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The DNA-binding transcriptional activator Gal4 and its regulators Gal80 and Gal3 constitute a galactose-responsive switch for the GAL genes of Saccharomyces cerevisiae. Gal4 binds to GAL gene UASGAL (upstream activation sequence in GAL gene promoter) sites as a dimer via its N-terminal domain and activates transcription via a C-terminal transcription activation domain (AD). In the absence of galactose, a Gal80 dimer binds to a dimer of Gal4, masking the Gal4AD. Galactose triggers Gal3-Gal80 interaction to rapidly initiate Gal4-mediated transcription activation. Just how Gal3 alters Gal80 to relieve Gal80 inhibition of Gal4 has been unknown, but previous analyses of Gal80 mutants suggested a possible competition between Gal3-Gal80 and Gal80 self-association interactions. Here we assayed Gal80-Gal80 interactions and tested for effects of Gal3. Immunoprecipitation, cross-linking, and denaturing and native PAGE analyses of Gal80 in vitro and fluorescence imaging of Gal80 in live cells show that Gal3-Gal80 interaction occurs concomitantly with a decrease in Gal80 multimers. Consistent with this, we find that newly discovered nuclear clusters of Gal80 dissipate in response to galactose-triggered Gal3-Gal80 interaction. We discuss the effect of Gal3 on the quaternary structure of Gal80 in light of the evidence pointing to multimeric Gal80 as the form required to inhibit Gal4.

Regulation of gene expression by promoter-specific DNA-binding transcription activator proteins is a common strategy cells use to modulate production of proteins adaptively. In the activated state, a transcriptional activator mediates a host of binding reactions to recruit and assemble RNA polymerase and associated transcriptional factors at the promoter. Typically, such transcriptional activators are inhibited or activated by signaling processes that involve ligand binding, protein-interaction cascades, and chemical modifications (1–7). Regulation of several well-studied transcriptional activators involves masking and unmasking of their activation domains through protein-protein interactions (8–12).

A prominent example is Gal4, the DNA-binding transcriptional activator of the GAL gene switch that controls expression of the galactose pathway genes in Saccharomyces cerevisiae (13–18). Alternative interactions among the three GAL gene switch proteins, Gal4, Gal80, and Gal3, determine whether the Gal4 transcriptional activation domain (Gal4AD) is masked or not masked by Gal80 (19, 20). In the absence of galactose, the Gal80 protein binds to a small peptide (amino acids [aa] 855 to 870) within the Gal80 self-association interactions (19, 20). Galactose converts Gal3 to a form that readily binds to Gal80 (21). It is well established that Gal3-Gal80 complex formation is required for the relief of Gal80 inhibition of Gal4AD and Gal4-mediated transcription activation of the GAL genes (22–25). These events occur rapidly, resulting in readily detectable GAL mRNA within 3 to 4 min of exposure to galactose (26–28).

Understanding exactly how galactose-activated Gal3 binding to Gal80 alters Gal80 to overcome inhibition of Gal4 has been a goal of researchers in the field for the past several years. Two questions concerning discrete mechanistic events in the GAL gene switch have driven such research. The first question is does Gal80 dissociate from Gal4 or remain associated with Gal4 in response to the Gal3-Gal80 interaction? On this issue, there is evidence in support of dissociation from our lab (19, 29, 30) as well as evidence for nondissociation from other labs (20, 31, 32). The second question is how does the binding of Gal3 to Gal80 alter Gal80 such as to relieve inhibition of Gal4? It is this second question that is the focus of the work presented here.

Just how the binding of Gal3 to Gal80 alters Gal80 might come down to simple competition between Gal4 and Gal3 for binding to Gal80 as has been proposed for the somewhat similar GAL gene switch of the distantly related yeast Kluyveromyces lactis (18, 33–35). In that system, the binding of K. lactis Gal1 (KlGal1), a Gal3 homologue, to KlGal80 overcomes its inhibition of KlGal4 activity (33, 36). The experimental evidence indicates that KlGal1 and KlGal4 binding to KlGal80 are mutually exclusive and that a heterotetrameric KlGal802-KlGal12 complex forms in response to galactose. Based on mathematical modeling of that system, it was suggested that two KlGal1 monomers somehow compete with KlGal80-KlGal4 dimer-dimer interactions (33). Possibly, in the S. cerevisiae GAL gene switch as well, it is simple competition between Gal80-Gal4 and Gal80-Gal3 binding events that mechanistically couples Gal3-Gal80 binding to activation of Gal4. The findings that overexpression of either Gal4AD or Gal3 relieves Gal80 inhibition of Gal4 in the absence of galactose (11, 15, 22, 37) and that increasing Gal80 concentration reverses the effect (38) are consistent with such a possibility. However, to date, there is no...
with the data of others pointing to a correlation between the Gal80 leading to relief of inhibition of Gal4. Our proposal is consistent Gal80. We propose that the Gal3-mediated decrease in Gal80 mul-
self-assemblies. Additionally, we report novel intranuclear clus-
showing that Gal3 interaction with Gal80 reduces levels of Gal80

plasmids used in this study

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**MATERIALS AND METHODS**

**Yeast strains and plasmids.** *S. cerevisiae* strain Sc952 [MATa ade1 leu2-3,112 ura3-52 trpl-1 HIII his3-D1 LacOx64:LEU2 (P_GAL1-GST)x8: Kan’ gal4::NAT] was used for the in vivo microcopy assays of Gal80-Gal80 and Gal80-Gal3 interactions. Sc952 was derived from yeast strain Sc902 (29) by deleting Gal4 with a deleter cassette PCR amplified from pJf6N (41) with primers OE174 (ATC ATT TTA AGA GAG GAC AGA) and OE175 (GAA GGT AAC CGG TGT GGT GGT TCA GAA TCT CTT AGC) and OE175 (GAA GGT AAC CGG TGT GGT GGT TCA GAA TCT CTT AGC). Protein extracts for the in vitro protein-protein binding assays were obtained from yeast strain Sc745 cells (MATa ade1 leu2-3,112 ura3-52 trpl-1 HIII his3-D1 MEL1 lys2::P_GAL1-HIS3 gal4::URA2). The plasmids used in this study were generated using standard molecular cloning and PCR techniques. A more detailed description for construction of each plasmid will be provided upon request. A list of all plasmids used in this study can be found in Table 1.

**Microscopy.** All microscopy experiments were carried out with a Nikon TE-2000U spinning-disk confocal microscope that was equipped with a 100×1.4-numerical-aperture (NA) objective lens (Nikon, Melville, NY), 488-, 514-, and 568-nm argon ion lasers, and a charge-coupled device camera (ORCA-AG; Hamamatsu, Bridgewater, NJ). The following protocol was used to observe the effect of Galactose on protein interactions in single cells. Cells were grown to mid-log phase in noninducing medium and immobilized in the Y04C microfluidics plates (CellASIC). The temper-
presence of 500 μM ATP and 25 mM galactose in a total mixture reaction volume of 30 μl at 4°C for 1 h. Formaldehyde was then added to the samples to a final concentration of 30 mM, and the samples were incubated at 4°C for an additional 2 h to cross-link the proteins. A volume of 10 μl of 4× SDS electrophoresis loading buffer was added to each sample, and the samples were incubated at room temperature for additional 10 min. Proteins were separated by 7.5% SDS-PAGE and visualized by Coo- massie blue staining.

**Discontinuous blue native protein gel electrophoresis.** Native gel electrophoresis was conducted by the Niepmann and Zheng procedure (46) that allows the separation of proteins according to their size, oligomeric state, and shape. A fixed amount of Gal80 (final concentration of 2.5 μM) was incubated with increasing amounts of Gal3 in the presence or absence of 2 mM ATP and 25 mM galactose at 4°C for 2 h. The samples were then mixed with gel loading buffer (100 mM Tris-Cl [pH 8.0], 40% glycerol, 0.5% Serva Blue G) and incubated for an additional 10 min at room temperature. The protein species in the samples were analyzed on a nondenaturing 4 to 16% polyacrylamide gradient gel. Histidine (final concentration of 100 mM; pH 8.0) and 0.002% Serva Blue G were added to the cathode buffer prior to electrophoresis. Catalase (230 kDa), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (143 kDa), and bovine serum albumin (BSA) (69 kDa) were corun as molecular markers. The gels were destained with several changes of 7.5% acetic acid and 5% ethanol. To check whether 6×His-Gal80−2 has a higher propensity to oligomerize compared to 6×His-Gal80, we repeated the above analyses using precast 4 to 20% blue native (BN)-polyacrylamide gels from Jule Inc. (Milford, CT, USA). The compositions of loading buffer, cathode buffer, and anode buffer, the method of destaining, and the molecular markers were kept the same as described above.

**RESULTS**

**Conditions favoring Gal3-Gal80 interaction reduce the level of HA-Gal80–Gal80-2mYFP complexes in yeast extracts.** To determine whether Gal3-Gal80 association reduces Gal80 self-association, we performed coimmunoprecipitation (co-IP) assays. Yeast extracts containing HA-Gal80 and Gal80-2mYFP and with or without galactose were incubated with *E. coli* extracts containing wild-type (WT) Gal3 or no Gal3 or a mutant Gal3 (H199R) that is defective in Gal80 binding (47). Approximately 2-fold-less Gal80-2mYFP was coprecipitated with anti-HA antibody (Ab)-conjugated agarose beads under conditions favoring Gal3-Gal80 interaction compared to conditions that did not (Fig. 1). Importantly, we detected Gal3 in the coprecipitates only in the presence of galactose. These results suggest a mechanism whereby Gal3 binding to Gal80 reduces Gal80 self-association and/or the stability of preexisting Gal80 oligomers.

**Increasing the concentration of Gal3 versus a fixed concentration of Gal80 leads to Gal3–Gal80 complexes at the expense of Gal80 oligomers.** Previously it was shown that Gal80 dimerizes very strongly (**K<sub>d</sub>** [dissociation constant] of 0.2 nM) and tetramerizes moderately strongly (**K<sub>d</sub>** = 50 nM) *in vitro* (40). To determine whether the reduction in Gal80 self-association interactions observed in our co-IP experiments reflects a direct impact of Gal3 binding on Gal80 oligomeric structures, we employed cross-linking and native polyacrylamide gel experiments. We could readily detect multiple bands on SDS-polyacrylamide gels corresponding to cross-linked Gal80 molecules in the absence of Gal3 (Fig. 2, lane 3), confirming oligomerization of Gal80 *in vitro*. While the mobility of the most prominent band was consistent with the molecular mass of the Gal80 dimer, the dimer is not necessarily the most populated quaternary Gal80 species under native conditions, as it requires only one cross-link to yield dimers but more than one to capture higher-order oligomers. Nevertheless, we also observed species with mobilities consistent with higher-order multimeric species. We nominally label these as trimeric (80<sub>3</sub>) and tetrameric (80<sub>4</sub>) complexes. However, their mobilities could also be reflective of tetrameric and hexameric species. Strikingly, the abundance of cross-linked Gal80 species decreased in response to increasing amounts of Gal3 (Fig. 2, lanes 4 to 9), as a new band corresponding to the expected size of a Gal3-Gal80 heterodimer appeared. Next, we wanted to verify that the gradual disappearance of Gal80 oligomers and the corresponding appearance of Gal3-Gal80 complex was a consequence of Gal3 binding to Gal80. We addressed this by performing similar cross-linking experiments using the Gal3-E116G mutant that does not bind to Gal80 (47). To compare
the mutant to WT Gal3, we had to use tagged Gal3 proteins, 6×His-Gal3-E116G and 6×His-tagged WT Gal3 (6×His-Gal3), because the mutant molecules could not be purified by binding to 6×His-Gal80. As expected, cross-linked 6×His-Gal80 oligomers were diminished in the presence of 6×His-Gal3 (see Fig. S1A in the supplemental material) but not in the presence of 6×His-Gal3-E116G (see Fig. S1B). These gels did not exhibit a distinct band corresponding to the cross-linked Gal3-Gal80 complexes like we observed for untagged Gal80 and Gal3 molecules. We do not know the basis for this. Perhaps 6×His-Gal3–6×His-Gal80 complexes migrate similarly to Gal80-Gal80 complexes and are obscured on the gel. Alternatively, the 6×His tags on Gal3 and/or Gal80 might inhibit cross-linking of the two species.

Native gels also showed a reduction in Gal80 self-association in response to formation of Gal3-Gal80 complexes. Two separate binding reaction series were performed in which the concentration of Gal3 was increased against a constant concentration (2.5 μM) of Gal80 (Fig. 3A). We detected Gal80 oligomers on 4 to 16% gradient nondenaturing polyacrylamide gels containing ATP and galactose (Fig. 3A). These oligomeric species of Gal80 diminished in response to increased levels of Gal3, while a new species appeared. The mobility of this newly appearing species corresponds to that expected of a Gal3-Gal80 heterodimer, the galactose/ATP-dependent Gal3-Gal80 complex first detected by analytical filtration chromatography (48). We cut out the newly appearing band

FIG 2 Cross-linking followed by SDS-PAGE shows that the levels of Gal80 multimers are reduced in response to Gal3-Gal80 interaction. Untagged Gal3 (3 μM) alone when incubated with 500 μM ATP and 25 mM galactose in 30 mM formaldehyde did not show any Gal3 oligomers (lane 1). A fixed amount of Gal80 (2 μM) was incubated with increasing amounts of Gal3 (lanes 4 to 9 have 0.3 μM, 0.6 μM, 1.2 μM, 3 μM, 6 μM, 12 μM Gal3, respectively) in the presence of 500 μM ATP, 25 mM galactose, and 30 mM formaldehyde at 4°C (lanes 4 to 9). The samples were resolved on a 7.5% SDS-polyacrylamide gel and were subsequently stained with Coomassie blue (n = 2) (Note that the Gal3 band in lane 6 is more than double that of the one in lane 5, which is not consistent with an increase from 0.3 μM to 0.6 μM. This discrepancy is likely due to a pipetting error.).

FIG 3 Native PAGE shows that the levels of Gal80 multimers decrease in response to Gal3-Gal80 complex formation. (A) 2.5 μM untagged Gal80 was incubated in series with increasing amounts of untagged Gal3 (0.4 μM, 1 μM, 2 μM, 4 μM, and 10 μM) in the presence of 2 mM ATP and 25 mM galactose at 4°C for 2 h. The samples were then incubated with loading buffer (100 mM Tris-Cl [pH 8.0], 40% glycerol, 0.5% Serva Blue G) for 10 min and subsequently resolved at 4°C on a 4 to 16% polyacrylamide gradient gel containing 2 mM ATP and 25 mM galactose (n = 2). (B) The band labeled 80-3 in panel A was cut out from the native gel. The peptides from this cutout band were analyzed in a 10% SDS-polyacrylamide gel. The positions of molecular mass markers (MM Markers) are indicated to the right of the gel. (C) The native PAGE in panel A was repeated with a Gal80S-2 mutant that is defective in Gal3 binding (n = 2).
binding sites (32 UASGAL sites total). This array is located close to
a locus. Gal80
2mYFP dissociates from DBD-Gal80 in a Gal3- and
galactose-dependent manner in live cells. Taken all together, the
results presented above constitute strong evidence that under in
vitro conditions, Gal3 association with Gal80 reduces Gal80 self-
association. To test whether Gal3 has such an effect in live yeast
cells, we set up a spinning-disk confocal microscopy-based exper-
iment that takes advantage of a previously developed UASGAL (up-
stream activation sequence in GAL gene promoter) array system
(29). The UAS
GAL
array in yeast S. cerevisiae consists of eight tandem
P
GAL1
-GST-1
ADH1
fusion genes that each have four Gal4
binding sites (32 UAS
GAL
sites total). This array is located close to
an array of 64 LacO elements at the genomic LEU2 locus. Gal80
fused to the Gal4 DNA-binding domain (DBD-Gal80), Gal80-
2mYFP, and Gal3-mCherry (Fig. 4; see Fig. S3A to C in the sup-
plemental material) from yeast ADH2 promoters were expressed in
the cells in which gal3 was deleted (Sc857) (Fig. 5C) or in the
gal3 deletion strain (Sc858) (Fig. 5D). Furthermore, when a
mutant of Gal3, Gal3
C-D368V
, which is capable of binding to
Gal80 in the absence of galactose (23), was expressed in the cells in
which gal3 was deleted (Sc858), we did not detect clusters in glyc-
erol-lactic acid medium (see Fig. S5C in the supplemental mate-
rial). On the other hand, the same host strain with a variant of Gal3
(Gal3D111C) that does not bind to Gal80 exhibited Gal80 clusters
even 2 h after the addition of galactose (see Fig. S5D). Thus, these
results revealed that there is a coalescence of Gal80-2mYFP within
subnuclear regions in the absence of galactose and that these
clusters are dispersed in response to galactose-triggered Gal3-Gal80
interaction.

**DISCUSSION**

The in vitro and in vivo studies presented here reveal for the first
time that the binding of Gal3 to Gal80 reduces the levels of dimers
and higher-order forms (oligomers or multimers) of Gal80. Our
in vitro data were obtained by using three approaches. One ap-
proach utilized co-IP of two differently tagged versions of Gal80 as
a measure of the various Gal80 self-association assemblies. The
other two approaches involved detection of Gal80 monomer, dimer,
and oligomer species by gel electrophoresis with or without
chemical cross-linking. All approaches allowed detection of Gal3-
Gal80 complexes and clearly demonstrated substantial reductions
in the levels of Gal80 dimer and multimer species under condi-
tions favoring the Gal3-Gal80 interaction.

Both native and formaldehyde-treated samples of Gal80 showed multiple forms of Gal80 with electrophoretic mobilities consistent with those of dimers and oligomers. These forms of Gal80 were diminished in response to increases in the concentra-
tion of Gal3 in the presence of galactose. In these experiments, we
noted discrepancies between the relative yields of various cross-
linked species observed on SDS-polyacrylamide gels and the yields of
the apparently corresponding species evident on native gels.
UASGAL and a dimer of Gal80-2mYFP binds to each DBD-Gal80 dimer. It is likely that a DBD-Gal80 dimer associates with each and strong dimerization of DBD and Gal80 (40, 44, 52) independently. In either case, our results establish that the interaction of Gal3 with Gal80 either competes with or destabilizes its ability to inhibit Gal4 comes from recent X-ray crystallographic studies from the Joshua-Tor lab. Their crystal structures for the dimeric and tetrameric forms of Gal80 and a Gal80-Gal4AD complex support the notion that Gal80 and Gal4 can interact to form a 2:2 heterotetramer (4). That work also identified an amino acid substitution (N230R) within the Gal80 dimerization surface that impairs Gal80 self-association also impairs Gal4AD-Gal80 inter-

In contrast to a previously proposed structural model (21), the results of our in vitro and in vivo experiments provide compelling evidence that Gal3 binding to Gal80 reduces the levels of Gal80 dimers and multimers. Mechanistically, how the binding of Gal3 to Gal80 brings about such a reduction is not clear. This could occur through destabilization of preexisting Gal80 multimers, competition with Gal80 self-association, or both.

What might be the biological relevance of this action of Gal3 on Gal80-Gal80-Gal80 self-association? Melcher and Xu obtained both in vitro and in vivo data documenting Gal80 self-association and implicating roles for Gal80 dimers and tetramers in inhibition of Gal4 (40). The results of their in vitro experiments revealed that Gal80 dimerizes with high affinity ($K_d \approx 0.1$ to $0.3$ nM), Gal80 dimers self-associate with a $K_d \approx 50$ nM, and Gal80 assembles into a 2:2 complex with a Gal4 dimer residing at its target UASGAL DNA site. Their in vivo experiments entailed measuring promoter activation as a function of the spacing between neighboring UASGAL sites. Their results indicated that Gal80 dimer-dimer interaction is stabilized under the natural spacings of multiple UASGAL sites located upstream of the very tightly repressed GAL1, GAL10, GAL7, and GAL2 genes (53). Such evidence provided a plausible explanation for why the MEL1 and GAL6 genes that have only one UASGAL site (53) show weaker repression (in the absence of galactose) and consequently, appreciable Gal4-dependent basal level of expression (i.e., weaker Gal80 inhibition) (40). Additional support for the notion that Gal80 self-association is important for its ability to inhibit Gal4 comes from recent X-ray crystallographic studies from the Joshua-Tor lab. Their crystal structures for the dimeric and tetrameric forms of Gal80 and a Gal80-Gal4AD complex support the notion that Gal80 and Gal4 can interact to form a 2:2 heterotetramer (4). That work also identified an amino acid substitution (N230R) within the Gal80 dimerization surface that impairs Gal80 self-association also impairs Gal4AD-Gal80 inter-

Such discrepancies are expected due to the inherently low cross-linking efficiency and the fact that a higher number of cross-links are required to capture trimers and tetramers relative to dimers. Nevertheless, these results highlight the occurrence of dimer and multimeric forms of Gal80 in the absence of galactose and document the emergence of Gal3-Gal80 complexes at the expense of Gal80 oligomers.

Our in vivo data were obtained using fluorescence microscopy of live yeast cells that contain a UASGAL array and express two interacting forms of Gal80, Gal80-2mYFP and DBD-Gal80. The Gal80-2mYFP is known to retain all normal Gal80 regulatory functions (29), and the DBD-Gal80 binds to the UASGAL site, Gal4, and Gal3 (44). Using such cells, we observed galactose- and Gal3-dependent dissociation of Gal80-2mYFP from UASGAL-associated DBD-Gal80. Gal3-mCherry association with DBD-Gal80 at the array followed the dissociation of Gal80-2mYFP closely. We do not know the detailed composition of the UASGAL array-associated DBD-Gal80-2mYFP complexes. Considering the high affinity of Gal4-DBD for the UASGAL site (3 nM [49]) (50, 51) and strongimerization of DBD and Gal80 (40, 44, 52) independently, it is likely that a DBD-Gal80 dimer associates with each UASGAL and a dimer of Gal80-2mYFP binds to each DBD-Gal80 dimer. It is also possible that a single dimer of DBD-Gal80 associated with a single UASGAL acts to nucleate binding of more than one dimer of Gal80-2mYFP through higher-order Gal80-2mYFP self-associations. In either case, our in vivo results establish that the interaction of Gal3 with Gal80 either competes with or destabilizes dimer-dimer and possibly higher-order, multimeric assemblies of Gal80. Given that at least a single DBD-Gal80 dimer is expected to remain at each UASGAL site within the array under galactose conditions, the Gal3-mCherry fluorescent dot we observe is likely due to either one or two Gal3-mCherry molecules bound to each DBD-Gal80 dimer.

FIG 5 Gal80-2mYFP forms nuclear clusters that dissipate in response to galactose-activated Gal80-Gal3 interaction. (A) Gal80-2GFP and Sec63-mCherry were expressed from genomic loci in Sc1029 cells. Sec63-mCherry marked the nuclear and cellular peripheries ($n > 10$). (B) Sc656 cells expressing Gal80 fused to two monomeric YFP molecules (Gal80-2mYFP) were grown to mid-log phase in medium containing glycerol-lactic acid and were loaded into Cellasic microfluidics plate chambers. Localization of Gal80-2mYFP in the uninduced cells was imaged. Galactose was then removed from the medium, and the same cells were imaged again at the indicated time points ($n > 5$). (C) The Gal80-2mYFP cells (Sc657) were grown to mid-log phase in glycerol-lactic acid-containing medium. Images were acquired either in the absence of galactose or after the cells were incubated in 2% galactose for 1 h ($n > 10$). (D) Genomic Gal80 was tagged with 2mYFP in gal3Δ cells (Sc838). The cells were prepared, and images were acquired as described above for panel C ($n > 5$).
action and Gal80 inhibition of Gal4 transcriptional activity (4). Taken together, the results from the Melcher and Joshua-Tor labs lend support to the idea that Gal80 self-association assemblies play a physiological role in the GAL gene switch. By extension, we propose that Gal3’s effect on the levels of Gal80 dimers and oligomers is an important mechanistic event in the galactose-triggered activation of Gal4.

Our discovery of Gal80 intranuclear clusters and their dissipation by galactose-activated Gal3 may well represent an additional illustration of Gal3’s effect on Gal80 self-assembly. Tracking of these clusters over time indicated that they are very dynamic assemblies. Our ability to image these Gal80 formations is no doubt an illustration of Gal3’s effect on the levels of Gal80 dimers and oligomers. Our ability to image these Gal80 formations is no doubt an important mechanistic event in the galactose-triggered activation of Gal4.

REFERENCES


