

The yeast Ada complex mediates the ligand-dependent activation function AF-2 of retinoid X and estrogen receptors

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Nuclear receptors can function as ligand-inducible transregulators in both mammalian and yeast cells, indicating that important features of control of transcription have been conserved throughout evolution. Here, we report the isolation and characterization of a yeast protein that exhibits properties expected for a coactivator/mediator of the ligand-dependent activation function AF-2 present in the ligand-binding domain (LBD, region E) of the retinoid X (RXR α) and estrogen (ER α) receptors. This protein is identical to Ada3, a component of the yeast Ada coactivator complex. We demonstrate that: (1) the region encompassing residues 347–702 of Ada3 interacts with the LBD of RXR α and ER α in a ligand-dependent manner in yeast; (2) this interaction corresponds to a direct binding and requires the integrity of the core of the AF-2 activating domain (AF-2 AD) of both RXR α and ER α ; (3) Ada3 as well as Ada2 and Gcn5, two other components of the Ada complex, are required for maximal AF-2 activity in yeast; and (4) Ada3 is able to enhance the AF-2 activity of RXR α and ER α when overexpressed in yeast and mammalian cells. Taken together, these data indicate that ligand-dependent transactivation by RXR α and ER α in yeast is mediated at least in part by the Ada complex, in which the Ada3 subunit directly binds to the holoreceptor LBD.

[Key Words: Transcriptional control; Ada2/Ada3/Gcn5; *Saccharomyces cerevisiae*; nuclear receptors; RXR α /ER α ; transcriptional intermediary factor; coactivator]

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Nuclear receptors (NRs) represent a large family of ligand-inducible transcriptional regulators that control complex developmental and homeostatic events in vertebrates by binding as homodimers or heterodimers to cognate DNA response elements present in target genes. NRs display a modular structure, with five to six distinct regions (denoted A–E/F; see Fig. 1A). The amino-terminal A/B region contains an autonomous activation function (AF-1), the highly conserved region C belongs to the DNA-binding domain (DBD), and the carboxy-terminal E region contains the ligand-binding domain (LBD), a dimerization surface and a ligand-dependent transcriptional activation function, AF-2 (for reviews and refs, see Mangelsdorf et al. 1995; Chambon 1996; Perlman and Evans 1997). The core of the AF-2 activating domain (AF-2 AD core; see Fig. 1A) has been characterized in the carboxy-terminal part of the E region and corresponds to the conserved amphipathic α helix 12 of the LBD. Upon

ligand binding, this helix is thought to fold back over the LBD to generate interacting surface(s) for the binding of transcriptional intermediary factors (TIFs, also denoted coactivators or mediators), whose effects ultimately result in the remodeling of the structure of the chromatin template and/or in the stimulation of initiation of RNA synthesis by the general transcription machinery (for reviews and references, see Chambon 1996; Wurtz et al. 1996; Glass et al. 1997).

Although the yeast *Saccharomyces cerevisiae* does not possess endogenous NRs, it has been shown that a number of NRs, including the estrogen receptor (ER, now designated ER α), the glucocorticoid receptor (GR), the retinoic acid (RA) receptors (RARs and RXRs), and the thyroid hormone receptor (TRs) can function as ligand-dependent transactivators in yeast (Metzger et al. 1988, 1992; Schena and Yamamoto 1988; Hall et al. 1993; Heery et al. 1993; and references therein). As in vertebrates (Chambon 1996 and references therein), the AF-1 and AF-2 of ER α , RAR α , and RXR α can activate transcription independently and synergistically in yeast (Metzger et al. 1988; White et al. 1988; Pierrat et al. 1992; Heery et al. 1993; and our unpublished results). Similarly, the transactivation potentials of ER α AF-1 and AF-2 are

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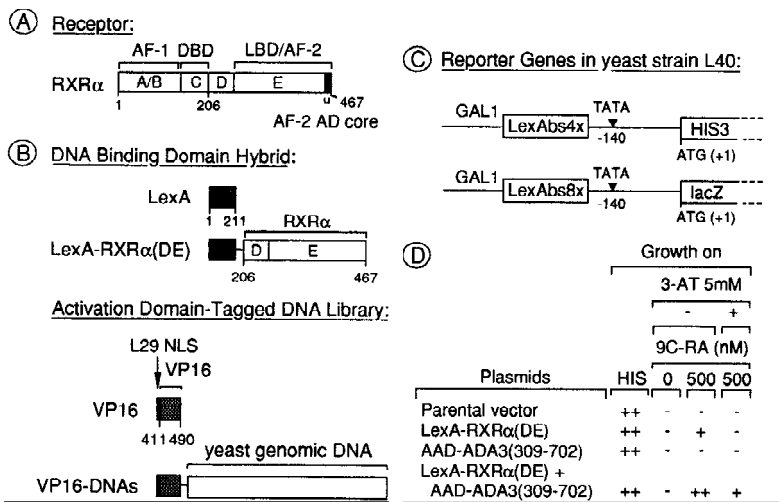


Figure 1. Identification of Ada3 by a two-hybrid screening for yeast proteins that interact with RXR α . (A) Schematic representation of mouse RXR α . Indicated are the various regions of the receptor (denoted A-E) that are conserved among members of the NR family. Transactivation domains (AF-1 and AF-2), DBD, and LBD are indicated. (Filled bar) Core motif of the AF-2 AD. (Numbers) Amino-acid positions. (B) Schematic representation of the LexA protein unfused or fused to the DE region of RXR α . The VP16 AAD-tagged *S. cerevisiae* genomic DNA library is represented below. The AAD tag also includes codons specifying the nuclear localization signal (NLS) of the yeast ribosomal protein L29. (C) Transcription of the integrated *HIS3* and *lacZ* reporter genes in the reporter strain L40 is driven by a chimeric *GAL1* promoter containing four and eight LexA-binding sites (LexAbs), respectively. (D) Growth complementation by interaction of RXR α (DE) with Ada3(309-

702). Yeast L40 cells expressing the indicated fusion proteins were plated on medium containing histidine (His) and on His-negative medium +/- 9C-RA and +/- 3-AT as indicated. Plates were incubated at 30°C for 2 days. (++) Wild-type growth; (+) weak growth; (-) no growth.

promoter-context-dependent in both animal and yeast cells (Tora et al. 1989; Berry et al. 1990; Metzger et al. 1992). Thus, some important features of the mechanism(s) by which these AFs stimulate transcription appear to have been conserved during evolution.

Several yeast proteins implicated in control of transcription have been shown to functionally interact with NRs. These factors include the Swi/Snf protein complex (Yoshinaga et al. 1992), Ssn6 (McDonnell et al. 1992), Sin3 (Nawaz et al. 1994), Spt6 (Baniahmad et al. 1995), Rsp5 and Spt3 (Imhof and McDonnell 1996). Mammalian homologs have been identified for Swi/Snf genes (Muchardt and Yaniv 1993; Chiba et al. 1994; Muchardt et al. 1995), Sin3 (Ayer et al. 1995), Spt6 (Segre et al. 1995), and Rsp5 (Imhof and McDonnell 1996). However, a physical interaction between yeast factors and NRs was observed only in the case of the Swi/Snf complex and Spt6, which have been reported to interact with the DBD of GR and the LBD of ER α , respectively (Yoshinaga et al. 1992; Baniahmad et al. 1995).

From the results of a two-hybrid screen for yeast proteins that can interact with the LBD/AF-2 of RXR α in a 9-*cis*-RA (9C-RA)-dependent manner, we report the identification of such a protein and show that it is involved in the mediation of transactivation by the AF-2 of RXR α and ER α in yeast. This protein is identical to the Ada3 subunit of the Ada coactivator complex that is important for transcriptional activation by specific yeast activators (Piña et al. 1993; Horiuchi et al. 1995; Grant et al. 1997 and references therein). We show that two other subunits of the Ada complex, Ada2 and Gcn5, are required in addition to Ada3 for full activation by the AF-2 of RXR α and ER α in yeast. Interestingly, human homologs of the Ada2 and Gcn5 proteins have recently been isolated, suggesting an evolutionary conservation of this complex (Candau et al. 1996). Thus, the Ada complex may play an important role in mediating the ligand-de-

pendent activation function AF-2 of certain NRs in yeast and higher eukaryotes.

Results

Yeast Ada3 interacts with nuclear receptors

The two-hybrid system (Fields and Sternglanz 1994) was used to identify yeast proteins that interact with the LBD/AF-2-containing region DE of RXR α in the presence of 9C-RA. The bacterial LexA DBD, fused amino-terminally to the DE region of RXR α [LexA-RXR α (DE); Fig. 1B] was expressed in the L40 yeast strain, which contains a *HIS3* reporter gene under the control of four LexA-binding sites (Fig. 1C; Vojtek et al. 1993). Addition of 500 nM 9C-RA allowed growth as a result of transactivation of the *HIS3* gene by LexA-RXR α (DE) on its own, whereas no growth was observed in the absence of ligand on plates lacking histidine (Fig. 1D). Addition of 5 mM 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of the *HIS3* gene product, suppressed growth in the presence of ligand (Fig. 1D).

A library of yeast genomic DNA fragments was constructed in pASV3, a yeast multicopy expression vector that directs the synthesis of polypeptides fused to the acidic activation domain (AAD) of the VP16 protein (Fig. 1B; Le Douarin et al. 1995b). L40 yeast cells expressing LexA-RXR α (DE) were transformed with this library. Approximately 4×10^6 yeast transformants (covering the library four times) were spread directly on histidine-negative plates containing 500 nM 9C-RA and 5 mM 3-AT. Colonies (232) were isolated and retested for activation of a second reporter gene, the *Escherichia coli lacZ* gene driven by a *GAL1* promoter containing eight LexA-binding sites (Fig. 1C). Plasmids were recovered from 110 clones exhibiting strong *lacZ* expression, amplified, and subjected to restriction analysis. DNA inserts from 15

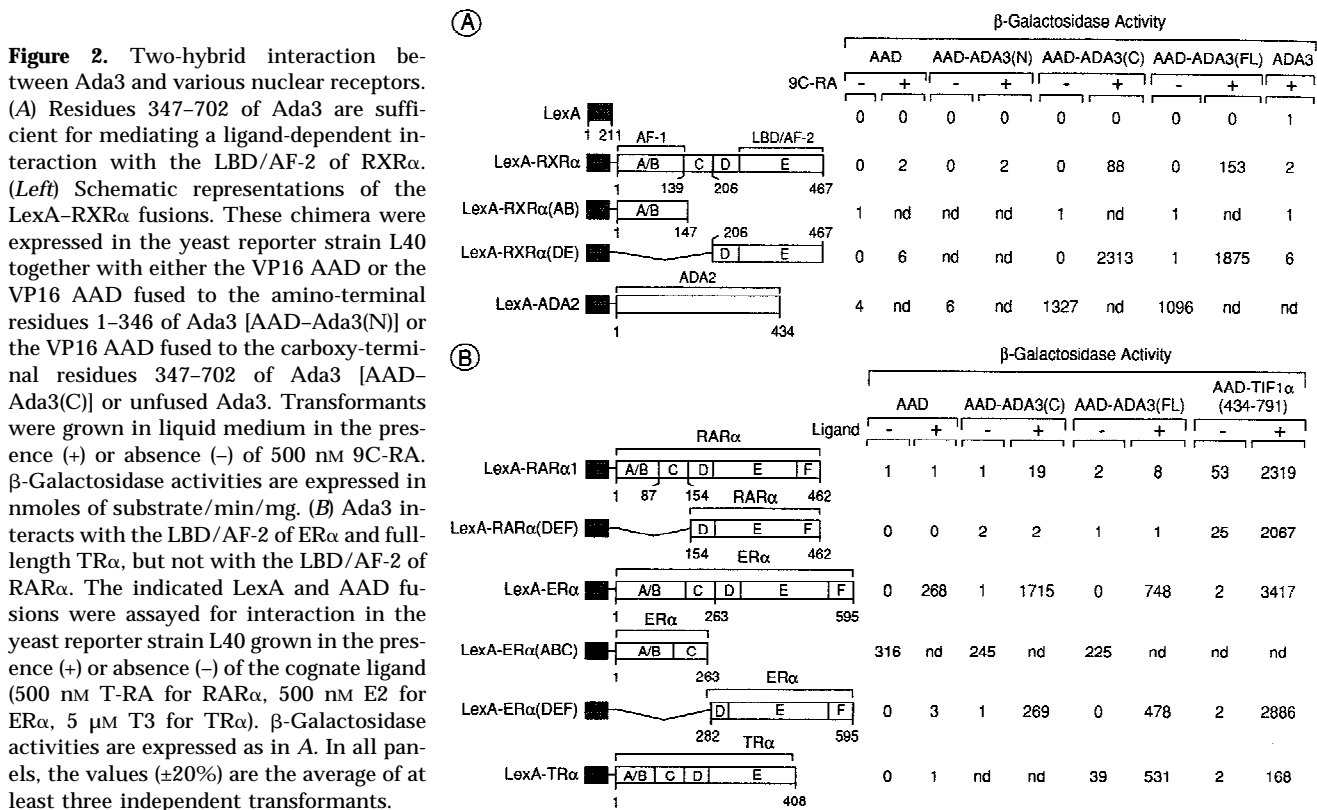
remaining unique library plasmids were sequenced. Sequence comparison with the NCBI and EMBL databases identified one of these DNA inserts as the sequence encoding amino acids 309–702 of Ada3 (Piña et al. 1993).

Residues 347–702 of Ada3 interact in a ligand-dependent manner with RXR α , TR α , ER α , but not RAR α

The entire coding sequence of RXR α fused to the LexA protein (LexA–RXR α ; Fig. 2A) was expressed in L40 cells with full-length (FL) or truncated Ada3, containing either the amino-terminal (N, amino acids 1–346) or the carboxy-terminal (C, amino acids 347–702) region of Ada3, fused to VP16 AAD [AAD–Ada3(FL), AAD–Ada3(N), and AAD–Ada3(C), Fig. 2A]. The coexpression of these Ada3 halves is known to complement a Δ ada3 strain as efficiently as full-length Ada3 (Horiuchi et al. 1995). In control assays, the LexA–RXR α fusion was assayed for activation with VP16 AAD or unfused Ada3. For comparison, assays were also performed with LexA–Ada2, which interacts with the carboxy-terminal region of Ada3 (Horiuchi et al. 1995). No increase in reporter activity was detected when LexA–RXR α was coexpressed with Ada3 or AAD–Ada3(N) (Fig. 2A). In contrast, 44- and 75-fold 9C-RA-dependent enhancements were observed in the presence of AAD–Ada3(C) and AAD–Ada3(FL), respectively (Fig. 2A), indicating that the carboxy-terminal moiety of Ada3 can functionally interact with RXR α in a 9C-RA-dependent manner. This

interaction was specific to the LBD/AF-2 of RXR α , since no β -galactosidase increase was obtained with LexA–RXR α (AB) containing the amino-terminal AB region of RXR α , which harbors the transcriptional activation function AF-1 (Fig. 2A), whereas coexpression of LexA–RXR α (DE) with AAD–Ada3(FL) or AAD–Ada3(C) resulted in a >300-fold 9C-RA-dependent increase in β -galactosidase activity (Fig. 2A). This increase was similar to that resulting from the interaction of Ada3 and Ada2 [see LexA–Ada2 and AAD–Ada3(FL) or AAD–Ada3(C); Fig. 2A].

LexA-fusion proteins containing sequences of RAR α , ER α , and TR α or deletion derivatives of these receptors (Fig. 2B) were also coexpressed with either AAD or AAD–Ada3 in the presence or absence of cognate ligands. For comparison, assays were also performed with AAD–TIF1 α (434–791), which contains the NR-interacting domain of TIF1 α , a putative mouse mediator of the ligand-dependent AF-2 of several NRs (Le Douarin et al. 1995a). A very weak increase in β -galactosidase was observed by coexpression of LexA–RAR α with either AAD–Ada3(FL) or AAD–Ada3(C) in the presence of T-RA (Fig. 2B), and no significant increase was observed in cells coexpressing LexA–RAR α (DEF) [Fig. 2B; under the same conditions, a high T-RA-stimulated activation was detected with AAD–TIF1 α (434–791)]. Thus, in contrast to RXR α , the LBD/AF-2 of RAR α cannot efficiently interact with Ada3. In the presence of estradiol (E2), LexA–ER α on its own activated the chimeric *GAL1* promoter (Fig. 2B). As expected (Metzger et al. 1992), the activation function



AF-1 was mostly responsible for this activation [compare LexA-ER α (ABC) to LexA-ER α (DEF); Fig. 2B]. Although poorly active on its own, the DEF region of ER α exhibited a significant E2-dependent interaction with the full-length and carboxy-terminal moiety of Ada3 (Fig. 2B), indicating that, like RXR α , the LBD of ER α can interact with Ada3. However, β -galactosidase activity resulting from this interaction was 6- to 10-fold lower than that corresponding to the LexA-ER α (DEF)/AAD-TIF1 α (434-791) interaction (Fig. 2B). In the case of LexA-TR α , the weak constitutive activation observed with Ada3(FL) was stimulated 16-fold on addition of thyroid hormone (T3) (Fig. 2B).

Mutations in the AF-2 activation domain core motif of RXR α and ER α impair interaction with Ada3 in vivo and in vitro

If Ada3 were involved in the mediation of transcriptional activation by NR AF-2, its ligand-dependent interaction with the corresponding LBDs should be affected by mutations in the core motif of the AF-2 activation domain (AD; helix 12 of the LBD), which have been shown to reduce AF-2 activity (but not ligand binding) in mammalian and yeast cells (Danielian et al. 1992; Ince et al. 1993; Durand et al. 1994; Heery et al. 1994) as well as in vitro interaction between NRs and putative mammalian

coactivators (e.g., CBP/p300, SRC-1 and TIF2/GRIP1; for review, see Glass et al. 1997). Several RXR α and ER α mutants carrying mutations in the AF-2 AD core motif (see Fig. 3) were tested for their ability to interact with Ada3 in the yeast two-hybrid assay. When compared with wild-type RXR α (DE), RXR α (DE)F455A/L456A and RXR α (DE)M459A/L460A were severely impaired in their ability to interact with Ada3(FL) and Ada3(C) (Fig. 3A). Similarly, a point mutant [ER α (DEF)M543A/L544A] as well as a mutant bearing an internal deletion of the core motif of ER α [ER α (DEF) Δ 535-547] showed no interaction with Ada3 (Fig. 3B). Thus, both RXR α and ER α require an intact AF-2 AD core motif to efficiently interact with Ada3 in the yeast two-hybrid system.

Binding assays between Ada3 and the LBD/AF-2s of RXR α , RAR α , and ER α were then performed in vitro with purified recombinant proteins. Purified *E. coli*-expressed histidine-epitope B10-tagged Ada3 was mixed with GST-receptor fusion proteins attached to glutathione-Sepharose beads (Fig. 3C-E). The matrix-associated Ada3 protein was revealed by Western blotting. In agreement with the two-hybrid data, a 9C-RA-dependent interaction was observed between the LBD/AF-2 of RXR α and Ada3 (cf. lanes 4 and 5 in Fig. 3C). As expected, the AF-2 AD core mutants GST-RXR α (DE)F455A/L456A (lanes 6,7) and GST-RXR α (DE)M459A/L460A (lanes 8,9) associated very poorly with Ada3. In contrast, as ob-

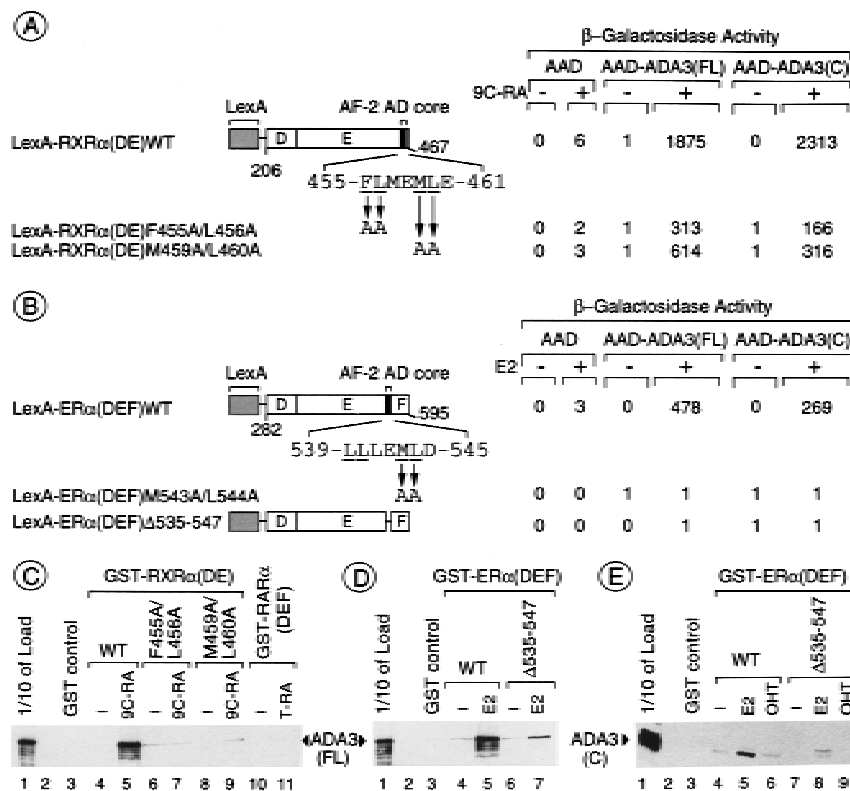


Figure 3. The AF-2 AD core motif is important for interaction between NRs and Ada3. (A) Point mutations in the conserved hydrophobic residues of the AF-2 AD core of RXR α impair interaction with Ada3. The indicated mutants of RXR α were fused to LexA and assayed for interaction with AAD-Ada3(FL) and AAD-Ada3(C) in the yeast reporter strain L40 grown in the presence or absence of 500 nM 9C-RA. β -Galactosidase activities are expressed as in Fig. 2A. (B) Deletion or point mutations in the AF-2 AD core of ER α abolish interaction with Ada3. L40 transformants expressing the indicated LexA and AAD fusions in the presence or absence of 500 nM E2 were treated as described in A. (C) Ada3 binds directly to the LBD of RXR α , but not of RAR α , in a ligand- and AF-2-integrity-dependent manner. Purified His-epitope B10-tagged Ada3 was incubated in a batch assay with control GST (lane 3), GST-RXR α (DE) wild type (WT; lanes 4,5), GST-RXR α (DE) mutated in the conserved hydrophobic residues of the AF-2 AD core motif (lanes 6-9), or GST-RAR α (DEF) (lanes 10,11) bound to glutathione-Sepharose beads, in the presence or absence of ligands (1 μ M 9C-RA for RXR α and 1 μ M T-RA for RAR α). Bound Ada3 protein was detected by Western blotting with the B10 antibody. (Lane 1) 1/10 the amount of input His-Ada3 fusion (arrow). (D) Ada3 binds directly to the LBD of ER α in a ligand- and AF-2-integrity-dependent manner. Binding assays were done as in C in the presence or absence of 500 nM E2. (E) E2 (500 nM; lane 5), but not OHT (1 μ M; lane 6), induces interaction between the ER α LBD and the carboxy-terminal moiety of Ada3.

of input His-Ada3 fusion (arrow). (D) Ada3 binds directly to the LBD of ER α in a ligand- and AF-2-integrity-dependent manner. Binding assays were done as in C in the presence or absence of 500 nM E2. (E) E2 (500 nM; lane 5), but not OHT (1 μ M; lane 6), induces interaction between the ER α LBD and the carboxy-terminal moiety of Ada3.

served in the two-hybrid assays, no significant interaction was detected between Ada3 and GST-RAR α (DEF) (lanes 10,11, Fig. 3C). Similarly to RXR α , agarose beads loaded with the LBD/AF-2 region of ER α retained Ada3 in a ligand-dependent manner [see GST-ER α (DEF)WT; lanes 4,5 in Fig. 3D], and this interaction was dependent on the integrity of the AF-2 AD core (cf. lanes 5 and 7, Fig. 3D). Essentially, the same results were obtained for interaction between the ER α DEF region and the carboxy-terminal moiety of Ada3 (Fig. 3E). Moreover, the anti-estrogen hydroxytamoxifen (OHT), which does not induce ER AF-2 activity in animal and yeast cells (Metzger et al. 1988, 1992; White et al. 1988; Berry et al. 1990), did not promote the binding of ER α to either Ada3(FL) or Ada3(C) (Fig. 3E, lanes 6,9 and data not shown), thus showing that both transactivation by AF-2 and receptor-Ada3 interaction require the same agonist-induced conformational changes in the LBD of ER α .

Ada3 acts as a coactivator for the AF-2 of RXR α and ER α in yeast

To determine whether Ada3 functions as a yeast mediator for the AF-2 of RXR α and ER α , the transcriptional activity of these AF-2s was tested in the absence of Ada3. The *ADA3* gene was disrupted in the PL3 reporter strain,

which contains a *URA3* reporter gene controlled by three estrogen response elements (EREs, Fig. 4A; Pierrat et al. 1992). Wild-type and Δ *ada3* PL3 strains were transformed with a high-copy-number plasmid YEp90 expressing [under the control of the phosphoglycerate kinase (PGK) promoter] either a chimeric receptor consisting of the DBD of ER α (ER.CAS, ER α amino acid residues 185–250) fused to the LBD/AF-2 of RXR α [RXR α (DE)-ER.CAS; Heery et al. 1993] or an amino-terminally truncated ER α derivative [ER α (CDEF), previously called HEG19; Berry et al. 1990] (Fig. 4A). The transcriptional activity of these proteins, as estimated by OMPdecase activity (the *URA3* product) in extracts from cells grown in the presence of increasing amounts of ligand, was reduced ~3- and 10-fold respectively, in the Δ *ada3* mutant when compared with wild-type cells (Fig. 4B,C). Thus, the AF-2 ligand-dependent activation functions of RXR α and ER α require Ada3 for full activity in yeast.

We also evaluated the effect of Ada3 overexpression on the AF-2 activity of RXR α and ER α . Overexpressing Ada3 in wild-type and Δ *ada3* strains resulted in an approximately two- and fivefold enhancement in the AF-2 activity of RXR α , respectively (Fig. 4D). Similar stimulatory effects were obtained on the AF-2 activity of ER α (Fig. 4D). Interestingly, a more drastic ~90-fold increase in the AF-2 activity of ER α was observed in the Δ *ada3*

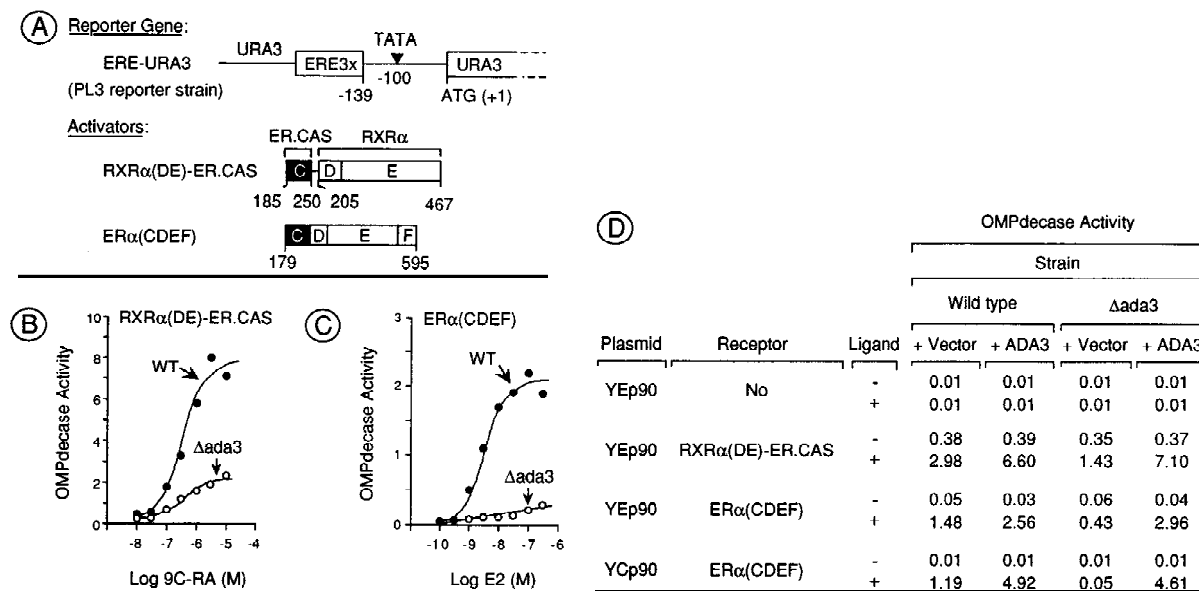


Figure 4. Effect of deletion or overexpression of Ada3 on the AF-2 activity of RXR α and ER α . (A) Schematic representation of the reporter gene and activators used in this study. The *ERE-URA3* reporter gene (integrated in the yeast strain PL3) was created by replacement of the poly[d(A-T)] sequences and the UAS_{Ura} site [from position -216 to -139 with respect to the ATG (+1) of the *URA3* gene] by three EREs (ERE3x) (Pierrat et al. 1992). (B, C) The ligand-dependent activation function AF-2 of RXR α and ER α requires Ada3 for full activity in yeast. Wild-type (WT) and Δ *ada3* PL3 strains were transformed with high-copy-number (YEp90) plasmids containing the receptor derivatives illustrated in A. Transformants were grown exponentially during five generations on selective medium containing uracil and ligand [9C-RA for RXR α (DE)-ER.CAS and E2 for ER α (CDEF)] at the concentrations indicated. OMPdecase assays were performed on each cell-free extract. Enzyme activity is expressed in nmole of substrate/min/mg protein. (D) Ada3 is a limiting coactivator for the AF-2 of RXR α and ER α . The indicated receptor derivatives were expressed from a high- (YEp90) or low- (YCp90) copy-number plasmid in the wild-type and Δ *ada3* PL3 strains in the presence (+) or absence (-) of ligand [500 nM 9C-RA for RXR α (DE)-ER.CAS and 500 nM E2 for ER α (CDEF)]. Cells were also transformed with an episomal expression vector (YE10) containing Ada3 (+ Ada3) or no insert (+ vector). OMPdecase activity is expressed as in B. The values (\pm 20%) are the mean of at least three independent experiments.

cells that express ER α (CDEF) from a low-copy-number plasmid (YCp90) in the absence or presence of Ada3 (Fig. 4D). This effect was strictly ligand dependent (Fig. 4D) and apparently promoter-specific, as no increase in the LexA-GAL1/lacZ reporter gene activity was observed when LexA-RXR α (DE) or LexA-ER α (DEF) was coexpressed with Ada3 in the L40 reporter strain (Fig. 2A and data not shown). To exclude the possibility that Ada3 had an effect on receptor expression levels, Western blot analyses were performed and no significant change in the expression levels of the RXR α and ER α receptor proteins was detected when Ada3 was deleted (not shown), indicating that, as reported (Henriksson et al. 1997), the activity of the PGK promoter is not Ada3-dependent. Taken together, these results indicate that Ada3 is a coactivator for the AF-2 of RXR α and ER α in yeast cells.

Integrity of the Ada complex is required for full activation of transcription by the AF-2 of ER α and RXR α

Ada3 forms a protein complex, the Ada complex, with other yeast proteins, including Ada2 and Gcn5 (see Discussion). We therefore investigated whether Ada3 could mediate the AF-2 activity of ER α through this complex. Deletions were introduced in the *ADA2* and *GCN5* genes of the yeast reporter strain PL3. Wild-type and mutant strains were transformed with the low-copy-number plasmid YCp90-ER α (CDEF), transformants were grown in the presence of hormone, and OMPdecase activity was determined. The reporter gene activity was approximately six- and fourfold reduced in Δ *ada2* and Δ *gcn5* strains, respectively (Table 1), indicating that the AF-2 of ER α requires functions of other components of the Ada complex for full activity. However, the AF-2 activity was reduced to a lesser extent in Δ *ada2* and Δ *gcn5* mutants than in the Δ *ada3* mutant (Table 1). Furthermore, we found that overexpression of Ada3 from a high-copy-number YEp10 vector suppressed the reduction of AF-2 activity in the Δ *ada2* and Δ *gcn5* strains (Table 1), indicating that Ada3 can function independently of Ada2 and

Gcn5, at least when overexpressed. Note also that, in contrast to Ada3, overexpression of Ada2 or Gcn5 in the wild-type strain did not enhance ER α AF-2 activity (Table 1). Thus, Ada3, but not Ada2 and Gcn5, appears to be a limiting cofactor for the AF-2 of ER α in yeast.

Similar to the ER α (CDEF) derivative, full-length ER α expressed from YCp90 exhibited a reduced transcriptional activity in mutant strains when compared to wild-type yeast (Table 1). The transcriptional activity of the chimeric RXR α (DEF)-ER.CAS expressed from the high-copy-plasmid YEp90 was also dependent on Ada2 and Gcn5 (Table 1). Taken together, our results indicate that the transcriptional activities of the AF-2s of ER α and RXR α are maximal when the Ada2, Ada3, and Gcn5 subunits of the ADA complex are all present.

Yeast Ada3 enhances transactivation by RXR α in Cos-1 cells and interacts with human Ada3

To investigate whether Ada3 might affect RXR α -dependent transcription in mammalian cells, RXR α and Ada3 expression vectors were transiently cotransfected into Cos-1 cells, together with a DR1-tk/CAT reporter, which contains a RXR response element (Durand et al. 1994). Cotransfection of increasing amounts of ADA3 stimulated up to approximately fivefold the 9C-RA-dependent transcription of the reporter in a dose-dependent manner, without inducing any significant activation in the absence of ligand (Fig. 5A). Thus, Ada3 can potentiate RXR α activity in cultured mammalian cells. Under similar conditions, a modest (~1.5- to 2-fold) increase in the E2-dependent transcription of a ERE-TATA-CAT reporter gene was observed when Ada3 was coexpressed with ER α (data not shown). Whether this could be related to differences in the reporter constructs is unknown. Note that similar observations have been made with putative mammalian coactivators, whose overexpression has been reported to enhance the AF-2 activity of only some of the NRs with which they interact (e.g., Voegel et al. 1996).

Interestingly, human homologs of the yeast Ada2 and

Table 1. Effect of deleting or overexpressing Ada2, Ada3, and Gcn5 on ER α and RXR α AF-2 activity

Strain	Transcriptional activity ^a					
	YCp90-ER α (CDEF)				YCp90-ER α (+ vector)	YEp90-RXR α (DE)-ER.CAS (+ vector)
	+vector	+Ada2	+Ada3	+Gcn5		
Wild type	1.19	0.78	4.92	0.88	10.65	2.98
Δ <i>ada2</i>	0.22	0.73	2.09	0.67	2.80	1.10
Δ <i>ada3</i>	0.05	0.05	4.61	0.17	1.30	1.43
Δ <i>gcn5</i>	0.34	0.43	2.54	1.25	4.20	1.60

^aThe indicated receptor constructs were expressed from a low (YCp90) or high (YEp90) copy number plasmid and tested for their ability to activate the *ERE-URA3* reporter gene (see Fig. 4A) in the isogenic PL3 strains wild type, Δ *ada2*, Δ *ada3*, and Δ *gcn5* grown in the presence of ligand [500 nM E2 for ER α constructs and 500 nM 9C-RA for RXR α (DE)-ER.CAS]. Cells were also transformed with an episomal expression vector (YEp10) containing Ada2, Ada3, Gcn5 or no insert. OMPdecase assays were performed on each cell-free extract. Enzyme activity is expressed in nmoles of substrate/min per mg protein. The values (\pm 20%) are the mean of at least three independent experiments.

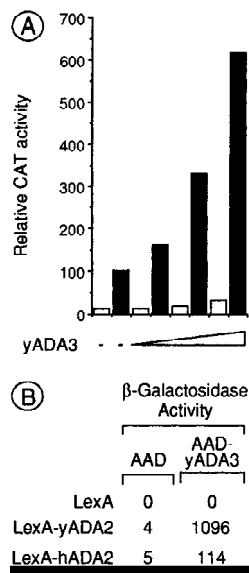


Figure 5. Yeast Ada3 (yAda3) stimulates the RXR α transcriptional activity in mammalian cells and interacts with hAda2 in a yeast two-hybrid assay. (A) Effect of yeast Ada3 on the RXR α transcriptional activity in transfected Cos-1 cells. DR1-tk/CAT reporter (1 μ g), RXR α (100 ng), and pCH110 (expressing β -galactosidase; 1 μ g) were transiently cotransfected into Cos-1 cells, together with increasing amounts of yeast Ada3 (1, 2, and 5 μ g). Cells were treated with control vehicle (\square) or 100 nM 9C-RA (\blacksquare). Values for CAT activities (\pm 20%) represent the averages of three independent duplicated transfections after normalization for the internal control β -galactosidase activity of pCH110. (B) Two-hybrid interaction between yeast Ada3 and hAda2. The indicated LexA and AAD fusions were introduced into the yeast reporter strain L40. β -Galactosidase activities are expressed as in Fig. 2A.

yeast Gcn5 proteins have recently been identified (hAda2 and hGcn5, respectively; Candau et al. 1996), suggesting an evolutionary conservation of the Ada complex. Because Ada2 is known to interact with Ada3 in the yeast Ada complex (Horiuchi et al. 1995), we investigated whether hAda2 could also interact with yeast Ada3 in the two-hybrid system (Ada3 in Fig. 5B). An \sim 20-fold increase in the reporter gene activity above the AAD control was obtained by coexpressing LexA-hAda2 and AAD-yeast Ada3. Although lower than that obtained by coexpression of LexA-Ada2 and AAD-yeast Ada3 (Fig. 5B), this stimulation suggests that yeast Ada3 and hAda2 may form a complex in mammalian cells, like Ada3 and Ada2 in yeast.

Discussion

Ada3, a coactivator/mediator for the ligand-dependent activation function AF-2 of certain nuclear receptors

The ligand-dependent activation function AF-2 of vertebrate nuclear receptors can stimulate transcription in yeast cells in the presence of their cognate ligand, thus suggesting that some of the basic features of the mecha-

nism(s) that mediate these effects have been conserved across eukaryotes (see introductory section for references). Here, we show that, in yeast, the regulatory protein Ada3 (Piña et al. 1993) is a coactivator for the activation function AF-2 of RXR α and ER α . Ada3 interacts functionally in yeast with the AF-2-containing LBD of RXR α and ER α in a ligand-dependent manner. In vitro binding assays demonstrate a direct physical interaction between purified ER α and Ada3 proteins in the presence of E2, but not of the anti-estrogen OHT. Similarly, RXR α interacts in a ligand-dependent manner with Ada3 in vitro. Importantly, both in yeast and in vitro, mutations within the RXR α and ER α AF-2 AD core motifs (helix 12), which inactivate AF-2, also impair Ada3 interaction. Finally, RXR α and ER α AF-2 activity is severely reduced in the absence of Ada3 and enhanced when Ada3 is overexpressed. Thus, Ada3 fulfills the criteria anticipated for a yeast coactivator (mediator) of the AF-2 of RXR α and ER α .

Ada3 can also interact with TR α in a hormone-dependent manner, but not with the AF-2-containing LBD of RAR α . This selectivity is consistent with previous studies on putative mammalian mediators, which revealed their differential interaction with the AF-2 ADs of various NRs in spite of the similarity of their respective AF-2 AD core motif (vom Baur et al. 1996; Thénot et al. 1997). Thus, the activity of the RAR α AF-2 constructs that can activate transcription in a RA-dependent manner in yeast (Heery et al. 1993) is probably not mediated by Ada3.

Role of the Ada complex(es) in activation of transcription by AF-2 of RXR α and ER α

Ada3 is found in vivo within multisubunit protein complexes of different size (\sim 0.2, 0.8–0.9, and 1.8–2 MD) and complexity that contain at least three to four additional proteins: Ada1, Ada2, Gcn5 (Ada4), and Ada5 (Grant et al. 1997; Horiuchi et al. 1997; Saleh et al. 1997). Direct and independent interactions have been observed in vitro between Ada2 and both Ada3 and Gcn5 (Horiuchi et al. 1995). In addition, immunoprecipitation assays and biochemical purification of Ada complexes have revealed further association of these three proteins with Ada1 and Ada5 (Marcus et al. 1996; Grant et al. 1997; Horiuchi et al. 1997). Here, we demonstrate that Ada2 and Gcn5, in addition to Ada3, are required for efficient ligand-dependent activation by the AF-2 of RXR α and ER α , indicating that the activity of these AF-2s is most probably mediated by an Ada complex through direct interaction between the liganded LBD and the Ada3 subunit. However, deletion of either the ADA2 or GCN5 gene reduces the transcriptional activity of the AF-2 of ER α to a lesser extent than that of the ADA3 gene. Moreover, Ada3 overexpression results in an increase of AF-2 activity even in Δ ada2 and Δ gcn5 deletion strains. Thus, Ada3 may mediate the ER α AF-2 activity through both Ada2/Gcn5-dependent and independent pathways.

Interestingly, *ada5* and *ada1* mutants exhibit more severe phenotypic defects and a broader spectrum of tran-

scriptionally affected genes than the *ada2* and *gcn5* mutants (Marcus et al. 1996; Horiuchi et al. 1997), suggesting that like Ada3, Ada1 and Ada5 possess additional functions that are independent of Ada2 and Gcn5 (see below). Several subunits of the Ada complexes have been shown to interact directly in vitro with the ADs of several activator proteins: Interactions have been reported between Ada2 and either VP16 (Silverman et al. 1994), Gcn4 (Barlev et al. 1995), Adr1 (Chiang et al. 1996), or GR (τ 1) (Henriksson et al. 1997), between Gcn5 and Adr1 (Chiang et al. 1996), and between Ada5 and VP16 (Marcus et al. 1996). We show here for the first time that Ada3 can also be the direct target for a transcriptional activator. Thus, it appears that, through interactions with activators, most if not all of the Ada subunits could be involved in the recruitment of Ada complexes to promoter regions. However, the various subunits of the Ada complex may have distinct coactivator functions, as they are not similarly involved in activation of transcription from different promoters. For example, activation through the *ADH1* and *CYC1 UAS1* yeast promoters is affected in Δ *ada1* and Δ *ada5*, but not in Δ *ada2*, Δ *ada3*, or Δ *gcn5* strains (Marcus et al. 1996; Horiuchi et al. 1997), whereas our present study indicates that Ada2 and Gcn5 are partially dispensable in the Ada complex recruited to the *URA3* promoter through interaction between the liganded LBD of ER α and Ada3.

How do Ada complexes mediate transcriptional activation by NRs?

Gcn5 possesses a histone acetyltransferase (HAT) activity (Kuo et al. 1996). Thus, the recruitment of Gcn5-containing Ada complex(es) might result in acetylation of histones, relieving some of their repressive effects on transcription. However, Gcn5 is responsible only for part of the transcriptional activation mediated by the Ada complex recruited by the ER, as the activity of ER α AF-2 is reduced to a lesser extent in a Δ *gcn5* strain than in a Δ *ada3* strain, while it is enhanced by overexpression of Ada3 in a Δ *gcn5* strain. Thus, Ada complexes recruited by liganded NRs might also act through additional targets, for instance through general transcription factors (GTFs). This possibility is supported by several recent findings. Interactions between the Ada complex and the GTF TATA-binding protein (TBP) have been reported (Barlev et al. 1995; Saleh et al. 1997), and both *ADA5* (*SPT20*) and *ADA1* have been shown to belong to the TBP class of *SPT* genes (Marcus et al. 1996; Roberts and Winston 1996; Horiuchi et al. 1997 and references therein). This class also includes TBP (*SPT15*), *SPT3*, *SPT7*, and *SPT8* (Eisenmann et al. 1992, 1994; Gansheroff et al. 1995). *SPT3* has been shown to interact genetically and functionally with TBP and TFIIA (Eisenmann et al. 1992; Madison and Winston 1997). Importantly, a native 1.8-MD Ada complex (named SAGA) has been recently isolated from yeast nuclei; it contains Spt7 and Spt3, and most probably Spt8, in addition to Gcn5, Ada2, Ada3, Ada1, and Ada5 (Grant et al. 1997; Horiuchi et al. 1997). Moreover, a physical interaction has been demon-

strated between Ada5/Spt20 and the four other Spt proteins, Spt3, Spt7, Spt8, and TBP (Grant et al. 1997; Roberts and Winston 1997). Thus, at least two mechanisms appear to be involved in the coactivator function of the subunits of Ada multiprotein complexes: remodeling of the chromatin structure through histone acetylation by Gcn5, via Ada2 and recruitment of the transcription machinery to promoter regions through interactions with TBP, and via Ada5 and/or Ada1 (see Fig. 6).

Taken together, these above findings and our present observations indicate that Ada3 could mediate the effect of the AF-2 of NRs to both the HAT Gcn5 and to TBP (and possibly other GTFs; see Fig. 6). This would readily account for our observations that ER α AF-2 activity is not fully suppressed in Δ *ada2* and Δ *gcn5* strains, and also that increased levels of Ada3 can enhance AF2 activity in the Δ *ada2* and Δ *gcn5* strains. After completion of this study, Henriksson et al. (1997) reported that the AF-1 amino-terminal activation domain τ 1 of GR interacts directly with Ada2 and requires Ada2, Ada3, and Gcn5 for full activity in yeast cells. Thus, at least two components of the Ada complex(es) can serve as coactivator/mediators for AF-1 and AF-2 of some NRs (see Fig. 6). The GR AF-1 (τ 1) is an AAD that resembles the AAD of VP16 (Tasset et al. 1990; Henriksson et al. 1997 and references therein). Therefore, like VP16 (Marcus et al. 1996), it may also interact with the Ada5 subunit of the Ada complex (see above). Thus, our present scheme would account for the observation of Henriksson et al. (1997) that an *ada3* deletion can cause a greater reduction in τ 1 activity than *ada2* and *gcn5* deletions (Henriksson et al. 1997). Interestingly, ER α and Ada3 could be coimmunoprecipitated from extracts of Δ *ada3* cells co-expressing an amino-terminally FLAG-tagged Ada3 and either full-length ER α or ER α (CDEF). However, this coimmunoprecipitation occurred irrespective of the presence of the ligand (E. vom Baur et al., unpubl.). These

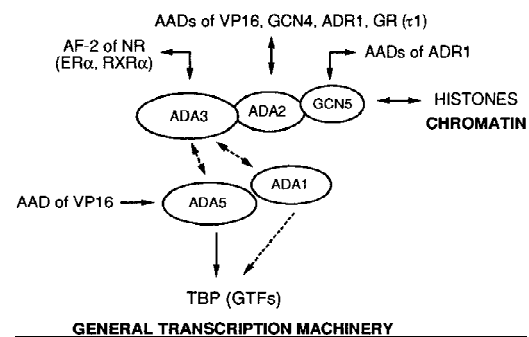


Figure 6. Schematic representation of the known interactions (double-headed arrows) between the ADs of transactivators and the subunits of the yeast Ada complex. Putative interactions between Ada3 and Ada5 and/or Ada1 are indicated by broken lines. Interactions between Ada5 (and possibly Ada1) and TBP, and between the HAT Gcn5 and the histone tails are also shown. It is postulated that transcriptional activation mediated by the Ada complex involves chromatin remodeling and/or recruitment of the general transcription machinery through interactions with GTFs.

observations suggest that, in addition to the ligand-dependent interaction with Ada3, ligand-independent interactions may exist between ER α and other components of the Ada complex.

Is there a vertebrate Ada3 homolog?

Human homologs of the yeast Ada2 and Gcn5 proteins have been identified (Candau et al. 1996). Yet, our attempts to isolate a mammalian homolog of Ada3 either by complementing the activation deficiency of the AF-2 of ER α in a Δ ada3 reporter strain or by using a two-hybrid screening aimed at isolating hAda2 interacting proteins, have been unsuccessful so far (E. vom Baur et al., unpubl.). Nevertheless, several lines of evidence suggest that a homolog of Ada3 might exist in higher eukaryotes. First, we have shown here that overexpression of yeast Ada3 in Cos-1 cells can enhance ligand-activated transcription by RXR α and ER α , whereas overexpression of human Ada2 enhances the τ 1 activation function of the GR (Henriksson et al. 1997). Second, we found that, similar to yeast Ada2, hAda2 can interact with yeast Ada3 in a two-hybrid assay, which suggests that Ada2-Ada3 interactions could be conserved in a putative vertebrate Ada complex. Third, a Gcn5-containing protein complex with an apparent size (molecular mass ~220 kD) similar to that of the smallest yeast Ada complex (Saleh et al. 1997; see above) has been purified from the protozoan *Tetrahymena*, which might contain the *Tetrahymena* homologs of the other yeast Ada proteins (Brownell et al. 1996). Interestingly, as in yeast cells (see above), coexpressed ER α or RXR α and FLAG-tagged yeast Ada3 could be coimmunoprecipitated from transfected Cos-1 cells, irrespective of the presence of the ligand (E. vom Baur et al., unpubl.), which suggests the possible existence of ligand-independent interactions between these nuclear receptors and other components of a putative vertebrate Ada complex.

Thus, Ada complexes might also exist in higher eukaryotes and, as in yeast, interact with transactivators to mediate their stimulatory effect on initiation of transcription through remodeling of the chromatin structure and/or recruitment of the general transcription machinery. In this respect, the Ada complex exhibits an interesting similarity with the mammalian and *Drosophila* CBP/p300 coactivator proteins, which do not exist in yeast cells. Indeed, like the Ada complex, CBP/p300 can interact directly with a number of transcriptional activators. CBP/p300 also interacts with the HAT P/CAF, which is related to Gcn5. Moreover, CBP/p300 possesses HAT activity on its own and, as the Ada complex, can interact with components of the general transcription machinery (for review, see Shikama et al. 1997 and references therein). The presence of Ada-like complexes in metazoan organisms, would obviously increase the number of combinatorial possibilities for controlling the multiple gene networks that are required for the realization of complex developmental and homeostatic programs in these organisms.

Materials and methods

Yeast strains, transformations, and media

S. cerevisiae L40 (a) strain [MATa, *his3* Δ 200, *trp1*-901, *leu2*-3,112, *ade2*, *LYS::(lexAbs)₄-HIS3*, *URA3::(lexAbs)₈-lacZ*] was a gift from S.M. Hollenberg (Vojtek et al. 1993). The reporter strain PL3 (α) [MAT α , *leu2*- Δ 1, *ura3*- Δ 1, *his3*- Δ 200, *trp1::(ERE)₃-URA3*] was described elsewhere (Pierrat et al. 1992). Δ ada2, Δ ada3, and Δ gcn5 deletion strains were generated in the PL3 (α) background according to the PCR-based disruption method described by Wach et al. (1994). Deletions were verified by PCR on genomic DNA and functional complementation. For all deletions, the full-length coding sequence of the deleted gene was replaced by the kanamycin-resistance gene. Yeast transformation was carried out by the lithium-acetate procedure and standard media were used for growth (Rose et al. 1990).

Plasmids

Details on individual plasmid constructs, which were all verified by sequencing, are available on request. Receptor cDNAs used in this study correspond to human RAR α 1 and ER α , mouse RXR α and chicken TR α . Yeast expression plasmids YEp10 (TRP1), YEp90 (His3) and YCp90 (His3), have been described elsewhere (Pierrat et al. 1992; Heery et al. 1994). AAD (VP16) fusion proteins were expressed from the multicopy plasmid pASV3 (Leu2) (Le Douarin et al. 1995b). All these plasmids express inserts under the control of the PGK promoter. LexA-fusion proteins were expressed from a derivative of the episomal plasmid pBTM116 (Trp1) under the control of the *alcohol dehydrogenase 1* (*ADH1*) promoter (Vojtek et al. 1993). Full-length *ADA2*, *GCN5*, and *ADA3* genes were isolated on the basis of published sequences by PCR on genomic DNA prepared from PL3. For transfection studies in mammalian cells, cDNAs were expressed from pSG5. The reporter genes *DR1-tk/CAT* and *ERE-TATA-CAT* have been described previously (Berry et al. 1990; Durand et al. 1994). For in vitro-binding assays, the indicated cDNAs were fused to GST in the pGEX2T plasmid (Pharmacia; vom Baur et al. 1996). Full-length Ada3 and Ada3(347-702) were cloned into the pET15bEpB10 plasmid, which directs the synthesis of 6 \times His-epitope B10 (region B of human ER α)-tagged fusion proteins in *E. coli*.

Two-hybrid screening

A yeast genomic VP16 fusion library was constructed by limited *Sau3AI* digestion of genomic DNA and subsequent insertion of fragments into a modified Leu2 pASV3 vector. The library was introduced by lithium-acetate transformation into the L40 reporter strain expressing LexA-RXR α (DE) from the Trp1 pBTM116m vector. Cells were spread directly on His⁻ Leu⁻ Trp⁻ plates containing 500 nM 9C-RA and 5 mM 3-AT. Two hundred thirty-two clones were isolated and then retested for β -galactosidase activity on permeabilized cells. Library plasmids from the positive isolates were recovered into *E. coli* HB101 (*leu2*⁻). Plasmids were subjected to restriction analysis, and unique inserts were sequenced.

Transactivation assays

Yeast transformants were grown for ~16 hr to a cell titer of 2–5 \times 10⁷ (exponential phase) in minimal medium supplemented with uracil and the required amino acids. When necessary, medium was supplemented with 500 nM of the appropriate ligands. PL3 cell-free extracts were prepared and assayed for

OMPdecase activity as described (Pierrat et al. 1992). L40 extracts were prepared and analyzed for β -galactosidase activity according to Rose et al. (1990). Transient transfections of Cos-1 cells, and CAT assays were performed as described (Durand et al. 1994).

Antibodies

Monoclonal antibodies (mAbs) B10 and F3 are directed against the B and F regions of human ER α , respectively, mAb 4RX is directed against the DE region of RXR α , and 2GV4 is directed against VP16 (see Le Douarin et al. 1995a and references therein). A polyclonal rabbit antiserum was raised against LexA expressed in *E. coli*.

In vitro binding assays

The assay was done as described previously (vom Baur et al. 1996). Briefly, GST or GST-fusion proteins were expressed in *E. coli* and purified on glutathione-Sepharose (Pharmacia). 6 \times His-epitope B10-tagged Ada3 fusions were expressed in *E. coli* and purified on Ni²⁺-chelating columns (Pharmacia). Purified proteins were quantified by Coomassie staining after SDS-PAGE separation and by Bradford protein assay. Glutathione-Sepharose beads were equilibrated with binding buffer (50 mM Tris-HCl at pH 7.5, 100 mM NaCl, 0.3 mM DTT, 10 mM MgCl₂, 8.7% glycerol, 0.1% NP-40), loaded with equimolar amounts of GST or GST fusions and washed. Appropriate ligands and purified His fusions were added to each reaction in the presence of BSA (fraction V, Sigma) to reduce nonspecific binding. Incubation was carried out at room temperature for 30 min. After three washes with binding buffer containing the appropriate ligands and BSA, the beads were dried, resuspended in SDS-loading buffer, and boiled for 10 min, and the proteins were analyzed by SDS-PAGE. Bound Ada3 was detected by immunoblotting with the mAb B10 (see above).

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