Transcriptional activating regions target a cyclin-dependent kinase

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Several yeast activators are phosphorylated by SRB10, a cyclin-dependent kinase associated with the transcriptional machinery. Sites of phosphorylation are found outside the activating region in each case, and the modification has different physiological consequences in different cases. We show here that certain acidic transcriptional activating regions contact SRB10 as assayed both *in vivo* and *in vitro*. The interaction evidently positions each activator, as it activates transcription, so that it gets phosphorylated by SRB10, and thus a common mechanism targets disparate substrates to the kinase.

Several yeast transcriptional activators become phosphorylated as they activate transcription (1–3). Phosphorylation has various consequences: it affects nuclear export of Msn2, degradation of Gcn4, inducer-dependent activation by Gal4, and the activity of Sip4 (1–3). In each case, the activator is phosphorylated on residues positioned outside its activating and DNA binding regions. For example, the most important sites phosphorylated in Gal4 (S690, S696, and S699) lie in the region between the DNA binding domain and the principal activating region (2). Nevertheless, under physiological conditions, both the DNA binding and the activating regions are required for these phosphorylations, just as they are required for activity of the activator (2, 4).

The kinase believed responsible for the phosphorylations described above is the cyclin-dependent kinase SRB10 complexed with the cyclin SRB11 (1–3). The SRB10/11 pair is part of a larger complex that includes SRB8 and SRB9, and that complex can, by some reports, associate with the RNA polymerase II mediator (5, 6). SRB10/11 is also believed to phosphorylate other substrates in the transcriptional machinery (5–7). How the various transcriptional activators are targeted to the SRB10 kinase has heretofore been unknown. Here, we show that the activating region of Gal4 contacts SRB10 as measured in binding assays performed *in vitro* and *in vivo*. Binding experiments performed *in vitro* indicate that other acidic activating regions also interact with SRB10.

Materials and Methods

Proteins. Purifications of recombinant CDK/cyclins were performed as described (6, 8). Yeast RNA polymerase II holoenzyme was purified as in ref. 9. GST-Gal4wt (840–874) and GST-Gal4mut (840–874) (which contains the mutations V864E and L868V) were purified as in ref. 10. TATA binding protein (TBP), Gal80, and other Gal4 derivatives were purified as described (10, 11).

For Fig. 1B, Gal4 derivative bearing the F856C substitution was overexpressed in BL21 (DE3) pLysS bacterial strain. Cells were lysed in buffer A (20 mM Hepes, pH 7.5/150 mM KCl/0.1% Nonidet P-40/20 μ M ZnCl₂/3 mM DTT/1 mM β -mercaptoethanol/10% glycerol/1 mM PMSF/0.5 mM benzamidine) containing 6 M urea. After centrifugation (15 min at 10,000 $\times$ g), the protein was purified by using a SP-Sepharose fast flow (Amersham Pharmacia) column under denaturing condi-

tions. The relevant fractions were concentrated and refolded by rapid dilution into buffer A without urea. The refolded protein was then dialyzed overnight at 4°C and concentrated by using centrprep-10 concentrators (Amicon). Protein aggregates were removed by Superdex G-75 size exclusion chromatography (in buffer A without urea), and fractions corresponding to 30 kDa were tested for ability to bind Gal4 sites and activate transcription *in vitro*.

Label-Transfer Photo-Induced Crosslinking. Labeling, modification, crosslinking, and label-transfer reactions with ¹²⁵I-labeled *N*-[4-(*p*-azidosalicylamido)butyl]-3'-(2'-pyridyldithio)propionamide (APDP) were performed as described (10, 11). Briefly, 8 pmol of radiolabeled Gal4 (F856C) derivative were incubated with 10 μ g of MonoS fraction of the holoenzyme (roughly estimated at 5 pmol, assuming $\approx$ 2 MDa molecular mass for the holoenzyme), and where noted the reaction was supplemented with 5 pmol TBP and/or 30 pmol Gal80. The reaction was carried out at 4°C in MTB buffer (50 mM Hepes, pH 7.5/100 mM potassium-glutamate/25 mM magnesium-acetate/5 mM EGTA/10% glycerol/0.01% Nonidet P-40). The crosslinking reaction was induced with a 5-min exposure to a 320/366-nm UV lamp, and the label was transferred by subsequent incubation with 20 mM DTT. The labeled bands were resolved by Tricine-SDS/PAGE and visualized with a Fuji BAS2000 phosphorimager.

N-(2-pyridyldithio)ethyl-4-azidosalicylamide (PEAS) is a heterobifunctional photo-reactive crosslinker (12). Radiolabeling of PEAS was identical to that described for APDP (10, 11). ¹²⁵I-PEAS conjugation to Gal4 derivative and the subsequent crosslinking reactions were performed under identical protein concentrations in the MTB buffer as described above for the experiments with ¹²⁵I-APDP-Gal4.

In Vitro Transcription/Translation and GST Pull-Down. Genes for SRB4, SRB10, MED2, and TBP were amplified by PCR and cloned into pSP72 (Promega). Proteins labeled with [³⁵S]methionine were produced by a T7 coupled transcription/translation system (Promega) and then incubated with GST-Gal4wt, a fusion protein comprising GST and a Gal4-activating region fragment (residues 840–870). The GST fusion and bound proteins were isolated by using glutathione-agarose beads as described (11). Fractions (1/10) of the input and the entire pellet were resolved by 4–20% acrylamide gradient SDS/PAGE. The products were visualized by autoradiography.

To further test interaction between SRB10/11 with the Gal4-activating region, 2 μ g of purified SRB10/11 or SRB10-3/11 (D290A mutant of SRB10 in ref. 5, currently renumbered as D304A) was incubated with 20 μ g of GST-Gal4 (840–874) fusion

Abbreviations: PEAS, *N*-(2-pyridyldithio)ethyl-4-azidosalicylamide; SPR, surface plasmon resonance; 5-FOA, 5-fluoroorotic acid; TBP, TATA binding protein; APDP, *N*-[4-(*p*-azidosalicylamido)butyl]-3'-(2'-pyridyldithio)propionamide.

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Fig. 1. Affinity crosslinking between an activating region of Gal4 and the RNA polymerase II holoenzyme. (A) Purified native Gal4 (1–100) + (840–881) with a solvent-accessible cysteine at residue 856 was conjugated with ^{125}I -APDP. The label-transfer crosslinking reaction with this modified protein was performed with the holoenzyme complex (lane 2), holoenzyme supplemented with TBP (lane 3), and holoenzyme and TBP in the presence of Gal80 (lane 4). The radiolabeled bands were resolved on an SDS/PAGE gel and visualized by phosphorimaging as described (10, 11). (B) Purified denatured Gal4 (1–100) + (840–881) was refolded and then conjugated to ^{125}I -PEAS/AET. We confirmed the ability of this protein to bind DNA and activate transcription *in vitro* (data not shown). UV-induced crosslinking reactions were then performed in the presence of Gal80 (lane 2), TBP (lane 3), holoenzyme supplemented with TBP (lane 4), and holoenzyme, TBP, and Gal80 (lane 5).

proteins for 3 h. GST pull-down experiments were performed as described (10). GST pull-down experiments were also performed with insect cell extracts containing recombinant SRB10 or SRB11. Insect cell extracts were prepared as described (8). Ovalbumin was added to each reaction mixture to serve as a control for specific precipitation. The GST fusions and bound proteins were isolated by using glutathione-agarose beads, resolved by 4–20% acrylamide gradient SDS/PAGE, and analyzed by Western blotting by ECL according to the manufacturer's directions (Amersham Pharmacia).

Surface Plasmon Resonance (SPR). DNA-bound Gal4 was tested for its ability to interact with various target proteins by SPR. The experiments were performed as described (10, 11). Baseline drift caused by solvent effects and nonspecific binding were simultaneously monitored in parallel channels containing either streptavidin surface alone or nonspecific DNA immobilized on the streptavidin surface of the chip. Binding of target proteins was tested at two densities (high and low) of a specific Gal4-binding oligonucleotide bearing two tandem sites (10, 11). This method provides a valuable tool to measure protein–protein interactions at concentrations typically used in transcription or kinase reactions. However, because of mass transport problems and related surface binding problems, it is an unreliable tool to determine precise affinity constants (10, 11, 13).

Split-Ubiquitin Assay. The *Saccharomyces cerevisiae* strain JD52a (14) was transformed with N_{ub} or N_{ub} -Gal4 derivatives (15) on the leucine-marked single copy plasmid PACNX- N_{ub} IBC, a derivative of the PADNX (16), and with the tryptophan marked SRB10- C_{ub} -Rura314 vector, which is based on the C_{ub} -Rura314 vector (17). The cotransformed yeast cells were dropped in serial 10-fold dilutions onto a plate selecting for the Trp and Leu auxotrophy in the presence or absence of 5-fluoroorotic acid (5-FOA).

Results

Interactions *in Vitro*. To identify proteins in the transcriptional machinery that interact with Gal4, we performed label-transfer photo-affinity crosslinking experiments *in vitro*. As described in ref. 10, we conjugated the radiolabeled crosslinker (^{125}I -APDP) to a cysteine residue at position 856 in a Gal4 derivative comprising the DNA binding domain (residues 1–100) fused to activating region II' (residues 840–881) (18). This modified Gal4 was incubated with the yeast RNA polymerase II holoenzyme. Crosslinking was induced by long-range UV light, and the label was transferred from the activating region to the crosslinked protein targets under reducing conditions. We have reported that in this experiment the modified Gal4 interacted with the mediator component SRB4; with TBP (where that protein was added along with the holoenzyme); and, in a control experiment, with the Gal4 inhibitor Gal80. Here, we identify SRB10 as another protein that interacts with the modified Gal4 in this experiment.

Fig. 1A shows that ^{125}I was transferred from Gal4 to a ≈ 60 -kDa protein and TBP, which was added along with the holoenzyme to the reaction. Addition of Gal80 to the crosslinking reaction significantly reduced the amount of label transferred both to TBP and the ≈ 60 -kDa protein; in this case, the predominantly labeled component was Gal80 (Fig. 1, lane 4). This is as expected because Gal80, by binding to the Gal4-activating region, masks the latter's interaction with the transcriptional machinery (11, 18). In the absence of UV, no crosslinking to target proteins was observed (data not shown). Although not evident from exposure of the gel in Fig. 1, SRB4, the protein we concentrated on in our previous report, was also labeled.

We repeated this experiment by using the Gal4 derivative purified under denaturing conditions. The denatured Gal4 derivative was refolded and then conjugated to another photo-inducible crosslinker, PEAS. This crosslinker has a shorter spacer between the photo-reactive and cysteine-reactive functional groups (15 vs. 20 Å for APDP) (12). ^{125}I -PEAS was conjugated to the cysteine at position 856 in Gal4 [(1–100) + (840–881)], and the experiment was performed as in Fig. 1A. Although the efficiency of label transfer was lower with the shorter crosslinker, the ≈ 60 -kDa protein, TBP and Gal80 were once again labeled (Fig. 1B). As is evident from Fig. 1, use of a Gal4 derivative purified in this fashion significantly reduced the background compared with that observed with Gal4 that had not been through a denaturation–renaturation cycle. That treatment evidently frees Gal4 of contaminants that copurify with the native activator.

Two components of the mediator, SRB10 and Med2, correspond in size to the labeled ≈ 60 -kDa target of Fig. 1, and deletion of either component impairs galactose-responsive gene expression (5, 19). We tested whether one or both of these proteins might interact with Gal4 as follows. The GST pull-down experiment of Fig. 2A shows that residues (840–874) of Gal4, fused to GST (GST-Gal4wt), interacted with *in vitro*-translated SRB10 but not with similarly prepared Med2. Fig. 2 also shows, consistent with previous findings, that the GST-Gal4wt fusion bound to *in vitro*-translated SRB4 and TBP. We purified the SRB10/11 complex from a baculovirus expression system (as described in ref. 8) and found that it bound to the Gal4-activating

Fig. 2. Gal4-SRB10 interaction studies *in vitro*. (A) *In vitro*-transcribed and -translated, ^{35}S -labeled SRB4, SRB10, Med2, and TBP were separately incubated with the fusion protein GST-Gal4wt (840-874). The GST fusion and bound proteins were isolated by using glutathione-agarose beads. Ten percent of the input and the entire pellet (output) were subjected to SDS/PAGE and analyzed by autoradiography. *In vitro*-translated and ^{35}S -labeled proteins were identified by their apparent molecular weights. (B) Recombinant SRB10/11 and SRB10-3/11 complex, purified from a baculoviral expression system, were separately incubated with GST-GAL4 (840-874) (GAL4wt) and with an otherwise identical protein bearing amino acid changes in the activating region (V864E and L868V) and called Gal4mut. This double mutation abolishes Gal4's ability to activate transcription. The GST control was included (Control), and ovalbumin (Ova) was added to each reaction mixture to serve as a control for specific precipitation. Bound proteins were subjected to SDS/PAGE as in A, and SRB10 and SRB10-3 were visualized by immunoblotting with an anti-SRB10 polyclonal antibody. (C) Insect cell extracts containing either SRB10 or SRB11 were incubated with proteins bearing GST fused to WT or mutant Gal4-activating region (840-874). GST pull-down and subsequent visualization of bound proteins were performed by using polyclonal antibodies against SRB10 and SRB11 as described in B. (D) SPR analysis was performed with purified recombinant SRB10/11 complex, TBP, and Gal80. In each case, ≈ 200 resonance response units of Gal4 (1-100) + rII' or a fragment comprising Gal4 (1-100) was prebound to a DNA fragment bearing a Gal4 DNA binding site immobilized on the surface of a flow cell. Target proteins were passed over this Gal4-DNA complex, and the change in resonance response units (ΔRU) upon binding of target proteins to the respective Gal4 derivative is presented.

Fig. 3. Gal4p interacts with SRB10 *in vivo* in the split-ubiquitin assay. Tenfold serial dilutions of yeast cells coexpressing Nub or Nub-Gal4 derivatives together with SRB10-Cub-Rura3 were spotted on a control plate lacking tryptophan and leucine (WL) or on plates additionally containing 5-FOA. Growth on either plate requires functions supplied by the plasmids, and the presence of FOA selects against Ura3.

region as determined by a GST pull-down experiment (Fig. 2B). That interaction was unaffected by a point mutation in SRB10 (SRB10-3) that eliminates its kinase activity (Fig. 2B). However, mutations in Gal4 that diminish its activating potential (V864E and L868V described in ref. 20) also significantly diminish its binding to the SRB10/11 complex (Fig. 2B). Neither GST alone nor ovalbumin (Ova) in Fig. 2B binds to the SRB10/11 complex. Fig. 2C shows that, in another GST pull-down experiment, Gst-Gal4wt bound SRB10, but not SRB11, in extracts of baculovirus-infected insect cells. Consistent with this result, coimmunoprecipitation experiments with anti-Flag antibodies directed at Flag-tagged SRB10 efficiently precipitated Gal4 (1-100) + (840-881) but not the Gal4 DNA binding domain alone Gal4 (1-100) (data not shown).

The data in Fig. 2D show that, as assayed by SPR, DNA-bound Gal4 (1-100) + (840-881) interacted with the SRB10/11 complex as avidly as it did with TBP (estimated K_D for TBP and Gal4-activating region interaction $\approx 10^{-7}$ M, as in ref. 18). SPR studies also showed that the SRB10/11 complex binds neither to the DNA binding domain of Gal4 nor to DNA under a variety of buffer conditions. Also, whether assayed by SPR, GST pull-down, or coimmunoprecipitation experiments, mutants bearing changes in the rII' activating region that impair activation *in vivo* [F856 and F869A (18) or V864E and L868V (20)] failed to interact detectably with SRB10.

Interactions *in Vivo*. Interaction between the activating region of Gal4 and SRB10 was detected *in vivo* by using the split-ubiquitin system (15). For these experiments, Gal4 (1-100), attached to one or another derivative of a Gal4-activating region fragment (Fig. 3), was fused to the N-terminal half of ubiquitin (N_{ub}) and expressed in yeast cells. These cells also contained a tripartite fusion protein comprising SRB10, the C-terminal half of ubiquitin (C_{ub}), and the enzyme Ura3 as indicated in Fig. 3. The fusion junction between C_{ub} and Ura3 includes an extra arginine residue, as a result of which the Ura3 is degraded when the two halves of ubiquitin are brought together as a consequence of interaction between their fused proteins (15, 21). Ura3 degradation, in turn, is reflected in an acquired ability to grow in the presence of 5-FOA.

As shown in Fig. 3, the N_{ub} fusion protein bearing the minimal activating fragment of Gal4 (residues 840-881) interacted efficiently with the C_{ub} fusion protein bearing SRB10, whereas the N_{ub} fusion bearing a much weaker activating region [Gal4 (840-857)] did not. Because the SRB10 fusion used here could be bound to a host-supplied SRB11, this experiment does not determine whether the activator interacts with the kinase alone, the 10/11 complex, or both.

Fig. 4. Activating regions from different activators bind SRB10. *In vitro*-translated, ^{35}S -labeled SRB10 was incubated with GST alone (control) or proteins comprising GST fused to one or another activating region as shown. The composition of each activating region is Gal4 (residues 840–874), AH (PGIELQELQELQALLQQ), VP16 (residues 420–490), p53 (residues 1–97), Gcn4 no. 4 (107–144). For details, see ref. 22. Fractions (10%) of the input and the entire pellet (output) were analyzed as in Fig. 2A.

Interaction with Other Activators. Acidic activating regions other than that of Gal4 also interact with SRB10. As shown in Fig. 4, the designed activating region AH, those from the viral and mammalian activators VP16 and p53, and that from the yeast activator Gcn4, all bound SRB10 as assayed in a GST pull-down experiment. For these experiments, [^{35}S]methionine-labeled SRB10 was generated by *in vitro* translation with rabbit reticulocyte extract as in Fig. 2A (see *Materials and Methods*). GST alone (control) did not interact detectably with SRB10 in this assay. The avidities of these interactions are in the order predicted by their activating strengths as assayed *in vivo* (22).

Discussion

Several assays indicate that the kinase SRB10 is a target of the transcriptional activating region of Gal4. *In vitro*, photo-crosslinking, coimmunoprecipitation, GST pull-downs, and SPR analyses detected interaction between the activating region of Gal4 and SRB10 when the latter was presented separated from other components, when part of the SRB10/11 complex, and when part of the holoenzyme. *In vivo*, the interaction was detected by the split-ubiquitin assay. Where tested, activating region variants deficient in activation were also deficient for interaction with SRB10. GST pull-down experiments indicated that other acidic activating regions (those found on the yeast activator Gcn4, the mammalian activator p53, the viral activator VP16, and the synthetic peptide AH) also interact with SRB10 and do so with relative affinities consistent with their activities *in vivo*.

The interaction of an activating region with SRB10, we suggest, brings the activator into apposition with the SRB10/11 kinase/cyclin pair so that the activator is phosphorylated. This form of targeting may be unusual; other CDK/cyclin pairs have been described as being targeted to substrates by binding interactions mediated by the cyclin (23, 24). Our results suggest an explanation for why, as observed some time ago, phosphorylation of Gal4 occurs concurrently with transcriptional activation *in vivo* (4) and requires that both its activating and DNA binding regions be intact. Thus, the activating region is required for apposition of the activator with the kinase, and the DNA binding region is required because, at physiological concentrations, cooperative binding of the activator and the transcriptional machinery (including SRB10) to DNA helps promote the reaction.

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