Gal3p and Gal1p interact with the transcriptional repressor Gal80p to form a complex of 1:1 stoichiometry

David J. TIMSON, Helen C. ROSS and Richard J. REECE¹

School of Biological Sciences, The University of Manchester, 2.205 Stopford Building, Oxford Road, Manchester M13 9PT, U.K.

The genes encoding the enzymes required for galactose metabolism in *Saccharomyces cerevisiae* are controlled at the level of transcription by a genetic switch consisting of three proteins: a transcriptional activator, Gal4p; a transcriptional repressor, Gal80p; and a ligand sensor, Gal3p. The switch is turned on in the presence of two small molecule ligands, galactose and ATP. Gal3p shows a high degree of sequence identity with Gal1p, the yeast galactokinase. We have mapped the interaction between Gal80p and Gal3p, which only occurs in the presence of both

ligands, using protease protection experiments and have shown that this involves amino acid residue 331 of Gal80p. Gel-filtration experiments indicate that Gal3p, or the galactokinase Gal1p, interact directly with Gal80p to form a complex with 1:1 stoichiometry.

Key words: Gal4p, galactose, gene expression, protein–protein interaction, yeast.

INTRODUCTION

Saccharomyces cerevisiae utilizes galactose via the enzymes of the Leloir pathway. When yeast cells are grown in the absence of galactose, the genes encoding the enzymes of the pathway (the *GAL* genes) are transcriptionally inert (reviewed in [1–3]). If the cells are switched to medium in which galactose is the sole carbon source, then the *GAL* genes are rapidly induced and transcribed at high levels [4]. The induction of the *GAL* genes is controlled by the interplay of three proteins: a transcriptional activator, Gal4p; a repressor, Gal80p; and a ligand sensor, Gal3p. Induction appears to occur as a result of a galactose- and ATP-dependent interaction between Gal3p and Gal80p [5–8]. The nature of the transcriptionally active complex remains unclear. It has been suggested that the association of Gal3p and Gal80p results in the formation of a transcriptionally active Gal4p–Gal80p–Gal3p complex [9]. It has also been suggested that the association of Gal3p with Gal80p results in the movement of Gal80p from the activation domain of Gal4p to a different part of the protein [10], and, in a different study, that the interaction between Gal3p and Gal80p occurs solely in the cytoplasm of yeast cells [11]. The apparent discrepancies between these data have yet to be resolved.

Gal3p is the ligand sensor of the *GAL* genetic switch [9]. The protein is highly related (73 % identity and 92 % similarity at the amino acid level) to Gal1p, the galactokinase of the Leloir pathway. Gal3p has no galactokinase enzymic activity itself [12], but the close relationship between Gal1p and Gal3p has been underlined by recent work showing that the insertion of just two amino acids from Gal1p into Gal3p turned the latter into a galactokinase [13].

In the present study, we set out to determine the site of interaction on Gal80p with Gal3p and the nature of the interaction between the two proteins. Our data show that both galactose and ATP are required to promote a direct physical interaction between Gal3p and Gal80p. The interaction involves amino acids in the C-terminal part of Gal80p.

MATERIALS AND METHODS

Protein expression and purification

His-tagged Gal1p, Gal3p, Gal80p and Gal80^{S-2}p were over-expressed in, and purified from, yeast as described previously [9], except that yeast cells were broken as described in [14]. Gal4p (amino acids 1–93 fused to amino acids 768–881) was over-expressed in *Escherichia coli* and purified as described previously [15].

Protease protection experiments

Gal3p and Gal80p were mixed in buffer A [50 mM Hepes/NaOH (pH 8.0), 150 mM NaCl, 10 % (v/v) glycerol and 1.4 mM 2-mercaptoethanol] at the concentrations stated in the absence or presence of ligands (galactose and ATP) as required, and incubated at 30 °C for at least 30 min. After this time, tosyl-lysylchloromethane ('TLCK')-treated α -chymotrypsin (Sigma) was added to a final concentration of 300 nM, and digestion allowed to proceed for 30 min at 30 °C. Products were analysed by SDS/PAGE [15 % (w/v) gels].

Analytical gel filtration

Gal3p (or Gal1p) and Gal80p were mixed in buffer A in the absence or presence of ligands (200 μ l total volume) and incubated at 20 °C for at least 30 min, prior to loading on to a Sephadex S75 column (bed volume, V_t , of 24 ml; Amersham Biosciences), which had been pre-equilibrated in buffer A containing the same ligand(s) at the same concentration as the protein mixture. The column was run at 0.5 ml/min and 0.5 ml fractions were collected. Proteins were precipitated from these fractions by acetone precipitation and analysed by SDS/PAGE.

The column was calibrated using a set of molecular-mass standards (Amersham Biosciences). The elution volume (V_e) for each standard protein was determined in buffer A along with the void volume of the column (V_0), which is equal to the elution volume of Blue Dextran. A standard curve was obtained by

Abbreviation used: BS³, bis(sulphosuccinimidyl) suberate.

¹ To whom correspondence should be addressed (e-mail Richard.Reece@man.ac.uk).

plotting K_{av} against the logarithm of the molecular mass, where K_{av} is given by $(V_e - V_o)/(V_t - V_o)$.

Protein–protein cross-linking

Gal4p (2 μ M) was mixed with either Gal80p (2 μ M) or Gal80^{S-2}p (2 μ M) in buffer A and incubated at 20 °C for 15 min. Bis(sulpho-succinimidyl) suberate (BS³; Pierce) is an amine cross-linking agent with a spacer arm of 1.1 nm [16]. It was dissolved in 5 mM sodium citrate buffer (pH 5.5) and then added immediately to protein solutions at a final concentration of 0.1 mM. The reactions were then incubated at 20 °C for 30 min. To quench the reaction, Tris/HCl (pH 7.5) was added to a final concentration of 25 mM, and the reaction was incubated at 20 °C for a further 15 min. Samples were analysed by SDS/PAGE.

Analytical methods

Protein concentrations were determined using the method of Bradford [17]. SDS/PAGE was carried out as described by Laemmli [18]. Acetone precipitation of protein samples was carried out by adding an equal volume of acetone and placing the samples at –20 °C for 30 min. Samples were then centrifuged at 15000 *g* in a bench-top microcentrifuge for 10 min at 20 °C, the supernatant was removed and the pellets were dried at 94 °C. Pellets were redissolved in 15 μ l of buffer A and an equal volume of SDS-loading buffer, prior to heating at 94 °C and loading on to an SDS/polyacrylamide gel. Protein sequencing was performed by Dr Linda Berry (School of Biological Sciences, University of Manchester, U.K.) using an Applied Biosystems 476A machine.

RESULTS

Gal3p protects Gal80p from proteolysis in the presence of galactose and MgATP

Protease protection experiments were used to determine the site of interaction between highly purified Gal3p and Gal80p. In the absence of Gal3p, Gal80p was digested by chymotrypsin into

three principal fragments of sizes 36, 25 and 12 kDa (Figure 1A, lane 2). This digestion pattern was not affected by the presence of either MgATP or galactose alone or together (Figure 1A, lanes 3–5). A similar digestion pattern of Gal80p was observed using a variety of proteases (results not shown).

In contrast, when Gal3p was digested under identical conditions, a greater number of protein fragments were produced, although less of the starting material was consumed (Figure 1A, lane 6). This pattern was not changed by the presence of either MgATP or galactose alone or together (Figure 1A, lanes 7–9). When the two proteins were mixed together and exposed to the protease, the digestion pattern was approximately additive (Figure 1A, lane 11). This was also the case when both proteins were mixed in the presence of either MgATP or galactose alone (Figure 1A, lanes 12 and 13). However, in the presence of both ligands (Figure 1A, lane 14), the 36 and 12 kDa bands (marked with an asterisk in Figure 1) from Gal80p were reduced markedly in intensity. Protein sequencing revealed that the 36 kDa band began at amino acid 4 and the 12 kDa at residue 332 of Gal80p (results not shown).

Since the ligands themselves did not affect the digestion patterns of the proteins on their own, the most likely explanation for this change was that the binding of Gal3p to Gal80p was preventing access to the digestion site on Gal80p by the protease, resulting in protection of Gal80p. Furthermore, this experiment suggested that both ligands are required to promote the interaction between Gal3p and Gal80p. The tolerance of the system to changes in the nucleotide ligand was tested (Figure 1B). MgADP was found to substitute effectively for MgATP, but MgAMP was ineffective in promoting binding and protection from protease digestion.

A 1:1 interaction between Gal80p and Gal3p requires both ligands

Gel filtration is a powerful technique for demonstrating protein–protein interactions *in vitro*. When Gal3p alone was applied to a gel-filtration column, it was eluted as a single symmetrical peak

Figure 1 Binding of Gal3p to Gal80p protects the latter from limited proteolytic digestion by chymotrypsin

(A) Gal3p (10 μ M) was mixed with Gal80p (4 μ M) in the presence of galactose (2.5 mM) and/or MgATP (2.5 mM) as indicated. Reaction mixtures were digested with chymotrypsin (300 nM) for 30 min at 30 °C, and then resolved by SDS/PAGE [15% (w/v) gel]. Lane M, molecular-mass markers. (B) ADP can substitute for ATP in promoting binding of Gal3p and Gal80p. Reaction mixtures were set up as above, except that MgATP was substituted with MgAMP or MgADP (both at 2.5 mM) as appropriate. Molecular-mass markers (in kDa) are shown on the left.

Figure 2 Gel-filtration analysis of Gal3p and Gal80p

(A) Gal3p (13 μ M), Gal80p (3 μ M), or a mixture of both, were applied to a Superdex S75 column (Amersham Biosciences). UV absorbance traces at 280 nm are shown alongside SDS/PAGE gels of acetone-precipitated samples from across the major peaks. The numbers on the gels correspond to the approximate elution volume (in ml). (B) A discrete Gal3p–Gal80p complex can be isolated by gel filtration in the presence of galactose (GAL) and ATP. Concentrations of proteins were as in (A). Ligands were added both to the sample, running buffer at a final concentration of 2.5 mM. Lane M, molecular-mass markers.

(Figure 2A), corresponding to a molecular mass of 46 kDa (actual molecular mass is 58 kDa). SDS/PAGE analysis of acetone-precipitated fractions from this peak confirmed that it corresponded to Gal3p elution. When a similar quantity of Gal80p was applied to the column, no visible peaks were observed. Gal80p was found to be present in small amounts across a wide range of elution volumes. This suggests that Gal80p does not exist as one single discrete entity in solution, and is consistent with the protein having a highly flexible conformation and/or existing in a variety of multimeric states. This result is consistent with recent observations of a variety of

multimeric states of Gal80p [19]. An alternative explanation is that Gal80p is binding to the column itself, but we disfavour this explanation, since the experiments were performed at relatively high salt levels (150 mM NaCl) and adherence to the column would result in an elution profile shifted to higher volumes, rather than the lower volumes observed.

When a mixture of Gal3p and Gal80p was applied to the column, a single peak was observed at exactly the same elution volume as was seen with Gal3p alone (Figure 2A). This peak contained almost exclusively Gal3p, as shown by SDS/PAGE analysis of acetone-precipitated fractions. A similar result was

Figure 3 Gal80^{S-2}p, a super-repressor mutant, is unable to bind to Gal3p, but does bind Gal4p

(A) Gal80^{S-2}p (2 μ M) was mixed with Gal3p (13 μ M) in the presence of galactose and MgATP (both at 2.5 mM). The mixture was applied to a Superdex S75 column pre-equilibrated in buffer containing both ligands at this concentration. (B) Gal80p and Gal80^{S-2}p, and Gal4p (all 2 μ M) were mixed as indicated in the absence or presence of the cross-linker BS³ (0.1 mM final concentration). Products were resolved by SDS/PAGE [10% (w/v) gel]. Lane M, molecular-mass markers.

seen when a mixture of the two proteins was applied in the presence of either galactose or MgATP (Figure 2B). However, when both proteins were mixed in the presence of both ligands, a new peak appeared. This peak corresponded to a molecular mass of 91 kDa, and SDS/PAGE analysis of fractions from it revealed the presence of both Gal80p and Gal3p. Visual inspection of the bands suggested that both proteins were present in approximately equal amounts (despite an excess of Gal3p being loaded). This, taken together with the apparent molecular mass, suggests that a complex containing one molecule of Gal3p and one molecule of Gal80p had been isolated, despite Gal80p existing in free solution as a variety of multimeric states [19].

Since Gal3p protects Gal80p from proteolytic digestion at amino acid residue 331, mutations in this region might be expected to disrupt the binding. Gal80^{S-2}p is a super-repressor

mutant carrying the point mutation Glu³⁵¹ $\rightarrow$ Lys [20]. Cells carrying this mutation are unable to induce the *GAL* genes even in excess galactose. The ability of this mutant protein to bind Gal3p was tested in the gel-filtration assay and was found to be unable to interact (Figure 3A). However, both the mutant and wild-type proteins could be cross-linked to Gal4p using the amine cross-linker BS³ (Figure 3B). The extent of cross-linking was not significantly different when the two proteins were compared, suggesting similar affinities for Gal4p. Interestingly, when the two forms of Gal80p were exposed to cross-linker in the absence of Gal4p, Gal80p, but not Gal80^{S-2}p, underwent cross-linking, which resulted in a doublet on the gel (Figure 3B, lane 3). This may suggest that Gal80p is amenable to intramolecular cross-linking, but that this ability is disrupted in the mutant protein. Additionally, although there is only a single amino acid changed

Figure 4 Interaction between Gal1p and Gal80p

(A) Binding of Gal1p to Gal80p protects the latter from proteolytic digestion. Gal1p (17 μ M) was mixed with Gal80p (5 μ M) in the presence of galactose and MgATP (both 2.5 mM) as indicated. Reaction mixtures were digested with chymotrypsin (300 nM) for 30 min at 30 °C, and then resolved by SDS/PAGE [15% (w/v) gel]. (B) A Gal1p–Gal80p complex can be isolated by gel filtration. A mixture of Gal1p (16 μ M), Gal80p (1 μ M), galactose (2.5 mM) and MgATP (2.5 mM) was applied to a Superdex S75 column equilibrated in running buffer supplemented with both ligands at 2.5 mM. Lane M, molecular-mass markers.

between the wild-type and mutant proteins, there may be significant structural alterations between Gal80p and Gal80^{S-2}p. However, any such changes only affect the ability of the protein to bind Gal3p and not its interaction with Gal4p.

Gal1p interacts with Gal80p in a similar manner to Gal3p

Gal1p was shown to bind Gal80p in both protease protection and gel-filtration experiments (Figure 4). The treatment of Gal1p with chymotrypsin yielded a large number of breakdown products (Figure 4A, lane 2). The combined digestion of Gal1p and Gal80p yielded an additive digestion pattern (Figure 4A, lane 4). The Gal80p-derived fragments (indicated by asterisks in Figure 4A, lane 4), overlapped with some of the Gal1p-derived fragments. It was still clear, however, that the protection of Gal80p from chymotryptic digestion required Gal1p, galactose and MgADP (Figure 4A, lane 5). MgATP was not tested in this experiment, as it is a substrate for the enzyme. In the presence of MgATP and galactose, a complex between Gal1p and Gal80p was isolated by gel filtration (Figure 4B). In this experiment, MgATP could be used, as the substrates were constantly replenished as the protein mixture moved down the column. Like the Gal3p–Gal80p complex, the Gal1p–Gal80p is a 1:1 stoichiometric complex, as judged by its apparent molecular mass (approx. 90 kDa) and the intensity of staining of the bands after SDS/PAGE (Figure 4B, lanes 8 and 9).

DISCUSSION

In order to understand how gene expression is switched on and off by systems such as the *GAL* genetic switch, it is necessary to know how the various protein and small molecule components of it interact and how these interactions influence each other. Both the protease protection and gel-filtration experiments show that there is a direct interaction between Gal3p and Gal80p in the presence of both ligands (ATP and galactose). It appears that there is an absolute requirement for the presence of both ligands in order to promote this interaction. Protease protection suggests that the interaction is centred at, or near, residue 331 in Gal80p.

This conclusion is supported by the fact that Gal80^{S-2}p, a super-repressor mutant, is unable to bind to Gal3p, although its interaction with Gal4p is unaffected. The nature of this mutation is a single amino acid substitution at residue 351, close to the site of interaction suggested by protease protection.

Gel filtration clearly indicates that the interaction between Gal3p and Gal80p results in the formation of a 1:1 complex. Furthermore, that the complex could be isolated by this method shows that it has a relatively long half-life. Even though the equilibrium has been disturbed at the moment the mixture of complexed and uncomplexed proteins enters the gel-filtration column, sufficient remains after the 20 min required for separation so that it could be detected. A similar complex could be detected between Gal1p, the galactokinase, and Gal80p, although the yield of the complex was low. It is possible that the Gal1p–Gal80p interaction is less strong than the one between Gal3p and Gal80p. It has been noted previously that approx. 40-fold more Gal1p than Gal3p is required to activate the *GAL* genetic switch *in vitro* [9].

Gal3p acts as the ligand sensor of the *GAL* genetic switch. In the present study, we show that both its ligands, galactose and MgATP, are required for interaction with Gal80p. This interaction has a 1:1 stoichiometry and requires no other proteins or small molecules to bring it about. The high degree of similarity between the galactokinase, Gal1p, and the ligand sensor, Gal3p, suggest that much of what we learn about one will apply to the other. Although Gal3p has never been shown to be a galactokinase, Gal1p will act as the ligand sensor in the *GAL* genetic switch both *in vivo* [12] and *in vitro* [9] and the present study). We have shown that Gal1p, when acting as a galactokinase proceeds via an ordered ternary-complex mechanism in which ATP binds first (D. J. Timson and R. J. Reece, unpublished work). It seems likely that this ordered ligand binding is preserved when Gal1p acts as a ligand sensor and also in Gal3p. We therefore conclude that *GAL* gene activation begins with the binding of ATP to Gal3p (or Gal1p). This induces a conformational change in the ligand sensor, creating a binding site for galactose. This Gal3p–ATP–galactose ternary complex is then capable of interacting with Gal80p, an event which relieves the inhibition of Gal4p and permits transcription to proceed.

We are grateful to members of the Reece laboratory for their comments on the manuscript. The work was funded by the Biotechnology and Biological Sciences Research Council and the Leverhulme Trust.

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Received 1 November 2001; accepted 30 January 2002