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Yeast ADA2 protein binds to the VP16 protein activation domain and activates transcription

(transcriptional adaptor/coactivators)

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ABSTRACT **Previously it was shown that yeast ADA2 protein is necessary for the full activity of some activation domains, such as VP16 and GCN4, *in vivo* and *in vitro*. These results suggest that ADA2 protein functions as a transcriptional coactivator or adaptor that bridges the interaction between certain acidic activation domains and the basal transcription machinery. Here we present two findings consistent with this model. (*i*) ADA2 protein interacts with a region of the VP16 acidic activation domain that requires *ADA2* for activity *in vivo*. (*ii*) ADA2 protein, when fused to a heterologous DNA-binding domain, can stimulate the activity of the basal transcription factors *in vivo*. This ability of ADA2 to activate transcription is mediated by *ADA3*, a gene with properties similar to *ADA2*. These findings suggest that ADA2 protein has at least some of the properties expected of a transcriptional adaptor.**

Several models have been proposed to explain eukaryotic transcriptional activation, including chromatin disruption by activators (1, 2) and interaction between activators and basal factors positioned at the TATA box (3). Direct interactions have been demonstrated between the basal transcription factors, TFIIB or TATA-binding protein (TBP), and acidic activation domains (4, 5). These interactions are disrupted by mutations in VP16 that prevent activation of transcription (6, 7). Further, mutations in TFIIB that prevent activation also prevent binding of VP16 (8). However, it has been clearly demonstrated that these interactions are not sufficient for the activation of transcription (9, 10), and many investigators have proposed that coactivators or adaptors must exist to bridge the interaction between activators and the basal machinery (11–14).

Tjian and colleagues (9) have shown that TBP is associated with a number of auxiliary factors (TAFs) that are necessary for activated transcription *in vitro*. In fact, it has been shown that $TAF_{II}110$ interacts with the activation domain of transcription factor SP1 (15) and $TAF_{II}40$ interacts with the C terminus of the VP16 activation domain. The significance of this interaction was demonstrated by the fact that anti-$TAF_{II}40$ antibodies blocked activation by GAL4–VP16 (16).

Likewise, *ADA2* was proposed to encode a transcriptional adaptor or coactivator because it is necessary for certain acidic activation domains to function *in vivo* and *in vitro* (17). The *ada2* mutant was originally isolated based upon its ability to grow in the presence of high levels of the toxic chimeric activator GAL4–VP16. The toxicity caused by overexpression of GAL4–VP16 correlates with its ability to activate transcription and to bind DNA. This result suggested that toxicity is due to the sequestration of essential general transcription factors by the activator at many sites throughout the genome. The collection of mutants resistant to GAL4–VP16 also includes *ADA3* (18) and *GCN5* (19), and mutations in these genes affect cell growth and activation of transcription in a manner similar to the *ada2* mutation.

The hypothesis that ADA2 protein, perhaps in concert with ADA3 and GCN5, constitutes a transcriptional adaptor makes two key predictions. (*i*) ADA2 protein should bind to those activation domains that require the integrity of *ADA2* for full activity. (*ii*) ADA2 protein should interact with one or more of the basal transcription factors. In this report we perform experiments that test directly whether ADA2 protein, or a complex containing ADA2 protein, binds to the VP16 activation domain. We also begin experiments to show that ADA2 interacts with the basal transcription factors *in vivo*. The results are consistent with the hypothesis that ADA2 protein is a transcriptional adaptor.

MATERIALS AND METHODS

Plasmids. pADA2HA was constructed by using PCR to engineer an in-frame *Bgl* II site at the C terminus of ADA2, as well as flanking *Hin*dIII sites. This PCR product was then ligated into the *Hin*dIII site of pDB20 (20). From this plasmid a *Sma* I–*Bgl* II fragment containing the *ADH1* promoter and *ADA2* was isolated and ligated into pHAH4 (21), which had been digested with *Sal* I, filled-in with Klenow fragment, and then digested with *Bgl* II; this results in the fusion of one copy of the 9-amino acid 12CA5 epitope from the influenza hemagglutinin (HA) protein to the C terminus of ADA2 (22). Likewise the pADA2-6HisL was created by using PCR to engineer six histidine codons fused to the C terminus of ADA2 and flanking *Hin*dIII sites; this gene was then cloned as a *Hin*dIII fragment into a version of pDB20LBglII (17) with a unique *Hin*dIII site.

To create the LexA–VP16 fusion constructs, the *Not* I site in pRS316 (23) was destroyed by cutting with *Not* I, filling-in with Klenow fragment, and religating, creating pRS316-Not. The P_{ADH}-lexA202-T_{ADH} cassette was then purified from pADH-lexA202 (18) as a *Sma* I–*Sal* I fragment and ligated into pRS316-Not, creating pARS/CEN-lexA202. Fragments encoding the various VP16 regions with flanking *Not* I sites were generated with PCR and cloned into the *Not* I site at the C terminus of lexA202.

LexA–ADA2 contains lexA residues 1–202 fused to the entire coding sequence of ADA2 (19). pADH-lexA202-HAP4 was described earlier (18). The 2μ ADA3 overexpression plasmid was constructed by using PCR to generate an *ADA3* gene with flanking *Bam*HI sites, which was then cloned into the *Bgl* II site of the ADH expression plasmid pDB20LBglII (17).

Coimmunoprecipitations. Δada2 strains carrying a 2μ plasmid expressing either ADA2HA (pADA2HA) or ADA2–6His (pADA2-6HisL) from the constitutive *ADH1* promoter were used to prepare nuclear extracts (24). Nuclear extracts were

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Abbreviations: TAF, TATA-binding protein associated factor; HA, hemagglutinin.

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diluted to ≈2 mg/ml in IP buffer [10% (vol/vol) glycerol/50 mM Hepes·KOH, pH 7.3/100 mM potassium glutamate, pH 7.3/0.5 mM dithiothreitol/6 mM MgOAc/1 mM EGTA/0.1% Nonidet P-40/protease inhibitors (11)]. Six micrograms of purified recombinant GAL4-(1–147)–VP16-(413–490) or -(413–456) was then added to 1 ml of extract and incubated for 3 hr at 4°C. After two centrifugations to clear the reactions, the extract was mixed with 25 μl of a slurry of protein A-agarose beads (Sigma) crosslinked to purified anti-HA monoclonal antibody (Berkeley Antibody, Richmond, CA) (25). After an overnight incubation at 4°C the beads were pelleted by centrifugation and washed six times with IP buffer. Then bound proteins were eluted from the antibody by incubation with HA peptide at 1 mg/ml (Berkeley Antibody) (26). Eluants were precipitated with 10% (vol/vol) trichloroacetic acid before SDS/PAGE and transfer to Immobilon-P membranes (Millipore). Immunoblots were probed with anti-HA or anti-VP16 antibody and visualized by using the Lumi-Phos chemiluminescent system (Boehringer Mannheim).

Yeast Manipulations. The lexA reporter plasmid is YEp21-Sc3423 (27) or pRBHis (19). Yeast manipulations and β-galactosidase assays were done as in Piña *et al.* (18). All strains were BWG1-7a and its derivatives or PSy316 (17, 18).

RESULTS AND DISCUSSION

To examine whether ADA2 protein binds VP16, we expressed ADA2 tagged with the HA epitope from the influenza virus (22) and determined whether this protein would coprecipitate with a chimera containing the DNA-binding domain of GAL4 fused to residues 413–490 of VP16 (28). Yeast extracts containing tagged or untagged ADA2 were prepared. The amount of ADA2 protein in both extracts was comparable, as measured by immunoanalysis with ADA2 antiserum (data not shown). The extracts and purified recombinant GAL4–VP16 were incubated together and mixed with protein A beads covalently coupled to monoclonal antibody against the HA epitope (25). After collecting and washing the beads, proteins that bound to the anti-HA antibody were eluted with a peptide consisting of the HA epitope and analyzed by immunoblotting (26, 29).

Fig. 1*A* shows a filter probed with the anti-HA antibody. Note a band of 49 kDa, the predicted size of ADA2HA, which was specifically bound to the beads. This protein was absent in the extract with untagged ADA2, verifying that the indicated band is ADA2HA. The same samples were probed with antibody to VP16 (Fig. 1*B*), revealing that the GAL4–VP16 chimera was bound to and eluted from the beads when mixed with extracts containing tagged ADA2, but not untagged ADA2. This experiment shows that GAL–VP16 binds to ADA2 or a yeast multi-protein complex containing ADA2.

To determine which portion of the GAL4–VP16 chimera interacts with ADA2, we repeated the coprecipitation experiment with a purified recombinant GAL4–VP16 derivative truncated at residue 456 of VP16. This very preparation shows considerable activity in a transcriptional activation assay *in vitro* (11). In experiments in which the full-length GAL4–VP16 bound to tagged ADA2 (Fig. 2*A*), the truncated protein did not bind to ADA2 at all (Fig. 2*B*). Thus, residues in VP16 carboxyl to position 456 are essential for binding to ADA2 protein.

To compare the binding *in vitro* of ADA2 protein to full-length but not truncated GAL4–VP16, with the ability *in vivo* of VP16 to activate transcription and respond to ADA2 protein, a set of VP16 derivatives was fused to the DNA-binding and dimerization domains of lexA (18). Previous experiments have indicated that VP16 could be separated into functional subdomains (16, 30, 31). We utilized a set of derivatives with domains extending from residue 413 to residues 450, 456, 460, or 470. Another derivative contained

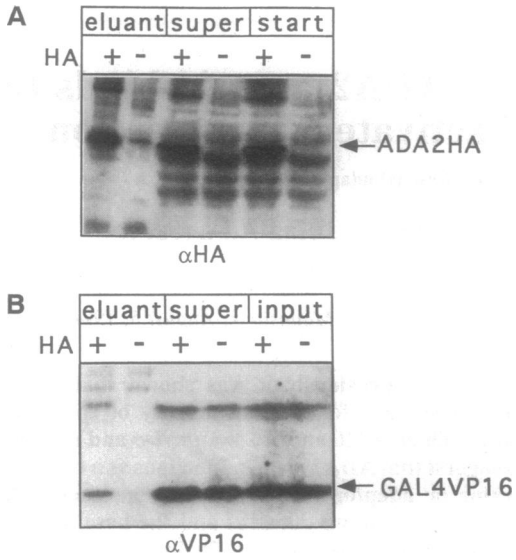

FIG. 1. GAL4–VP16 specifically coimmunoprecipitates with ADA2. In brief, extracts containing either HA-tagged or untagged ADA2 were mixed with recombinant GAL4–VP16 and incubated with agarose beads covalently coupled to the anti-HA monoclonal antibody. After the beads were collected and washed, proteins bound to the antibody were eluted with HA peptide, subjected to gel electrophoresis, and analyzed on the immunoblots shown. (*A*) Immunoblot probed with anti-HA antibody (Berkeley Antibody). The lanes labeled + contain ADA2HA extract, whereas the lanes labeled − contain untagged ADA2 extract. Lanes labeled start contain material before the immunoprecipitation, and the lanes marked super contain supernatant that did not bind to the beads. The material eluted with the HA peptide was loaded in the lanes marked eluant. The faint band in the eluant − lane that runs slightly slower than ADA2 is a small amount of IgG heavy chain that has leached off the agarose beads. (*B*) Immunoblot from the same experiment that was probed with anti-VP16 antibody (from S. Berger, J. Culp, and Brian Hellmig, SmithKline Beecham). The lanes are marked as in *A*.

VP16 residues 452–490. We were unable to use a construct containing lexA fused to residues 413–490 of VP16 because this construct was toxic in *ADA2+* (but not *ada2−*) cells (data not shown). However, previous experiments have shown that a GAL4 fusion to this region of VP16 activated transcription *in vivo* and was reduced 5- to 10-fold in *ada2* mutant cells (17). The ability of the above lexA fusions to activate transcription *in vivo* was monitored by a lacZ reporter containing a single lexA operator site, in both *ADA2+* and Δ*ada2* strains.

The 413–470 domain of VP16 was active and highly dependent on ADA2 (Fig. 3). The activity of the 413–470 moiety was abolished by mutation of the critical Phe-442 residue to alanine (32). Importantly, the 413–456 moiety was inactive *in vivo*, even though the protein was present at the same levels as the 413–470 fusion protein (data not shown). Thus, the inability of the 413–456 moiety to bind to ADA2 protein *in vitro* correlates with its inability to activate transcription *in vivo*. Other derivatives, truncated at residues 460 or 450, were also inactive. These assays define an ADA2-dependent VP16 activation domain encompassing Phe-442 and extending to a boundary between residues 460 and 470.

Interestingly, the carboxyl moiety of VP16 (containing residues 452–490), shown previously to contact the *Drosophila* $TAF_{II}40$ as well as replication protein A (16, 33), was also active *in vivo* but showed a minimal requirement for ADA2 protein (<2-fold) (Fig. 3). This result is consistent with earlier findings that some activation domains (e.g., GCN4) show a strong dependence on ADA2 protein, whereas others (e.g., GAL4 and HAP4) do not (18). In all, these data suggest that VP16 protein contains at least two functional activation

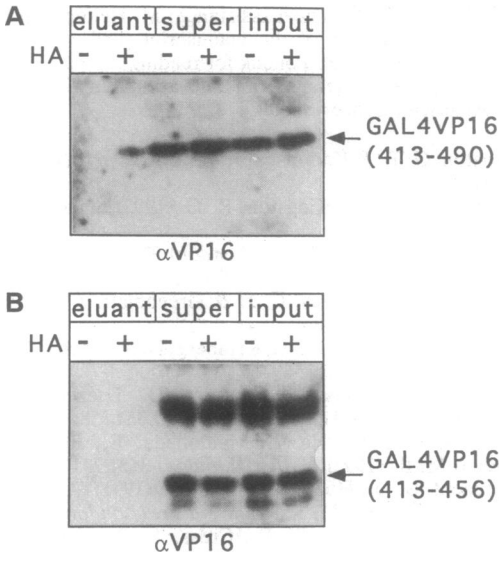

FIG. 2. The full VP16 activation domain, but not a version truncated at residue 456, coimmunoprecipitates with ADA2. (*A*) Results of a coimmunoprecipitation experiment with ADA2 and the full VP16 activation domain (residues 413–490) were analyzed by immunoblot probed with anti-VP16 antibody. (*B*) Immunoblot from an experiment identical to that in *A*, except ADA2 was coimmunoprecipitated with a version of VP16 truncated at residue 456 and then analyzed with anti-VP16 antibody. The high-molecular-weight band in *B* represents GAL4–VP16 dimers that, presumably, have become cross-linked in that preparation. The lanes are marked as in Fig. 1.

domains; one is the ADA2-dependent domain delineated above, whereas the other is a very potent, ADA2-independent, activation domain at the C terminus.

Given that ADA2 binds to VP16 and that this binding appears important for activation *in vivo*, we considered two possible mechanisms. (*i*) If VP16 activity were down-regulated by the binding of a repressor or by internal repression, ADA2 might potentiate activation by preventing repression. (*ii*) ADA2 might directly activate the basal factors, for example, by protein–protein contact. By the latter model, ADA2 protein itself should possess the ability to activate transcription. We therefore tested activation by a lexA–ADA2 chimera (containing all of ADA2 protein), which retained the ability to complement an *ada2* deletion mutation. This chimera was expressed from the strong *ADH1* promoter on a 2μ plasmid.

lexA–ADA2 was active, although only ≈10% as active as the lexA–VP16-(413–470) construct (Fig. 4). This activity decreased to background levels when a similar reporter missing the lexA binding site was used (data not shown). Further, the activity depended on the dose of lexA–ADA2—i.e., activity was reduced 3-fold when the chimera was expressed on a low-copy plasmid (data not shown). A further indication of the significance of the activity of lexA–ADA2 is its unique dependence on *ADA3*, a second gene identified by the selection for GAL4–VP16-resistant mutants (17). This dependence was demonstrated in two ways. (*i*) The activity of lexA–ADA2 was reduced in a Δ*ada3* strain. To show that this effect was specific, we tested a fusion of lexA to the HAP4 activation domain. As shown in Fig. 4 *Lower* and previously demonstrated, the activity of lexA–HAP4 was only slightly reduced in the Δ*ada3* strain (18).

However, the above requirement of lexA–ADA2 for ADA3 is not unique because certain activators, such as GCN4, also require ADA3 for full activity (18). Therefore, we carried out a second test of ADA3-dependence by overexpressing ADA3 from a 2μ plasmid. In this case the activity of lexA–ADA2 was augmented ≈5-fold above the levels observed in a wild-type

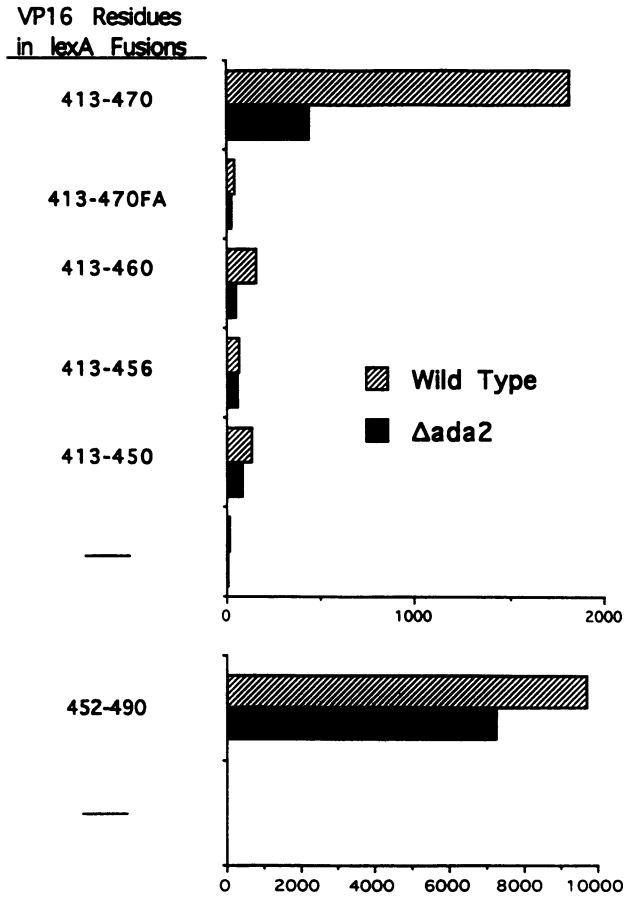

FIG. 3. VP16 residues 413–470 comprise a potent, and ADA2-dependent, activation domain. (*Upper*) A wild-type strain (BWG1-7a) and an isogenic Δada2 strain were transformed with the indicated lexA–VP16 fusion plasmid and a lacZ reporter with one lexA-binding site upstream of the *CYC1* promoter (17, 27). Fusions contain lexA residues 1–202 and the indicated residues of VP16. The entry labeled 413–470FA has a Phe-442 → Ala substitution (32). (*Lower*) Activity of a lexA fusion with the C-terminal portion of VP16 is ADA2 independent. —, No part of VP16 was fused to the DNA-binding domain. β-Galactosidase activity was determined, and the specific activity is indicated on the *x* axis. Mean values are shown; three to six independent experiments were done for each fusion construct, and the SDs were <25%.

strain, but the activity of lexA–HAP4 (Fig. 4) and lexA–GCN4 (data not shown) was not affected at all.

Because the activity of lexA–ADA2 was sensitive to both a decrease and an increase in ADA3 levels, we infer that it reflects a true aspect of ADA2 function. Further, our findings suggest that ADA2 and ADA3 proteins are part of a heteromeric complex. We imagine that only a small percentage of the highly expressed lexA–ADA2 is complexed with the other ADAs and that this fraction is preferentially diverted away from the lexA site to *bona fide* yeast promoters. Expression of high levels of ADA3 protein would increase the number of ADA complexes available to bind to the lexA site. If the ADA complex is required for stimulating transcription, then the observed stimulation of lexA–ADA2 activity by overexpression of ADA3 protein would be expected.

In summary, our findings show that ADA2 protein, or a complex containing ADA2 protein, binds to VP16 protein and that this binding is important for activation *in vivo*. Further, they show that ADA2 itself can activate transcription and that this activity is highly regulated by the level of ADA3 protein in cells. These findings are consistent with the hypothesis that ADA2 protein, perhaps as a part of a complex, functions as a transcriptional adaptor linking activators

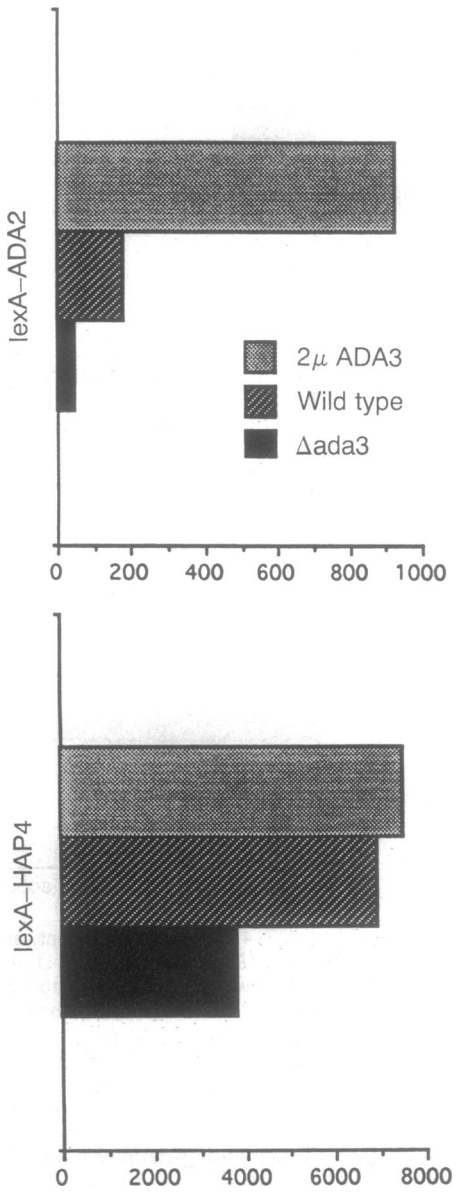

FIG. 4. LexA–ADA2 is an ADA3-dependent transcriptional activator. (*Upper*) ADA3 regulates the activity of the lexA–ADA2 fusion. (*Lower*) Activity of lexA–HAP4 is relatively insensitive to ADA3 levels. The *x* axis indicates β-galactosidase specific activity. The mean of three to five independent experiments is shown; SDs were <25%.

and basal factors at promoters. Interestingly, the cysteine-rich region at the N terminus of ADA2 protein shares significant similarity to a mammalian protein, CREB-binding protein (CBP), which binds to the active, phosphorylated form of transcription factor CREB and may itself be a coactivator (34). It will be important to determine the identity of all components in a probable ADA complex and to ascertain how ADA2 protein stimulates the basal factors.

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