LBDs.

Keywords: activation function/GRIP1/ligand-dependent activation/NR box/transcription intermediary factors

Introduction

Nuclear receptors (NRs) represent a family of ligand-inducible transcription factors which trigger complex events during development, differentiation and homeostasis. They control gene expression upon binding of small hydrophobic ligands, such as steroid and thyroid hormones, vitamin D and retinoids. All NRs display a modular structure, with five to six distinct regions, termed A–F. The N-terminal A/B region contains the activation function AF-1, which can activate transcription constitutively. Region C encompasses the DNA-binding domain (DBD), which recognizes cognate *cis*-acting elements. Region E

contains the ligand-binding domain (LBD), a dimerization surface and the ligand-dependent transcriptional activation function AF-2 (reviewed in Gronemeyer and Laudet, 1995; Kastner *et al.*, 1995; Mangelsdorf and Evans 1995; Mangelsdorf *et al.*, 1995; Beato *et al.*, 1995; Chambon, 1996).

In transiently transfected cells, both AF-1 and AF-2 of several NRs activate transcription in a promoter- and cell-dependent manner (Tora *et al.*, 1989; Berry *et al.*, 1990; Nagpal *et al.*, 1992, 1993). These findings, together with the transcriptional interference/squelching observed between the AFs of steroid receptors (Bocquel *et al.*, 1989; Meyer *et al.*, 1989; Tasset *et al.*, 1990), led to the concept of transcriptional mediators/intermediary factors (TIFs), which mediate AF activity to the transcriptional machinery and chromatin template. Several putative coactivator TIFs for NR AF-2s have been characterized (for recent reviews, see Chambon, 1996; Horwitz *et al.*, 1996; Glass *et al.*, 1997). In particular, Le Douarin *et al.* (1996) have demonstrated that a 10 amino acid fragment of TIF1 α is necessary and sufficient to mediate interaction with retinoid X receptor (RXR) in a ligand- and AF-2 integrity-dependent manner. Notably, within this TIF1 α fragment, these authors identified a LxxLLL motif, termed the NR box, whose integrity is required for interaction with NRs, and pointed out that this motif is conserved in several other putative coactivators (Le Douarin *et al.*, 1996). TIF1 α and several other putative coactivators do not, or only very poorly, stimulate transactivation by NRs in transiently transfected mammalian cells. In contrast, the TIF2/SRC-1 family (Oñate *et al.*, 1995; Voegel *et al.*, 1996), the CBP/p300 family (Chakravarti *et al.*, 1996; Hanstein *et al.*, 1996; Kamei *et al.*, 1996; Smith *et al.*, 1996; for reviews see Eckner, 1996; Janknecht and Hunter, 1996; Shikama *et al.*, 1997) and the androgen receptor coactivator ARA70 (Yeh and Chang, 1996) have been shown unequivocally to enhance AF-2 activity.

In addition to binding NRs, CBP/p300 can also interact directly with SRC-1 (Kamei *et al.*, 1996; Yao *et al.*, 1996), and both CBP and p300 have been shown to exert histone acetyltransferase (HAT) activity (Bannister and Kouzarides, 1996; Ogryzko *et al.*, 1996). Moreover, CBP/p300 can recruit p/CAF, which is itself a nuclear HAT (Yang *et al.*, 1996). However, apart from interacting with coactivators in a ligand-dependent manner, NRs have also been shown to interact, often in a ligand-independent fashion, directly or indirectly with components of the transcriptional machinery, such as TFIIB, TBP, TAFs or TFIIF (Baniahmad *et al.*, 1993; Jacq *et al.*, 1994; Schulman *et al.*, 1995; May *et al.*, 1996; Mengus *et al.*, 1997; Rochette-Egly *et al.*, 1997).

We have reported previously that the 160 kDa human nuclear protein TIF2 exhibits all of the properties expected for a *bona fide* coactivator/TIF/mediator of NR AF-2; it

interacts directly with the LBDs of several NRs in an agonist- and AF-2-integrity-dependent manner *in vitro* and *in vivo*, harbours an autonomous activation function, relieves NR autosquelching, and enhances the activity of steroid NR AF-2s when overexpressed in transiently transfected mammalian cells (Voegel *et al.*, 1996). However, even though TIF2 interacts in an agonist- and AF-2 integrity-dependent manner with the retinoic acid and retinoidX receptors (RAR and RXR), no stimulation of RAR/RXR-induced transcriptional activation could be observed in mammalian cells overexpressing TIF2 under the experimental conditions used (Voegel *et al.*, 1996). Hong *et al.* (1996) originally described a partial cDNA of the mouse homologue of TIF2, named GRIP1, and recently reported the isolation of a full-length GRIP1 cDNA (Hong *et al.*, 1997). Their results have confirmed our previous observations and, furthermore, using the yeast *Saccharomyces cerevisiae* as model system, they have shown that transcriptional activation by the thyroid hormone receptor (TR), RAR and RXR could also be stimulated by GRIP1 co-expression, which suggests that TIF2/GRIP1 could be a general coactivator for NRs (Walfish *et al.*, 1997).

Here we show that TIF2 contains an NR interaction domain (NID) and two autonomous activation functions (AD1 and AD2). Moreover, we performed a detailed mapping of both the NID and the AD1, resulting in the identification of (i) three redundant LxxLL motifs in the NID and (ii) a CBP interaction domain (CID), which is identical with AD1. The present results are discussed in view of the integration of TIF2 function in the sequence of events leading to activation of target gene transcription by NRs.

Results

The TIF2 nuclear receptor interaction domain comprises three binding modules, each containing the NR box motif LxxLL

We have demonstrated previously that a fragment of TIF2 which encompasses residues 624–1287 (TIF2.1; Voegel *et al.*, 1996) interacts in an agonist- and AF-2-integrity-dependent manner with several NRs *in vitro* and *in vivo*, and stimulates the transcriptional activity of several NR AF-2s, most likely via the activation function which was identified in this fragment. To delineate further the TIF2 NID, we studied the interaction between a series of TIF2 deletion mutants and the oestrogen receptor (ER) or RAR α LBDs, using GST fusion protein-based *in vitro* assays. In both cases, a NID was mapped to the central region of TIF2 (amino acids 624–869 in mutant TIF2.5; see Figure 1A and B). The agonist-dependent TIF2–NR interaction was also observed on DNA-bound NRs (C.Zechel, unpublished observation). No additional NID could be identified in the N- or C-termini of TIF2 (Figure 1A and B; mutants TIF2.0, TIF2.2 and TIF2.7). In contrast, SRC-1, a paralogue of TIF2, apparently harbours two distinct non-contiguous NIDs located in the central and C-terminal regions (Oñate *et al.*, 1995; Yao *et al.*, 1996; Zhu *et al.*, 1996).

To delineate further the TIF2 NID, TIF2.5 was C-terminally truncated to Pro775, yielding TIF2.34 which also interacted with ER and RAR α LBDs in a ligand-

dependent manner (Figure 2A and B). Upon further truncation to Ser697, the resulting mutant TIF2.35 still interacted with both ER and RAR α LBDs but, surprisingly, a ligand-dependent interaction was also found with TIF2.36 (Figure 2A and B), thus indicating that the TIF2 NID is composed of at least two autonomous NR-interacting modules.

An alignment of the TIF2 NID amino acid sequence present in TIF2.34 with the corresponding region of SRC-1 (Oñate *et al.*, 1995) revealed three highly conserved regions (Figure 2A). Interestingly, all three contain the motif LxxLL (Figure 2C), originally identified in the so-called NR box of TIF1 α as the LxxLLL motif, which is also present in RIP140 (Cavallès *et al.*, 1995) and TRIP3 (Lee *et al.*, 1995) (see Le Douarin *et al.*, 1996 and Figure 2C). Importantly, 10 amino acid peptides comprising the TIF1 α or RIP140 NR boxes were sufficient for functional interaction with RXR in a ligand- and AF-2 AD-integrity-dependent manner, and mutation of the leucines at position 4 and 5 (LL \rightarrow AA) of the TIF1 α LxxLLL motif abrogated TIF1 α –RXR interaction (Le Douarin *et al.*, 1996). The functionality of the RIP140 NR box was confirmed recently (Heery *et al.*, 1997).

To investigate the functional significance of the three TIF2 NID motifs, the LL \rightarrow AA mutation was introduced in the context of both the full-length TIF2 and TIF2.1. Mutation of all three motifs (TIF2m123, TIF2.1m123; see Figure 2A; numbers following 'm' refer to the mutated motifs) abrogated both the ligand-induced binding of TIF2 or TIF2.1 to ER, RAR α and RXR α (Figure 2D, quantitation in Figure 2E, white bars) and the TIF2- or TIF2.1-dependent stimulation of ligand-induced transactivation by ER and RXR α AF-2s (Figure 2E, black bars). TIF2.1 constructs in which two NR box motifs were mutated still exhibited both ER binding and stimulation of AF-2 activity, in particular when the NR box motif II was intact, suggesting that the three NR box-containing modules are, at least in part, functionally redundant (Figure 2D and E; TIF2.1m12, m13 and m23). This redundancy was obvious when only one NR box motif was mutated; in contrast to TIF1 α , which contains only one NR box (Le Douarin *et al.*, 1996), mutation of a single TIF2 NR box did not abrogate ER binding and stimulation of ER AF-2 activity. All three mutants (TIF2.1m1–m3) bound to ER and stimulated oestradiol-dependent transactivation by ER with efficiencies similar to TIF2.1 itself (Figure 2D and E). In the case of RAR α and RXR α , the mutations had, in general, a more deleterious effect on receptor LBD binding and stimulation of AF-2 activity than in the case of ER (Figure 2D and E). This may possibly reflect a weaker interaction between TIF2 and either RAR α or RXR α than with ER. However, in spite of exhibiting in general a lower activity, the patterns of NR binding and stimulation of AF-2 activity of the NR box mutants were similar for ER, RAR α and RXR α , as mutation of motif II was always more detrimental in double mutants than mutation of motifs I and III (see Figure 2E). Importantly, for both ER and RXR α , we observed a good qualitative correlation between the effect of any of the various mutations on TIF2.1–receptor binding *in vitro* and TIF2.1-mediated stimulation of AF-2 activity (Figure 2E; note the significant reduction in both parameters for ER/TIF2.1m12, ER/TIF2.1m23 and RXR/TIF2.1m2 despite

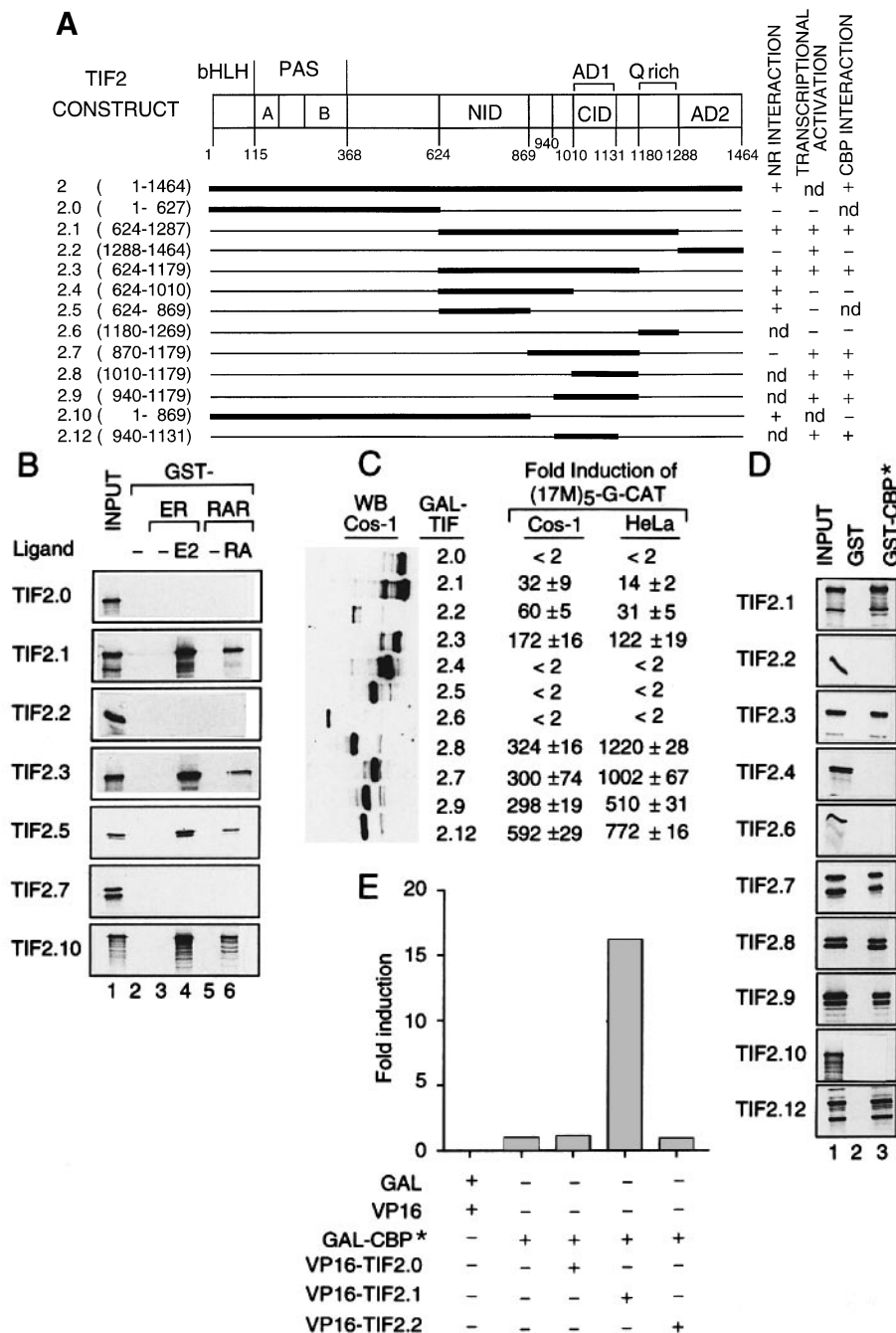


Fig. 1. Mapping of TIF2 domains. (A) Schematic representation of functional domains identified in TIF2 [NID, nuclear receptor interaction domain; CID, CBP interaction domain; AD1 and AD2, two autonomous activation functions; bHLH, sequence similarity with basic helix-loop-helix motifs; PAS A, B, sequence similarity with the Per Arndt-Sim (PAS) motifs; Q rich, a glutamine-rich sequence]. The various TIF2 constructs are denoted; expressed residues are given in parentheses. Bold lines indicate expressed sequences. Constructs that score positive or negative for NR interaction, transactivation or CBP binding are identified on the right by '+' and '-' signs respectively; nd, not determined. (B) Mapping of the NID of TIF2. GST pull-down experiments were performed with ³⁵S-labelled *in vitro* translated TIF2 polypeptides and bacterially produced GST, GST-hER α (DEF) and GST-hRAR α (DEF) in the presence or absence of 1 μ M of the cognate ligand (E2, oestradiol for ER; RA, all-*trans*-retinoic acid for RAR). (C) Analysis of the transcriptional activity of GAL-TIF2 fusion proteins. Cos-1 and HeLa cells were transfected with 3 μ g of plasmids expressing different regions of TIF2 fused to the DNA-binding domain of the yeast transcription factor GAL4 together with 1 μ g of the (17m)₅-G-CAT reporter plasmid. Fold inductions above the GAL4 DBD value are indicated. The mean and standard deviation of at least three experiments are shown. A representative Western blot, illustrating the expression levels of the GAL4-TIF2 fusion proteins, expressed from 10 μ g of the corresponding expression vectors, is shown on the left. The blot was revealed with mouse monoclonal antibodies 2GV3 and 3GV2 specific for the GAL4 DBD domain (White *et al.*, 1992). (D) Mapping of the CID of TIF2. GST pull-down experiments were performed with ³⁵S-labelled *in vitro* translated TIF2 polypeptides and bacterially produced GST and GST-CBP* (expressing CBP residues 1872-2165). (E) Two-hybrid analysis of the CBP-TIF2 interaction in mammalian cells *in vivo*. HeLa cells were transfected with 0.2 μ g of the GAL4 or GAL4-CBP* expression vectors together with 0.2 μ g of the VP16 or VP16-TIF2 expression vectors in the presence of 1 μ g of (17m)₅-TATA-CAT reporter plasmid. Fold induction relative to the activity displayed by GAL-CBP* in the absence of a VP16 fusion protein is indicated. The mean of three experiments is shown; in each case, values varied by <20%.

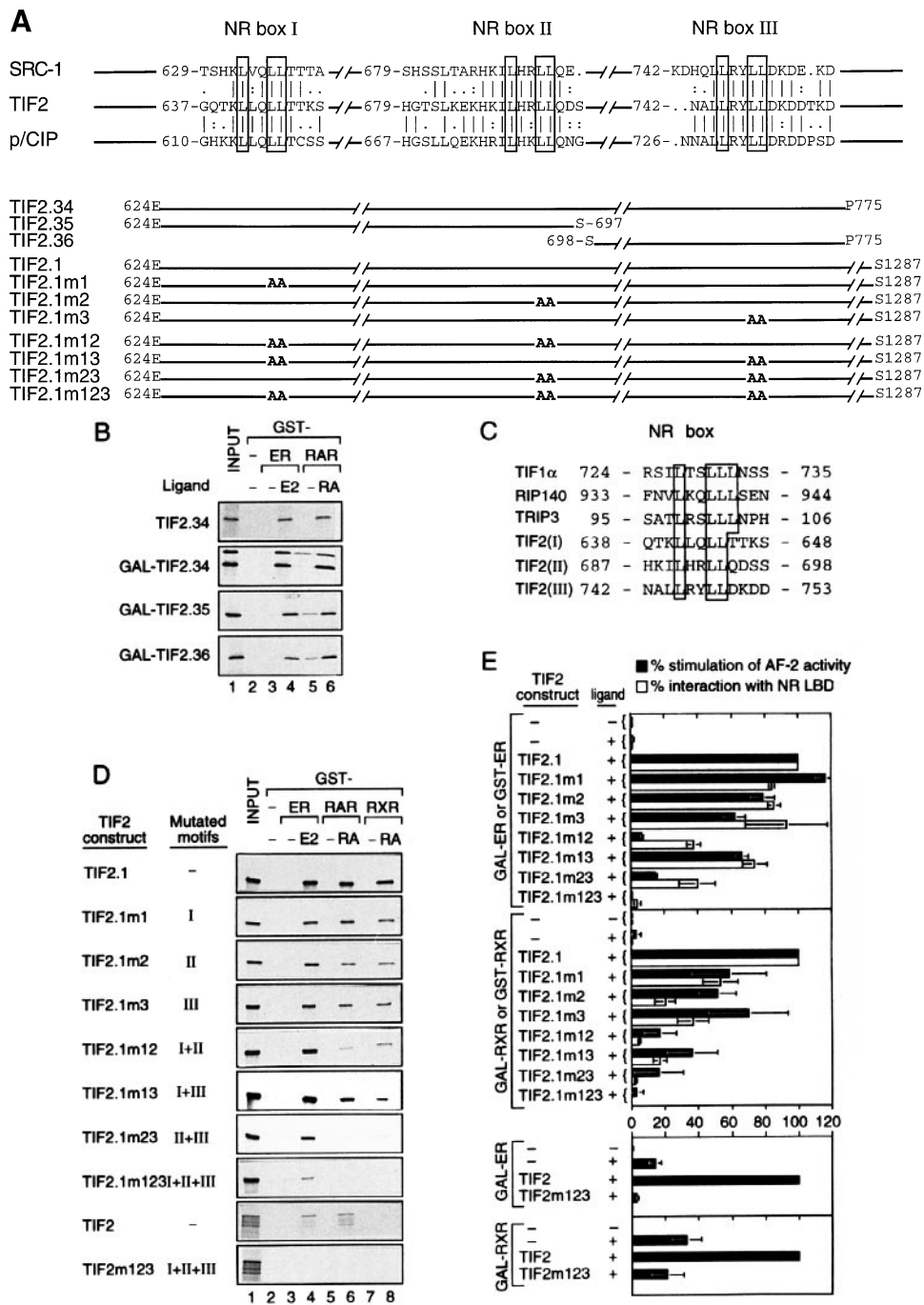


Fig. 2. Mapping of the TIF2 NID. (A) Alignment of the TIF2 NID with the corresponding regions of SRC-1 and p/CIP, and description of NID mutations. The three conserved regions are displayed with the corresponding amino acid numbers of hTIF2 (GenEMBL accession No. X97674), full-length hSRC-1 (F-SRC-1; Takeshita *et al.*, 1996; accession No. U59302) or p/CIP (Torchia *et al.*, 1997; GenEMBL accession No. AF000581); the leucines pertaining to the three NR box motifs (I, II and III) are boxed. The various deletion and leucine→alanine point mutation constructs are denoted. (B and D) Interaction of TIF2 NID mutants with NRs *in vitro*. GST affinity chromatography experiments were carried out with ³⁵S-labelled *in vitro* translated GAL4 DBD fusions of TIF2 deletion mutants (B), TIF2 or TIF2.1 point mutants (D) and bacterially expressed GST and GST fusions of the ER(DEF) (\pm 1 μ M E₂), RAR(DEF) (\pm 1 μ M all-*trans* RA), and RXR(DE) (\pm 1 μ M 9-*cis* RA). For quantification of point mutant interactions, see below. (C) Alignment of the NR boxes identified in several cofactors. The conserved leucines (cf. LeDouarin *et al.*, 1996) are boxed. For the TRIP3 sequence, see DDBJ/EMBL/GenBank accession No. L40410 and Lee *et al.*, 1995. (E) Effect of TIF2 NID point mutations in TIF2 and TIF2.1 on the stimulation of NR AF-2 activity. Cos-1 cells were co-transfected with 1 μ g of the (17m)₅-TATA-CAT reporter, 0.2 μ g of Gal-hER α (DEF) or Gal-mRXR α (DE), and 2.5 μ g of the TIF2.1 wild-type or mutated fragments or 0.25 μ g of the wild-type or mutant TIF2, as indicated. The reporter gene activation relative to the TIF2.1 (top panels) or TIF2 (bottom panels) wild-type activity and in the presence ('+') of 1 μ M estradiol or 9-*cis*-RA, respectively, is indicated for each mutant (black bars); for comparison, *in vitro* binding of the respective mutants relative to TIF2.1 wild-type binding in the presence of ligand is indicated by the white bars. Each bar represents the mean value obtained from at least three interaction or at least four transactivation experiments, respectively; standard deviations are indicated. The expression levels of TIF2 mutants in the cells were verified by Western blot (not shown) with mouse monoclonal antibody 3Ti3F1, which is directed against an epitope outside the mutated area.

some quantitative differences; in all other cases there is also a good quantitative correlation between NR binding and stimulatory activities of TIF2.1 mutants within the variations of the assays), supporting a mechanism whereby the stimulation of AF-2 activity by TIF2 involves TIF2–NR interaction through the NR holo-LBD–TIF2 NR box interface(s).

TIF2 contains two autonomous transcription activation functions

Transient transfection assays with a GAL4 reporter plasmid and chimeras containing various TIF2 fragments linked to the GAL4 DBD demonstrated the presence of two autonomous transcription activation domains in the C-terminal 460 amino acids of TIF2, termed AD1 and AD2 (delineated by mutants TIF2.8, TIF2.12 and TIF2.2 in Figure 1A and C). The N-terminal AD1 (amino acids 1010–1131), which is present in TIF2.1, showed a stronger activity than the C-terminal AD2 (amino acids 1288–1464) (compare TIF2.8 and TIF2.12 with TIF2.2 in Figure 1A and C). The weaker activity of AD2 (relative to AD1) could be due to a lower expression level of the GAL–TIF2.2 fusion protein (compare with GAL–TIF2.8 and GAL–TIF2.12 in Figure 1C). Both TIF2 activation functions were active in Cos-1 and HeLa cells (Figure 1C). However, the minimal AD1 (TIF2.8) and AD2 (TIF2.2) constructs exhibited some cell-specific activities, as GAL–TIF2.8 was more active in HeLa than in Cos cells, whereas the opposite was observed for GAL–TIF2.2 (Figure 1C). Interestingly, the glutamine-rich region of TIF2 could neither activate transcription on its own when fused to the GAL4 DBD (Figure 1A and C; mutant TIF2.6) nor was it required for transcriptional activation by AD1 or AD2. No activation function could be detected in the N-terminal part of TIF2 (see Figure 1A and C; mutant TIF2.0).

We conclude from these data that the NID and the two transcription activation functions of TIF2 correspond to distinct modular domains, since TIF2.5 can bind to NRs, but cannot activate transcription, whereas TIF2.2 and TIF2.8 cannot bind NRs but are able to activate transcription (Figure 1A).

In TIF2 the activation domain-1 is indistinguishable from the CBP interaction domain

Recently, CBP and p300, originally identified as coactivators of the transcription factor CREB, were shown to act as general integrators of multiple signalling pathways, including activation via agonist-bound RAR α and TR (for reviews and references, see Eckner, 1996; Janknecht and Hunter, 1996; Glass *et al.*, 1997; Shikama *et al.*, 1997). Furthermore, it was reported that SRC-1, which belongs to the same gene family as TIF2, interacts with CBP and p300 (Hanstein *et al.*, 1996; Kamei *et al.*, 1996; Yao *et al.*, 1996). Using GST fusion protein-based interaction and animal cell-based two-hybrid assays, we therefore analysed whether TIF2 could also interact with CBP. In the two-hybrid system, only the central TIF2.1 fragment, but not the N-terminal TIF2.0 or the C-terminal TIF2.2 fragments (Figure 1A), scored positive for interaction with GAL–CBP* (containing amino acids 1872–2165 of CBP, which encompass the SRC-1-interacting domain of CBP; Figure 1E). A GST–CBP* fusion protein was expressed in

Escherichia coli and used for pull-down assays with *in vitro*-translated TIF2 polypeptides (Figure 1D). TIF2 did interact with CBP and, interestingly, the CID apparently overlapped the AD1 activation domain of TIF2 (compare Figure 1A, C and D; mutants TIF2.8 and TIF2.12). The interaction of TIF2 with CBP was direct, as a purified *E.coli*-expressed TIF2.1 protein also interacted with GST–CBP* (data not shown). Only this region of TIF2 interacted significantly with GST–CBP*, thus suggesting that the TIF2 AD1 activity may originate from the recruitment of CBP. Deletion mutants encompassing further N-terminal regions (Figure 1A and D; mutants TIF2.10 and TIF2.4) or the C-terminal AD2 activation domain (Figure 1A and D; mutant TIF2.2) did not bind to GST–CBP*. Furthermore, TIF2.2 also did not interact with full-length CBP (data not shown), suggesting that the activity of TIF2 AD2 is mediated by (a) factor(s) distinct from CBP.

To investigate whether the CID of TIF2 could be separated from the AD1 activation domain, the ability of a series of GAL–TIF2 truncation mutants to activate a GAL4 reporter was compared with their ability to interact with the GST–CBP* protein *in vitro* (Figure 3). TIF2.13 (which encompasses Pro1011–Ser1122) exhibited potent transcriptional activity, comparable with that of larger TIF2 fragments (compare Figures 1 and 3). Removal of 26 C-terminal (TIF2.15) or 20 N-terminal (TIF2.18) amino acid residues reduced transcriptional activity only weakly (Figure 3A and B). Note that TIF2.13 also interacted with CBP *in vivo*, as shown by two-hybrid assay in transfected mammalian cells (Figure 4C).

While the internal deletion of residues Asp1061–Ala1070 (TIF2.19) had only a minor effect on the ability of TIF2.13 to transactivate, deletion of the Glu1071–Leu1080 segment (mutant TIF2.20) significantly reduced TIF2 AD1 transcriptional activity. These residues belong to a sequence predicted to fold into an α -helical structure which is highly conserved between TIF2 and SRC-1 (H1, grey bar in Figure 3A). The involvement of this region in transactivation was confirmed by the analysis of mutants TIF2.21–TIF2.31 (Figures 3A and B). All constructs containing the TIF2 wild-type sequence from Glu1071 to Ile1096 stimulated transcription, whereas even a deletion of only some of these residues significantly reduced, but did not abrogate, transcriptional activation. On its own, this α -helical H1 peptide transactivated very poorly, and had to be incorporated into additional upstream and/or downstream TIF2 sequences to generate significant transcriptional activity (Figure 3A and B; compare mutants TIF2.13, TIF2.21 and TIF2.31, and data not shown). In particular, an adjacent predicted α -helical region (H2, grey bar in Figure 3A) contributes significantly to TIF2 AD1 activity, since deletion of this region severely reduced AD1-dependent transactivation (compare TIF2.18 and TIF2.21 in Figure 3A and B). Moreover, the H2 region can apparently function even when H1 is partially deleted (mutant TIF2.20), suggesting that the AD1 surface may be composed of partially redundant structural elements. Importantly, in all cases, AD1 activity coincided with CBP interaction, since transcriptionally inactive constructs did not interact with CBP (TIF2.24, TIF2.27 and TIF2.29 in Figure 3A–C), while transcriptionally active mutants also bound CBP.

To investigate whether the leucine motif (LLxxLxxxL)

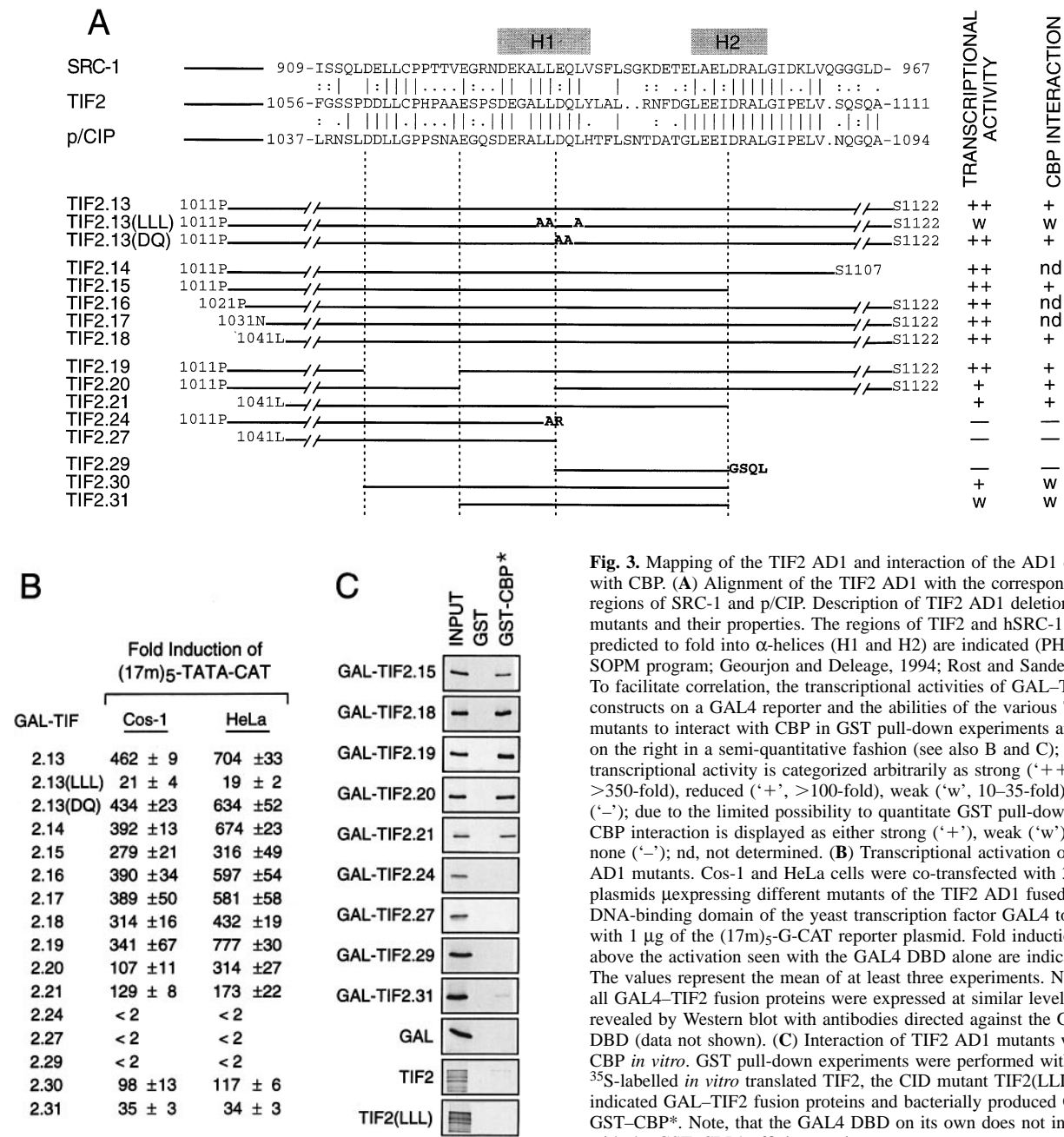


Fig. 3. Mapping of the TIF2 AD1 and interaction of the AD1 domain with CBP. (A) Alignment of the TIF2 AD1 with the corresponding regions of SRC-1 and p/CIP. Description of TIF2 AD1 deletion mutants and their properties. The regions of TIF2 and hSRC-1 predicted to fold into α -helices (H1 and H2) are indicated (PHD and SOPM program; Geourjon and Deleage, 1994; Rost and Sander, 1994). To facilitate correlation, the transcriptional activities of GAL-TIF2 constructs on a GAL4 reporter and the abilities of the various TIF2 mutants to interact with CBP in GST pull-down experiments are given on the right in a semi-quantitative fashion (see also B and C); transcriptional activity is categorized arbitrarily as strong ('++', >350-fold), reduced ('+', >100-fold), weak ('w', 10–35-fold), none ('-'); due to the limited possibility to quantitate GST pull-down data, CBP interaction is displayed as either strong ('++'), weak ('w') or none ('-'); nd, not determined. (B) Transcriptional activation of TIF2 AD1 mutants. Cos-1 and HeLa cells were co-transfected with 3 μ g of plasmids μ expressing different mutants of the TIF2 AD1 fused to the DNA-binding domain of the yeast transcription factor GAL4 together with 1 μ g of the (17m)₅-G-CAT reporter plasmid. Fold inductions above the activation seen with the GAL4 DBD alone are indicated. The values represent the mean of at least three experiments. Note that all GAL4-TIF2 fusion proteins were expressed at similar levels, as revealed by Western blot with antibodies directed against the GAL4 DBD (data not shown). (C) Interaction of TIF2 AD1 mutants with CBP *in vitro*. GST pull-down experiments were performed with ³⁵S-labelled *in vitro* translated TIF2, the CID mutant TIF2(LLI) or the indicated GAL-TIF2 fusion proteins and bacterially produced GST or GST-CBP*. Note, that the GAL4 DBD on its own does not interact with the GST-CBP* affinity matrix.

in the H1 region is required for both AD1 transcriptional activity and interaction with CBP, we introduced point mutations into full-length TIF2 and TIF2.13, converting the three conserved hydrophobic leucines to alanines [TIF2.13(LLI) in Figure 3A and TIF2(LLI)]. Interestingly, this mutation dramatically reduced AD1 activity [compare GAL-TIF2.13 and GAL-TIF2.13(LLI) in Figure 3B], while a mutation of the adjacent Asp-Gln sequence, which is also conserved [Figure 3A; TIF2.13(DQ)], had very little, if any, effect (Figure 3B). Again, AD1 activity (Figure 4A) and interaction with CBP *in vivo* (Figure 4C), as well as *in vitro* (Figure 4D), were correlated, since GAL-TIF2.13(DQ), which transactivated as efficiently as wild-type GAL-TIF2.13, interacted strongly with CBP, whereas the transcriptionally inactive GAL-TIF2.13(LLI) interacted very weakly with

CBP. Notably, TIF2(LLI) was also unable to interact efficiently with CBP (Figure 3C), supporting a critical role for these leucine residues in shaping the CID also in the context of the full-length TIF2 protein. Finally, co-expressed TIF2(LLI) partially impaired the stimulatory effect of TIF2 on the ligand-induced ER AF-2 activity of GAL-ER (Figure 4B), suggesting that the integrity of these leucines is required for at least part of the transcription stimulatory effect exerted by TIF2. That TIF2(LLI) still exhibited some stimulatory activity on ER AF-2 is not surprising in view of the existence of the second activation domain AD2 (see above). In addition, we presently also do not exclude that although mutating the three leucines abrogates TIF2-CBP interaction *in vitro*, *in vivo* this mutation may not be sufficient to inhibit fully the formation of a complex comprising, for example, holo-NR-CBP-

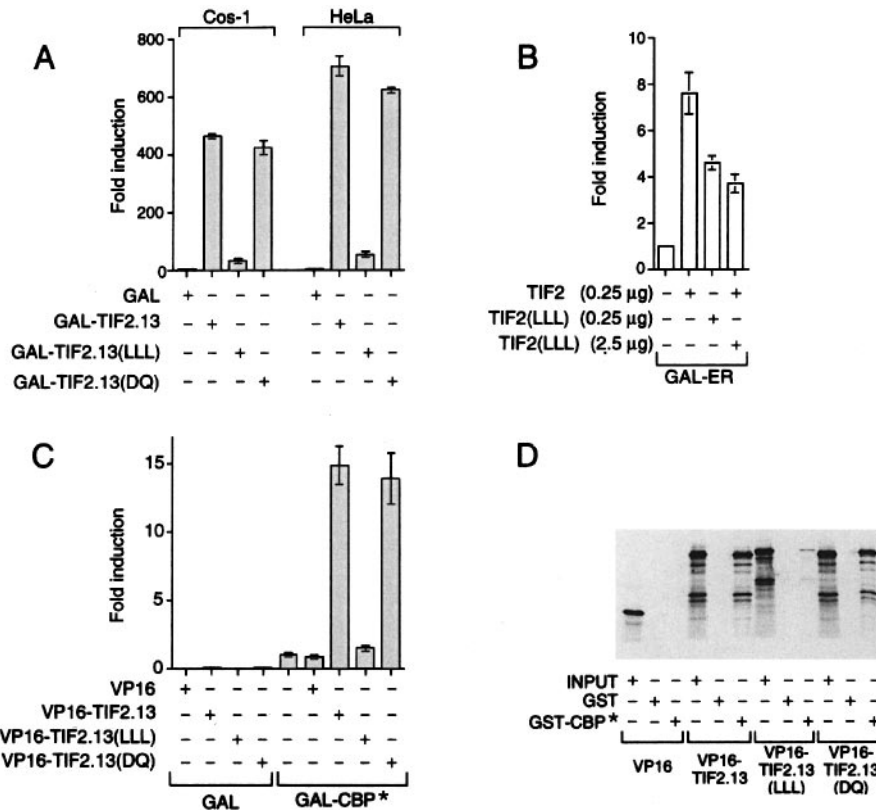


Fig. 4. Identification of TIF2 AD1 mutants which are impaired in both transcriptional activation and interaction with CBP. **(A)** Transcriptional activation by TIF2.13 and TIF2.13 mutants. Cos-1 and HeLa cells were co-transfected with 3 µg of plasmids expressing the TIF2.13 region and the indicated TIF2.13 mutants fused to the DNA-binding domain of the yeast transcription factor GAL4 together with 1 µg of the (17m)₅-G-CAT reporter plasmid. Fold inductions above the GAL4 DBD 1-fold value are indicated. The mean and standard deviation obtained from at least four experiments are shown. The expression levels of the GAL4-TIF2.13 fusion proteins were confirmed by Western blotting (data not shown). **(B)** The CID mutant TIF2(LLI) is partially deficient in stimulating ligand-induced ER AF-2 activity and its co-expression decreases the TIF2-dependent stimulation. Cos-1 cells were co-transfected with 1 µg of (17m)₅-TATA-CAT reporter, 0.2 µg of GAL-ERα(DEF) ('GAL-ER') and the indicated amounts of TIF2 constructs in the presence of 1 µM oestradiol. Mean and standard deviation of five experiments are represented as fold induction of the oestradiol-induced GAL-ER activity. Note that TIF2(LLI) contains an intact AD2 function. **(C)** Interaction of TIF2.13 wild-type and TIF2.13 mutants with CBP in mammalian cells revealed by two-hybrid analysis. HeLa cells were transfected with 0.2 µg of GAL4 or GAL-CBP* expression vectors together with 0.2 µg of VP16 or VP16-TIF2.13 expression vectors in the presence of 1 µg of (17m)₅-TATA-CAT reporter plasmid. Data are represented as fold induction of the activity seen with GAL-CBP* alone. The mean and standard deviation obtained from 10 experiments are shown. The expression levels were confirmed by Western blotting with antibodies directed against GAL4 DBD and VP16 AAD (data not shown). **(D)** Interaction of TIF2.13 wild-type and TIF2.13 mutants with CBP *in vitro*. GST pull-down experiments were performed with ³⁵S-labelled *in vitro* translated VP16-TIF2.13 polypeptides and bacterially produced GST and GST-CBP*. Note that the VP16 activation domain on its own does not interact with GST-CBP*.

TIF2, which may be stabilized by further protein-protein interactions. Together, the above results indicate that (i) CBP mediates the AD1 activity of TIF2, (ii) the H1 motif is critically involved in, but not sufficient for, efficient CBP binding and AD1 activity and (iii) the integrity of a leucine-rich motif within H1 is required for an efficient CID/AD1 function.

TIF2 expression in yeast strongly enhances the AF-2 activity of ER, RARα or RXRα, but has no effect on ER AF-1 activity

The observation that animal transcriptional activators, such as the human ER (Metzger *et al.*, 1988), are also active in the yeast *S.cerevisiae* demonstrated that the basic principles of transcriptional enhancement have been conserved from yeast to man. We therefore investigated whether TIF2 could enhance transcriptional activation by various NR constructs expressed in *S.cerevisiae*. Both NRs and TIF2.1 were expressed from multicopy plasmids in the yeast strain PL3(α), which contains a *URA3*

reporter gene under the control of three oestrogen response elements [(ERE)₃-URA3; Pierrat *et al.*, 1992].

As expected from previous studies (Metzger *et al.*, 1988, 1992; Pierrat *et al.*, 1992, 1994), the full-length ER (HEG0) induced orotidine-5'-monophosphate decarboxylase (OMPdecase) activity in a ligand-dependent manner (Figure 5, lanes 1 and 3). Interestingly, the transcriptional activity of ER was enhanced further by co-expression of the TIF2.1 fragment (Figure 5, compare lanes 3 and 4). In the absence of hormone, TIF2.1 had no significant effect on ER-induced transcriptional activation (Figure 5, compare lanes 1 and 2). Essentially the same results were observed for HEG19 which is devoid of the N-terminal region A/B, indicating that TIF2 exerts its effect on the ligand-dependent ER AF-2 (Figure 5, lanes 5-8). In contrast, neither the AF-1 activity of HE15 (which encompasses the ER regions A, B and C; Kumar and Chambon, 1988) nor the AF-2a activity of the HE179-338 construct (Pierrat *et al.*, 1994) were stimulated by co-expressing TIF2.1 (Figure 5, lanes 9-12). This is in

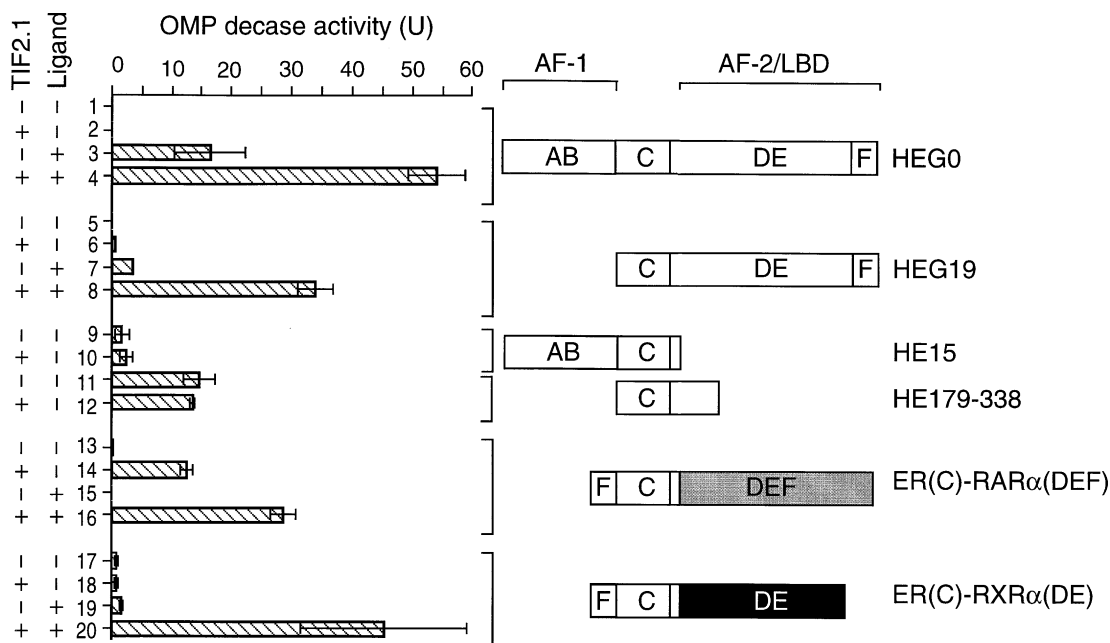


Fig. 5. The TIF2.1 coactivator fragment efficiently stimulates the ligand-dependent AF-2s of ER, RAR and RXR in yeast. No stimulatory effect of TIF2.1 on the isolated AF-1 of ER (HE15) is observable. Plasmids expressing different regions of hER α (white), hRAR α (grey) and mRXR α (black) fused to the ER DBD [hER α (C)] were introduced into the yeast reporter strain PL3(α) together with TIF2.1 as indicated. Transformants were grown in the presence or absence of 1 μ M of the cognate ligand (oestradiol for ER, all-*trans*-RA for RAR, 9-*cis*-RA acid for RXR). OMPdecase activities determined on each cell-free extract are expressed in nmol/min/mg protein; the mean and standard deviation of at least four experiments are shown. The ER(C)-RAR(DEF) and ER(C)-RXR(DE) expression vectors contain N-terminally the ER(F) epitope tag for Western blot detection; this peptide is known not to exert any transcriptional activity.

agreement with the results obtained in mammalian cells and with the observation that an intact LBD is required for TIF2.1 to interact with the ER (Voegel *et al.*, 1996).

TIF2.1 also stimulated the AF-2 activity of the liganded RXR α (DE) region in yeast (Figure 5, compare lanes 19 and 20; Heery *et al.*, 1993). This enhancement was ligand-dependent; no activation via the RXR α (DE) region was observed when the ER(C)-RXR α (DE) chimera was co-expressed with TIF2.1 in the absence of ligand (Figure 5, compare lanes 18 and 20). Again these observations parallel those made in HeLa and Cos-1 cells (see Figure 6D).

Surprisingly, even in the absence of ligand, and in contrast to the observations made with ER and RXR α , TIF2.1 very efficiently enhanced transactivation by the RAR α AF-2 (Figure 5, lanes 13 and 14). The addition of retinoic acid further increased this transcriptional activation (Figure 5, lanes 14 and 16). Note that, as previously reported (Heery *et al.*, 1993), both RAR α and RXR α AF-2 on their own poorly activated transcription from the *URA3* reporter.

The isolated TIF2 NID inhibits the AF-2 activity of several NRs in transfected cells

As previously shown, overexpression of the TIF2.1 fragment, which contains both the NID and AD1 functions, stimulates ER AF-2 activity in Cos-1 cells (Figure 6A, lanes 2 and 3; Voegel *et al.*, 1996). This stimulation was due to a direct interaction between the ER LBD and the NID of TIF2, as is apparent from the observation that overexpression of the TIF2.5 mutant (which contains the isolated NID, but lacks AD1; see Figure 1A) prevented the stimulatory effect of TIF2.1 (Figure 6A, compare lanes 3 and 4). Note that in the presence of TIF2.1, TIF2.5

overexpression decreased the transactivation by the ER AF-2 even below the level observed in the absence of TIF2.1 (Figure 6A, compare lane 4 with lane 2). This level of activity presumably originates from endogenous Cos-1 coactivators, thus suggesting that these mediators either correspond to endogenous TIF2s or interact with the ER holo-LBD through surfaces which are identical to, or in the direct vicinity of, the TIF2 NID interaction surface. Indeed, the ligand-induced ER AF-2 activity could be rescued from TIF2.5 inhibition and further enhanced (Figure 6B, lane 3) by co-expressing TIF2 (lane 5) or TIF2.1 (lane 4). Moreover, SRC-1 could also relieve the inhibition by TIF2.5 (data not shown), suggesting that TIF2 and SRC-1 may interact with a common, or adjacent, surface(s) on the ER LBD.

We previously reported an agonist-dependent interaction of TIF2 with RAR and RXR LBDs, which was dependent on the integrity of the NR AF-2 AD core, but failed to observe a stimulatory effect of TIF2 on the transcription activation of a (17m)₅-globin-promoter-CAT reporter by GAL-RAR LBD or GAL-RXR LBD fusion proteins (Voegel *et al.*, 1996). Since this failure was likely to be due to the presence of sufficient amounts of endogenous mediators for achieving maximal transactivation from this reporter gene, we modified the transfection conditions and used a reporter construct bearing a minimal promoter. A clear TIF2 and TIF2.1 stimulatory activity for RXR α AF2 was observed in HeLa and Cos-1 cells when using the (17m)₅-TATA-CAT reporter (Figure 6D, compare lanes 3–6 and 11–14). This stimulatory effect was less marked with RAR α AF-2 and could be observed reproducibly only with the TIF2.1 fragment in Cos-1 cells (Figure 6E, compare lane 10 with lanes 13 and 14; note that TIF2.1

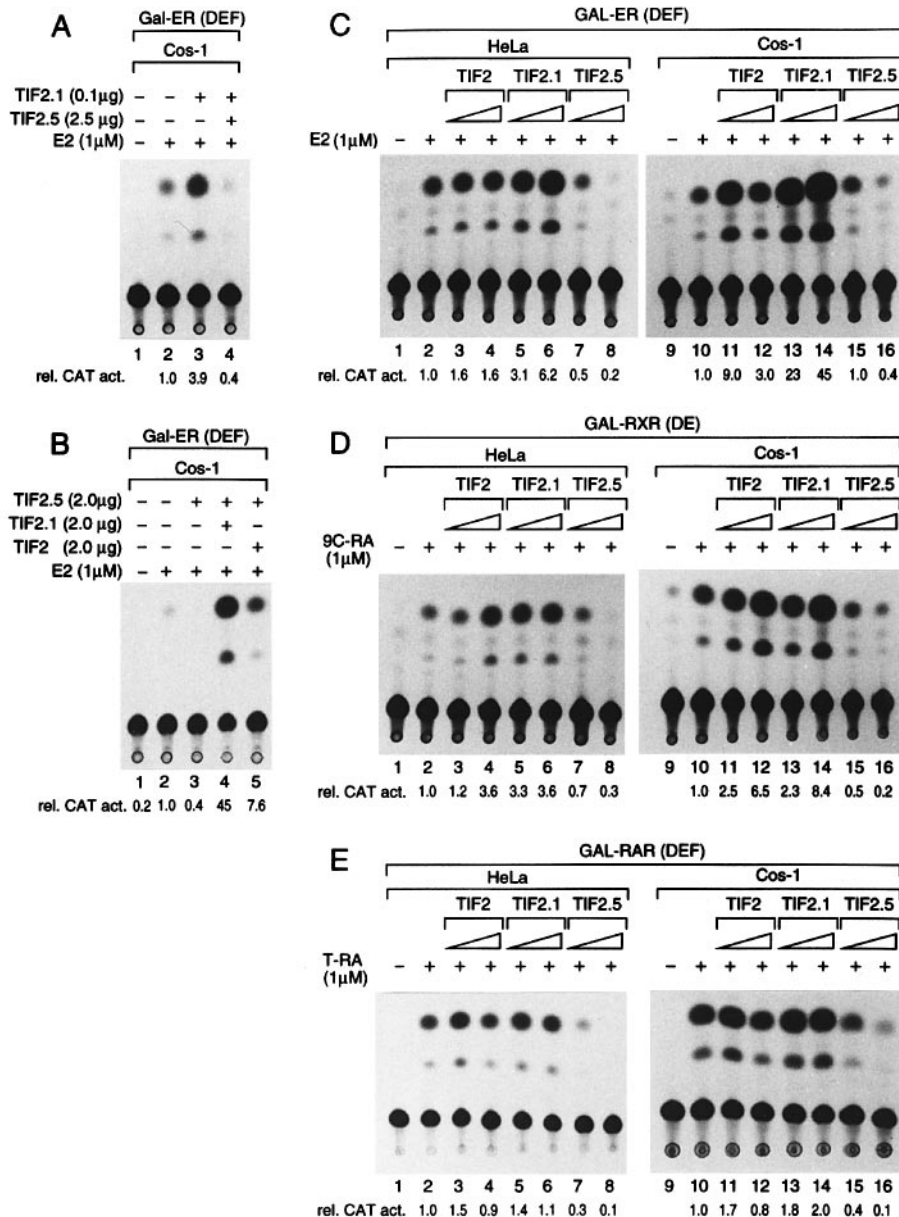


Fig. 6. Co-expression of the isolated NID of TIF2 inhibits the ligand-induced activity of the transcription activation function AF-2 in the ER, RXR and RAR LBDs, while TIF2 and TIF2.1 stimulate NR AF-2 activity. (A) Expression of the NID-containing TIF2.5 lacking the AD1 and AD2 activation functions reverses the stimulatory effect of the potent coactivator fragment TIF2.1. Cos-1 cells were co-transfected with 1 μg of the (17m)₅-TATA-CAT reporter and 0.2 μg of GAL-ERα(DEF) expression vector in the presence or absence of 1 μM oestradiol. Where indicated, 0.1 μg of TIF2.1 and 2.5 μg of TIF2.5 expression vectors were co-transfected in addition. (B) Co-expressed TIF2 or TIF2.1 rescue the ligand-induced ER AF-2 activity from TIF2.5-mediated repression. Cos-1 cells were co-transfected with 1 μg of (17m)₅-TATA-CAT reporter, 0.2 μg of GAL-ERα(DEF) and the indicated amounts of the TIF2 constructs. (C-E) Full-length TIF2 and the coactivator fragment TIF2.1 enhance, whereas TIF2.5/NID blocks, the ligand-induced AF-2 activity of the ER, RXR and RAR LBDs. Cos-1 and HeLa cells were co-transfected with 1 μg of the (17m)₅-TATA-CAT reporter and 0.2 μg of the expression vector encoding the respective GAL DBD fusion of hERα(DEF), mRXRα(DE) or mRARα(DEF) in the presence or absence of 1 μM ligand (E2, oestradiol; 9C-RA, 9-*cis*-RA; T-RA, all-*trans*-RA), together with 0.25 or 2.5 μg of TIF2, TIF2.1 and TIF2.5 expression vectors. (A-D) The mean value of induction obtained from the quantitation of at least three experiments (relative to the respective receptor LBD activity in the absence of recombinant TIF2) is indicated below each panel. Similar expression levels for TIF2.1 and TIF2.5 were verified routinely by Western blotting with mouse monoclonal antibody 3Ti3C11 directed against a region of TIF2.5 (not shown).

and TIF2.5 are expressed at a >10-fold higher level than TIF2; data not shown).

Assuming that TIF2 or coactivators recognizing the TIF2-interacting surface on NR LBDs mediate the AF-2 function of NRs, the NID-containing TIF2.5 should exert its inhibitory activity not only on ER, but also on other NRs, independently of the cellular context. We therefore

analysed the effect of TIF2.5 on the AF-2 activity of ER, RXRα and RARα in HeLa and in Cos-1 cells (Figure 6C-E). In all cases, TIF2.5 expression led to a dose-dependent inhibition of the NR AF-2 activity, indicating that the endogenous mediators were competed out by the isolated overexpressed TIF2 NID, and strongly suggesting that TIF2 or transcriptional intermediary factors recogniz-

ing the same or overlapping surfaces mediate NR AF-2 activity in these transfected cells (Figure 6C–E, compare lane 2 with lanes 7 and 8; lane 10 with lanes 15 and 16).

Discussion

TIF2 is a coactivator for the AF-2s of nuclear receptor holo-LBDs in transfected animal and yeast cells

We have reported previously that TIF2 fulfils all of the criteria that are expected for a coactivator of the ER AF-2 functions (Voegel *et al.*, 1996). We show here that TIF2 can also be a coactivator for non-steroid receptors, as the AF-2 activity of both RAR α and RXR α was activated by TIF2 overexpression in both animal and yeast cells. This enhancement of AF-2 activity, which was particularly strong in yeast cells, has also been observed recently for GRIP1, the mouse homologue of TIF2 (Hong *et al.*, 1997). These observations suggest that yeast cells contain coactivators which only poorly mimick the action of mammalian NR coactivators. As yeast cells apparently do not contain a CBP homologue, it will be interesting to investigate which yeast factor(s) mediates the activity of TIF2. Note in this respect that ER(C)–TIF2.1 and LeXA DBD–TIF2.1 are strong transactivators in yeast and that the LLL mutation in TIF2.13 impairs AD1 activity in both mammalian and yeast cells (our unpublished results), suggesting a similar recognition of the TIF2 AD1 surface by presently unknown yeast factor(s).

Interestingly, the expression of TIF2 in yeast led to a marked stimulation of transactivation by the unliganded ER(C)–RAR α (DEF), which was not observed with ER or RXR α unliganded LBDs. Structural studies have revealed that binding of the ligand results in a conformational change of the LBD, which generates the surface(s) for coactivator binding (Renaud *et al.*, 1995). Our present result, therefore, suggests that a high level of coactivators might, in the absence of ligand, drive the LBD of some receptors into a holo-LBD-like conformation, thus giving rise to ligand-independent transcriptional activity. By analogy, one could speculate that high levels of co-repressors could ‘lock’ NR LBDs in the apo-LBD conformation. It would therefore be interesting to investigate whether high levels of co-regulators might lead to constitutive activity (even in the presence of antagonists) or, conversely, to lack of inducibility of NRs in some pathological states.

The NID between NRs and TIF2 comprises three partially redundant modules each containing the NR box motif LxxLL

Our present structure–function analysis reveals that TIF2 contains a single NID. In contrast, the other TIF2 family member, SRC-1, was reported to contain two NIDs (Oñate *et al.*, 1995; Yao *et al.*, 1996; Zhu *et al.*, 1996). However, only one of the two SRC-1 NIDs is most probably homologous to the TIF2 NID characterized here (see Figure 2A). The TIF2 NID is composed of three modules, and we have shown that the C-terminal and the two N-terminal modules can bind in a ligand-dependent manner to the NRs tested in this study, suggesting that each module can mediate NR binding independently. Interestingly, these modules contain the NR box motif LxxLL originally recognized as the motif LxxLLL (Figure 2C) within a 10

amino acid NR-binding peptide of TIF1 α that was critical for TIF1 α –NR interaction and conserved in RIP140 and TRIP3 (Le Douarin *et al.*, 1996 and references therein; see also Results). Moreover, the TIF1 α and RIP140 modules were shown to interact functionally with NRs (Le Douarin *et al.*, 1996). Confirming our original observations, the implication of NR box motifs in NR–coactivator binding and their presence in a number of different coactivators was pointed out in two subsequent reports (Heery *et al.*, 1997; Torchia *et al.*, 1997). Note that all three TIF2 NR box motifs described here are conserved in the recently discovered TIF2 paralogue p/CIP (Torchia *et al.*, 1997).

In contrast to TIF1 α , for which mutation of leucines to alanine at positions 4 and 5 of its single NR box motif (LL \rightarrow AA) abrogates NR binding, mutation of all three motifs is required in the TIF2 NID to abrogate NR binding, indicating that each of these motifs can contribute to a TIF2 surface that interacts with a cognate surface of NR holo-LBDs. That the NR boxes of TIF2 exhibit functional redundancy is supported by the observation that the LL \rightarrow AA mutation in any of the three TIF2 NID motifs apparently did not (in the case of the ER) or only weakly (in the case of RAR α and RXR α) reduce the efficiency of NR interaction. Moreover, in the TIF2.1 environment, any single intact NR box motif on its own (i.e. when the two other motifs were mutated) was sufficient for interaction with the holo-ER LBD, although only motif II on its own could bring about a nearly wild-type NR binding efficiency. In contrast, for RAR α and RXR α interaction, mutants with single intact NR boxes were five (box II) to 20 (box I or III) times less efficient than the wild-type TIF2 containing the three NR boxes.

Crystallographic studies will be necessary to distinguish between two possible models, in which the three NR box motifs (i) contribute to the formation of a tripartite NID surface that specifically recognizes a cognate holo-NR LBD surface, or (ii) belong to independent surfaces which each can interact, albeit with different efficiencies, with the same holo-NR surface. The second model could allow TIF2 to interact cooperatively with both partners of NR homo- or heterodimers, thus rendering transactivation by NRs sensitive to small variations in TIF2 levels. Furthermore, for both ER and RXR α , the effects of NR box mutations on NR binding and stimulation of AF-2 activity were correlated, thus supporting the conclusion that the transcriptional effect of TIF2 involves the formation of an NR box–NR LBD interaction interface.

The motif LxxLL has been found in a number of other NR coactivators (see above), thus suggesting some similarity in the mode of NR–coactivator interactions. However, this does not exclude NR-specific modulation of these interactions, as the NR box-surrounding sequences are highly variable.

TIF2 contains both a CBP-mediated and a CBP-independent activation function

The two TIF2 activation functions (AD1 and AD2) apparently operate through different transcriptional activation cascades. While the TIF2 AD1 activation domain could not be separated by mutational analysis from the TIF2 domain which interacts *in vitro* and *in vivo* with a region of the CBP surface which also mediates SRC-1 binding

(Kamei *et al.*, 1996), neither this region nor full-length CBP interacted with the TIF2 AD2. We currently are attempting to define the mechanism mediating TIF2 AD2 activity. That the two TIF2 activation functions may operate through distinct pathways is also suggested by the differential cell specificity of the minimal fragments exhibiting AD1 (e.g. TIF2.8, TIF2.7, TIF2.9 and TIF2.12) and AD2 activity (TIF2.2). While TIF2.2 is more active in Cos-1 than in HeLa cells, all of the minimal fragments containing AD1 are more active in HeLa cells. It is tempting to speculate that the coactivator activity of TIF2 may be modulated cell-specifically by the differential efficiency of its two ADs.

The core of AD1 (TIF2.31) on its own is a very poor transactivator and binds CBP only weakly, thus requiring additional surrounding sequences to generate a fully active (i.e. efficient CBP-binding) surface. However, mutational analysis of the AD1 core in the context of a strong activator fragment (TIF2.13) reveals the critical importance of transactivation and CBP binding *in vivo* and *in vitro* of three leucine residues (Figure 4). These leucines belong to a fully conserved LLxxLxxxL motif, embedded in a highly conserved region of all three members of the TIF2 coactivator family (Figure 3A; Torchia *et al.*, 1997), which is distinct from the LxxLL NR box motif.

Role of TIF2 in nuclear transactivation by nuclear receptor AF-2

The overall picture emerging from recent studies on the mechanisms by which nuclear receptors modulate target gene transcription involves three subsequent steps, (i) the ligand-induced transconformation of the NR LBD, which results in (ii) the dissociation of co-repressors and formation of NR-coactivator complexes, which themselves or (iii) through interaction with additional downstream factors (e.g. CBP, p300, p/CAF) modulate the acetylation status of core histones and, thus, chromatin condensation/decondensation (see Introduction). Histone acetylation on its own is, however, insufficient for transcription activation (Wong *et al.*, 1997), and a simultaneous or subsequent fourth event comprises the direct and/or indirect recruitment of elements of the transcription machinery (e.g. TFIIB, TBP, TAFs, TFIIF; Baniahmad *et al.*, 1993; Jacq *et al.*, 1994; Schulman *et al.*, 1995; May *et al.*, 1996; Mengus *et al.*, 1997; Rochette-Egly *et al.*, 1997). Note that such interactions do not need to be ligand-dependent, if the primary function of the liganded LBD (AF-2) is to regulate DNA accessibility through chromatin remodeling. Indeed, most of the reported interactions between NRs and general transcription factors occur in a ligand-independent manner.

Within this sequence of events, TIF2 can apparently fulfil at least two mediator functions: (i) as a 'bridging factor' between the AF-2 function of NRs and CBP via its AD1 activation domain and (ii) as a transcriptional mediator through as yet unknown CBP binding-independent mechanisms via its AD2 function. We have not been able to detect HAT activity of bacterially expressed TIF2 fragments under conditions where bacterially expressed and purified yeast GCN5 was highly active (our unpublished results).

Finally, it is important to stress that our present data demonstrate that TIF2 interacts with NRs through a surface

(NID) that is critical for NR AF-2 activity, as the ligand-induced transcription of several NR AF-2s was blocked by the isolated NID (TIF2.5) and could be rescued by co-expressing TIF2, TIF2.1 or SRC-1. This observation clearly establishes that, at least in transfected cells, TIF2, SRC-1 or other coactivators which interact with an overlapping, if not identical, holo-LBD surface, are essential in mediating the NR AF-2 activation function. This is in keeping with the presence of three NR box motifs in the TIF2 NID, and of at least one conserved LxxLL NR box motif in all bona fide coactivators described to date. It will be challenging to develop compounds that block the NR box-NR interaction as an alternative to NR antagonists for the use in endocrine therapies; in view of the divergent environment, different number and functional redundancy of coactivator NR boxes, it may be possible to identify compounds that interfere in a receptor-selective manner with coactivator binding. Similarly, blocking the interaction between coactivators and CBP (e.g. by expressing the TIF2 CID, cognate synthetic peptides or functionally equivalent compounds) may allow inhibition of CBP-mediated, as well as the revealing of CBP-independent, pathways in NR signalling.

Materials and methods

Plasmids

All recombinant DNA work was performed according to standard procedures (Ausubel *et al.*, 1993). GST fusions and GAL4 DBD fusions of NRs have been expressed from the following previously described plasmids: GAL (GAL4 DBD, pG4MpolyII, amino acids 1-147; Tora *et al.*, 1989), GAL-ER(DEF) [GAL4DBD-hER α (DEF), GAL-ER(147/282), amino acids 282-595; Webster *et al.*, 1989], GAL-RXR(DE) [GAL4DBD-mRXR α (DE), amino acids 206-466; Allenby *et al.*, 1993], GAL-RAR(DEF) [GAL4DBD-mRAR α (DEF)-hER α (F), amino acids 154-462 of RAR plus 553-595 of ER; Allenby *et al.*, 1993], GST (pGEX2T; Pharmacia), GST-ER [pGEX2T-hER α (DEF), also called pGEX2T-HE14G, amino acids 282-595], GST-RXR [pGEX2T-mRXR α (DE), amino acids 205-467] and GST-RAR [pGEX2T-hRAR α (DEF), amino acids 153-462] (all LeDouarin *et al.*, 1995a). The reporter plasmids (17m)₅-TATA-CAT (May *et al.*, 1996) and (17m)₅-G-CAT [(17m)₅- β -globin-CAT; Durand *et al.*, 1994] each contain five copies of the GAL4 response element in front of a simple TATA motif or of the β -globin promoter, respectively, upstream from the CAT reporter gene.

In yeast, the hER α constructs were expressed from the following YEp90-based plasmids: HEG0 (hER α , YEp90-HEG0, amino acids 1-595), HE15 (YEp90-HE15, amino acids 1-282), HEG19 (YEp90-HEG19, amino acids 179-595) (all Pierrat *et al.*, 1992) and HE179-338 (YEp90-HE179-338; Pierrat *et al.*, 1994). From the yeast multicopy plasmid pBL1 (LeDouarin *et al.*, 1995b), which codes for ER(F)-epitope-tagged ER(C) fusions, the following plasmids were expressed: ER(C)-RAR(DEF) [pBL1-hRAR α (DEF), amino acids 154-462] and ER(C)-RXR(DE) [pBL1-mRXR α (DE), amino acids 205-467] (both vom Baur *et al.*, 1996).

TIF2 was expressed in yeast from the multicopy plasmid pAS3 (gift from B.LeDouarin), which is a derivative of YEp90 containing the *LEU2* marker. TIF2 constructs for transient transfection and *in vitro* translation were obtained by PCR amplification of the indicated regions of TIF2 followed by subcloning in pSG5 (Green *et al.*, 1988). The GAL4(1-147) and VP16 chimeras were constructed by PCR amplification of the indicated regions of TIF2 followed by subcloning in pG4MpolyII (Tora *et al.*, 1989) and NVPI6 (Nagpal *et al.*, 1993), respectively. For *in vitro* binding assays, the indicated cDNAs were fused to GST in the pGEX-2TK plasmid (Pharmacia). GAL-CBP* and GST-CBP* were obtained by subcloning a PCR-amplified fragment coding for amino acids 1872-2165 of mCBP in pG4MpolyII or pGEX-2TK, respectively. Details concerning the plasmid constructions, all of which were verified by sequencing, are available on request.

GST pull-down assays with [³⁵S]methionine-labelled proteins

DNA was transcribed and translated *in vitro* using the TNT T7-coupled reticulocyte lysate system (Promega) following the instructions of the manufacturer. The reticulocyte lysate containing the ³⁵S-labelled protein (2 µl) was then incubated as described (vom Baur *et al.*, 1996) with GST, GST-CBP* or GST-hERα(DEF) and GST-hRARα(DEF), in the presence or absence of 1 µM E2 and all-*trans*-retinoic acid (T-RA), respectively. Bound proteins were recovered in SDS sample buffer and revealed by fluorography (Amplify, Amersham) of SDS-polyacrylamide gels.

Transactivation assays

Yeast PL3(α) (Pierrat *et al.*, 1992) transformants were grown exponentially in the presence or absence of ligand for about five generations in selective medium containing uracil. Yeast extracts were prepared and assayed for OMPdecase activity as described previously (Pierrat *et al.*, 1992). Transient transfections of HeLa and Cos-1 cells and CAT assays were performed as described (Bocquel *et al.*, 1989). Quantitative data on CAT reporter expression were obtained either by phosphorimager analysis (BAS2000, Fuji) of ¹⁴C-labelled CAT reaction products separated by thin-layer chromatography, or using the CAT ELISA kit (Boehringer Mannheim). In all cases, CAT activities were normalized to the β-galactosidase concentrations resulting from co-transfection of 1 µg of pCMVβGal (gift from T.Lerouge) as internal control.

Western blotting and antibodies

Expression levels of recombinant proteins in transfected cells were determined by standard SDS-PAGE and subsequent semi-dry transfer (MilliBlot, Millipore) to nitrocellulose membranes. Proteins were revealed by chemiluminescent Western blot (SuperSignal, Pierce). The following mouse monoclonal antibodies were used: 3Ti3F1 directed against a TIF2 epitope within residues 940–1010, 3Ti3C11 against an epitope between residues 624 and 869, 2GV3 and 3GV2 (White *et al.*, 1992) against the GAL4 DBD, and 2GV4B7 (gift from Y.Lutz) against the VP16 activation domain.

Acknowledgements

We thank A.Pornon and Cathie Erb for help in transfection, OMPdecase assays and GST pull-down experiments, and J.M.Garnier and his team for construction of the TIF2 NR box mutants. We thank D.Bonnier, I.Cheynel, G.Eisenmann and F.Granger for technical help; Y.Lutz for antibody production; I.Davidson and his collaborators for the (17m)5-TATA-CAT reporter; B.Le Douarin, E.vom Baur and R.Losson for yeast strains and plasmids; T.Lerouge for the pCMVβGal plasmid; M.-J.Tsai for SRC-1 and R.Goodman, P.Angel and C.K.Glass for CBP vectors; and the DNA, peptide synthesis and sequencing, cell culture, photography and artwork services for help. J.J.V., M.J.S.H. and M.T. are recipients of C.I.E.S., Boehringer-Ingelheim and H.F.S.P. fellowships, respectively. This work was supported by funds from the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, the Centre Hospitalier Universitaire Régional, the Association pour la Recherche sur le Cancer, the Fondation pour la Recherche Médicale, the Ministère de la Recherche et de la Technologie, the Collège de France, the International Human Frontier Science and the EC BIOMED (contract BMH4-96-0181 to H.G.) Programmes, and Bristol-Myers-Squibb.

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Received June 26, 1997; revised October 28, 1997;
accepted October 29, 1997

Note added in proof

In parallel with the present work, Kalkhoven *et al.* [(1998) *EMBO J.*, **17**, 232–243] identified a similar functional domain structure in the TIF2 orthologue SRC-1. The human homologue of mouse p/CIP, named AIB1/RAC3/ACTR, has recently been identified by three groups independently [Anzick *et al.* (1997), *Science*, **277**, 965–968; Li *et al.* (1997), *Proc. Natl Acad. Sci. USA*, **94**, 8479–8484; Chen *et al.* (1997), *Cell*, **90**, 569–580].