The tetratricopeptide repeats of Ssn6 interact with the homeo domain of $\alpha 2$

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The tetratricopeptide repeat (TPR) is a 34-amino-acid degenerate sequence motif that is found in a large variety of proteins, both prokaryotic and eukaryotic. TPRs are usually found in tandem arrays of up to 16 copies. In this paper we identify a direct interaction between the TPRs of Ssn6, a general transcriptional repressor, and $\alpha 2$, a cell-type regulator in *Saccharomyces cerevisiae*. Five of the Ssn6 TPRs were tested individually, and all were found to interact specifically with $\alpha 2$. These results suggest a model for TPR-protein interactions and for the role that a tandem array of TPRs may have in mediating transcriptional repression.

[Key Words: Tetratricopeptide repeat; homeo domain; Ssn6; $\alpha 2$; transcriptional repression; repeated amino acid motif]

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The tetratricopeptide repeat (TPR) motif is a 34-amino-acid domain found in tandem arrays in a large number of proteins. This family now numbers almost 30 proteins and includes members with very diverse cellular functions (for review, see Lamb et al. 1995). TPR-containing proteins are known to have roles in cell-cycle progression, protein degradation, transcription, RNA splicing, chromosome segregation, and protein import into mitochondria and peroxisomes (Lamb et al. 1995), and they have been found in organisms ranging from bacteria to humans. In all of the proteins tested, the TPRs have been found to be required for biological function (Hirano et al. 1990; Schultz et al. 1990; Sikorski et al. 1991, 1993; Lamb et al. 1994; Chen et al. 1995; Tzamarias and Struhl 1995). In spite of the obvious importance of the TPR motif, little is known of its molecular role.

Four years ago, when the TPR motif was first recognized, it was suggested to mediate protein-protein interactions (Goebl and Yanagida 1991). A number of recent papers indicates that this is the case (Brocard et al. 1994; Lamb et al. 1994; Chen et al. 1995; Terlecky et al. 1995; Tzamarias and Struhl 1995). Questions remain, however, as to how these interactions occur. For example, TPRs nearly always exist in tandem arrays, although the reason for this arrangement is not known, nor is it known what types of protein structures are recognized by the TPRs. It is also not known whether or not a single TPR constitutes an independent protein-protein interaction domain.

An attractive and reasonably well-understood biological context in which to study TPR-protein interactions involves the *Saccharomyces cerevisiae* TPR protein Ssn6. Ssn6 has been termed a general transcriptional repressor, as it is responsible for repressing a number of

different gene types in yeast (Keleher et al. 1992). In the cell, Ssn6 is complexed with Tup1 (Williams et al. 1991; Tzamarias and Struhl 1995), and this complex is recruited to the promoters of the genes they repress by sequence-specific DNA-binding proteins (Keleher et al. 1992; Komachi et al. 1994; Tzamarias and Struhl 1994). Once at a promoter, an as-yet-undefined mechanism leads to gene repression. Ssn6 contains 10 copies of the TPR motif found in a tandem array at the amino terminus of the protein (Schultz et al. 1990; Sikorski et al. 1990), and the region of Ssn6 that contains the TPRs is essential for its function (Schultz et al. 1990). Several putative non-TPR-containing target proteins of Ssn6 have been identified; Ssn6 interacts with Tup1 (Williams et al. 1991; Tzamarias and Struhl 1995) and has been proposed to interact with the DNA-binding proteins $\alpha 2$, Rox1, Mig1, and the Crt repressor (Nehlin et al. 1991; Keleher et al. 1992; Zhou and Elledge 1992; Balasubramanian et al. 1993; Treitel and Carlson 1995; Tzamarias and Struhl 1995). Moreover, it is known that the amino terminus of Ssn6, which includes the TPRs, plays an important role in mediating these examples of repression (Schultz et al. 1990; Tzamarias and Struhl 1995), yet the means by which the TPRs function is not well understood. Do the TPRs interact directly with DNA-binding proteins? How is this region able to interact with many different proteins? Is one TPR responsible for binding one DNA-binding protein, or do all the TPRs contribute to binding a particular protein?

In this paper we have concentrated on one potential binding partner of Ssn6, $\alpha 2$, a DNA-binding protein that has been well characterized genetically, biochemically, and structurally (for review, see Johnson 1992). $\alpha 2$ is a member of the highly conserved family of homeo do-

main proteins. The cocrystal structure of the $\alpha 2$ homeo domain bound to DNA demonstrates a high degree of similarity between this homeo domain and the numerous others from metazoans (Wolberger et al. 1991).

In this study we identify a direct interaction between the homeo domain of $\alpha 2$ and the TPRs of Ssn6, and we address two models for how TPRs might mediate protein–protein interactions. The first model proposes that each TPR mediates a specific interaction with a target protein and predicts that $\alpha 2$ should bind to one (or a few) of the TPRs. The second model proposes that TPRs function more like the repeats of the microtubule-binding protein Tau, in that each TPR repeat would have a weak, but specific, affinity for the target protein and the overall affinity between proteins would rely on several repeats working together (Butner and Kirschner 1991). By this second model we would expect $\alpha 2$ to interact with most or all of the TPRs independently. We find that the second model describes more accurately the interaction between the TPRs of Ssn6 and the homeo domain of $\alpha 2$.

Results

$\alpha 2$ binds directly to the TPRs of Ssn6

To test the hypothesis that TPRs mediate protein–protein interactions, we sought first to identify a direct interaction between Ssn6 and one of its suspected binding targets, $\alpha 2$. An affinity column was constructed in which a purified fusion protein containing the amino terminus of Ssn6 and glutathione *S*-transferase (GST) was immobilized on glutathione agarose. This fusion contains TPRs 1–10 as well as 45 additional amino acids on each side of the TPR array (GST:Ssn6, Fig. 1), and this region of Ssn6 has been shown to be sufficient for Ssn6 function in vivo (Schultz et al. 1990). Purified $\alpha 2$ was passed over this column, the column was washed, and the bound material eluted in high salt. As can be seen in Figure 2A, $\alpha 2$ was retained by the column matrix (as evidenced by its depletion from the flowthrough fractions) and was eluted from the column matrix by high salt.

That the interaction of $\alpha 2$ with GST:Ssn6 is specific is demonstrated by the following three observations. First, when a bacterial extract overexpressing $\alpha 2$ is passed over the column, the flowthrough fractions are depleted of $\alpha 2$ but contain most of the bacterial proteins present in the load (Fig. 2B). Thus, of the ~35 proteins that can be visualized by Coomassie staining, only $\alpha 2$ is observed to be depleted from the material passed over the column. Second, $\alpha 2$ is not depleted from a bacterial extract when the extract is passed over a column containing immobilized GST (data not shown). Third, $\alpha 2$ does not bind to a GST–TPR fusion if the TPR is derived from a protein other than Ssn6 (see below). These results indicate that $\alpha 2$ interacts with Ssn6 and not with the GST component of the fusion protein.

To prove that the TPRs are mediating this $\alpha 2$ –Ssn6 interaction we constructed a GST fusion containing TPRs 1–9 but lacking the 45 amino acids amino- and carboxy-terminal to the TPR region (GST:1–9, Fig. 1). Again, $\alpha 2$ was seen to be depleted specifically from a

Figure 1. (A) Schematic diagram showing the structure of full-length Ssn6. The polyglutamine repeats (solid box) and a stretch of repeated poly(glutamine-alanine) residues (lined box) are also indicated. Only the region containing the TPR domain is essential for the function of Ssn6 (Schultz et al. 1990). (B) Schematic diagrams of the GST:TPR fusions used in these experiments.

bacterial extract when passed over the column matrix (Fig. 2C). These results demonstrate that an array of nine TPRs is sufficient for the specific recognition and retention of $\alpha 2$ from a mixture of different proteins.

Many individual TPRs are able to bind $\alpha 2$

To test the hypothesis that a particular TPR was responsible for interacting with $\alpha 2$, we made several other GST fusions, which subdivided the region containing the TPRs: GST:1–7, GST:1–3, GST:3–9, and GST:6–8 (Fig. 1). Bacterial extracts containing $\alpha 2$ were passed over these columns and, in all cases, $\alpha 2$ was depleted specifically from the extract (Fig. 3A–D) and was eluted in high salt. This observation indicates that several binding sites for $\alpha 2$ are spread throughout the TPRs.

Next we tested whether single TPR repeats were able to bind specifically to $\alpha 2$. The fusions GST:1–2, GST:3, GST:6, GST:7, GST:8, and GST:9 (Fig. 1) were constructed, and $\alpha 2$ was specifically retained by and eluted from all six of the individual GST:TPR fusions tested (Fig. 4A–F). These results indicate that most—and perhaps all—of the TPRs of Ssn6 are competent to bind $\alpha 2$. However, the ability of a column to bind $\alpha 2$, as judged by the extent of depletion of $\alpha 2$ from the extract, appears to increase as the number of TPRs in the fusion increases [cf. $\alpha 2$ binding to GST:TPR 1–9 (Fig. 2C) to GST:3–9 (Fig. 3C), GST:1–2, and GST:3 (Fig. 4A,B)].

The interaction of $\alpha 2$ with TPRs is specific for the TPRs of Ssn6

From the above results it is clear that the TPRs of Ssn6 are able to bind $\alpha 2$ specifically from a mixture of pro-

Figure 2. $\alpha 2$ binds directly to the TPRs of Ssn6. (A) Coomassie blue-stained SDS gel showing the results of passing purified $\alpha 2$ over columns bearing the GST:Ssn6 fusion. Flowthrough fractions are loaded sequentially from left to right. The lane designated Col. Bed represents a sample of the column bed boiled in Laemmli buffer after the elution step. (B) Coomassie blue-stained SDS gel demonstrating that $\alpha 2$ is specifically depleted from a bacterial extract when passed over a column bearing the fusion GST:Ssn6. (C) Coomassie blue-stained SDS gel showing the results from passing bacterial extract overexpressing $\alpha 2$ over a column bearing the fusion GST:1-9. Note that this fusion lacks 38 of the 45 amino acids amino-terminal to the TPRs, as well as all residues carboxy-terminal to TPR9. In A and C the first elution fraction was not loaded on the gel. In B, the amount of GST:Ssn6 resin used in the experiment is roughly fivefold less than the amount of fusion-bound resin used in A and B. As a result $\alpha 2$ binding to the column in B saturated earlier, and so $\alpha 2$ is present in the flowthrough fractions. $\alpha 2$ is indicated by an arrow in A, B, and C. In Figs. 2-4 and 6, the depletion profile of $\alpha 2$ from the flowthrough fractions was reproduced consistently with each trial, although the elution efficiency of $\alpha 2$ varied from experiment to experiment with often less than full recovery of the amount of $\alpha 2$ bound to the column. Thus, the depletion of $\alpha 2$ from the flowthrough fractions rather than the amount of $\alpha 2$ present in the eluate should be compared from figure to figure.

teins. These results do not, however, address whether the interaction between $\alpha 2$ and the TPRs is specific for the TPRs of Ssn6. To address this question we tested whether a TPR from another *S. cerevisiae* protein, which is not suspected of interacting with $\alpha 2$ in vivo, binds $\alpha 2$ in vitro. We chose TPR5 from the protein Pas10. Pas10 is a peroxisomal import receptor (Van der Leij et al. 1993; Brocard et al. 1994). Its TPRs mediate an interaction with the -SKL signal peptide (Brocard et al. 1994; Terlecky et al. 1995). TPR5 was chosen because it is the only TPR of Pas10 that is less related in sequence to the TPRs of Ssn6 than the TPRs of Ssn6 are to each other. The net charge of Pas10-TPR5 is identical to that of Ssn6-TPR6, which interacts with $\alpha 2$ (Fig. 4C). When a crude extract containing $\alpha 2$ was passed over a GST:Pas10-TPR5 column, the $\alpha 2$ was found in the flowthrough lanes (Fig. 5, Flowthrough). In addition, $\alpha 2$ was not detected in the eluate from these columns (Fig. 5, Eluate). (This experiment was performed simultaneously with the experiment of Fig. 4C in which $\alpha 2$ was bound by TPR6 of Ssn6.) These results show that $\alpha 2$ does not bind to all TPRs and confirm that the interaction between the TPRs of Ssn6 and $\alpha 2$ is specific.

The homeo domain of $\alpha 2$ interacts with the TPRs of Ssn6

Next we determined what part of the $\alpha 2$ protein interacted with the TPRs of Ssn6. $\alpha 2$ (210 amino acids total) was digested with chymotrypsin to produce two fragments: 1-102 (amino-terminal) and 132-210 (carboxy-terminal) (Sauer et al. 1988). This latter fragment contains the homeo domain. A mixture of these two fragments was passed over a GST:1-9 column, and the results are shown in Figure 6A. The amino-terminal fragment of $\alpha 2$ flowed through the column, and no amino-terminal fragment was eluted in high salt. The carboxy-terminal fragment of $\alpha 2$ showed the opposite behavior: It was depleted from the flowthrough and reappeared in the elution fractions (Fig. 6A). This result shows that the TPR-binding determinant of $\alpha 2$ lies within or very near the homeo domain and that it is distinct from the part of $\alpha 2$ that interacts with Tup1 (Komachi et al. 1994).

The carboxy-terminal chymotryptic fragment includes, in addition to the homeo domain, a short carboxy-terminal tail. To test the ability of the homeo domain per se to interact with Ssn6, purified $\alpha 2$ homeo domain that lacked the tail (129-189) was passed over the GST:1-9 column (Fig. 6B). The homeo domain was depleted from the flowthrough fractions and eluted in the high salt wash, confirming that the binding site for the TPRs of Ssn6 lies within the homeo domain of $\alpha 2$.

The TPRs of Ssn6 interact preferentially with the $\alpha 2$ homeo domain

As the homeo domain is a highly conserved structure, we next tested whether the TPRs of Ssn6 exhibited specificity for the homeo domain of $\alpha 2$. The *Drosophila* engrailed (en) homeo domain and the $\alpha 2$ carboxy-terminal

Figure 3. Several different groups of TPRs can bind $\alpha 2$. Coomassie blue-stained SDS gels show the results of passing bacterial extracts overexpressing $\alpha 2$ over GST:1-7 (A), GST:1-3 (B), GST:3-9 (C), and GST:6-8 (D). $\alpha 2$ is indicated by an arrow in each of the panels. Flowthrough fractions are loaded sequentially from left to right. The elution lane represents a pool of the peak fractions containing protein, and the lane designated Col. Bed represents a sample of the column bed boiled in Laemmli buffer after the elution step. Wash fractions were not loaded on these gels. In B and C the flowthrough lanes represent every other fraction collected rather than a pool of two successive fractions as in A and D.

fragment were mixed together and loaded over a GST:1-9 column, and the flowthrough and elution fractions were assayed for the presence of the two proteins. As shown in Figure 6C, the $\alpha 2$ carboxy-terminal fragment is efficiently retained by the column. The en homeo domain, although depleted in the first flowthrough fraction and present in the eluate lane, is also present in the remainder of the flowthrough fractions. The presence of the en homeo domain in the flowthrough fractions as $\alpha 2$ continues to be depleted indicates that although the TPRs of Ssn6 are capable of interacting with the en homeo domain, they have a higher affinity for the $\alpha 2$ homeo domain than for the distantly related en homeo domain.

Discussion

The results of this paper demonstrate a direct interaction between two highly conserved protein motifs. One of these, the homeo domain, has been studied extensively. The other, the TPR, occurs in tandem arrays and is less well understood. The homeo domain studied in this work is found in $\alpha 2$, a DNA-binding protein involved in determining *S. cerevisiae* cell type, and the TPRs are in Ssn6, a general repressor of transcription that is recruited to DNA by $\alpha 2$. We observed that each of the single TPR units of Ssn6 tested in these experiments is competent to interact specifically with $\alpha 2$, suggesting that a TPR protein can interact in several different orientations with its target protein. This idea is analogous to the manner in which the Tau repeats have been proposed to bind microtubules (Butner and Kirschner 1991).

The homeo domain of $\alpha 2$ interacts with the TPRs of Ssn6

The homeo domain is thought of primarily as a DNA-binding module, but like most other DNA-binding domains, it is also known to interact with other proteins: For example, the homeo domain of the human Oct-1 protein interacts with the viral *trans*-activator VP16 (Stern et al. 1989; Stern and Herr 1991; Lai et al. 1992; Pomerantz et al. 1992) and the homeo domain of the yeast cell-type determination protein a1 interacts with the carboxy-terminal tail of $\alpha 2$ (Mak and Johnson 1993). The cocrystal structure of the homeo domain of a1 interacting with the carboxy-terminal tail of $\alpha 2$ has recently been solved (Li et al. 1995). It demonstrates that one way these interactions can occur is through the packing of an α -helix (the $\alpha 2$ tail) against helices 1 and 2 of the homeo domain. It has been suggested that the Oct-1/VP16 interaction may occur through a similar mechanism (Baxter et al. 1994; Li et al. 1995), and it is conceivable that the TPR-homeo domain interaction described in this paper also utilizes this mechanism. In any case, it seems likely that the convergence of two widespread protein motifs, the TPR and the homeo domain, to carry out a universal cellular function, transcriptional repression, is likely to be found in other organisms as well.

All of the single TPRs tested are capable of interacting with $\alpha 2$

As mentioned in the introductory section, there are two obvious models for how a repeated sequence motif such as the TPRs might function to mediate several different

Figure 4. $\alpha 2$ binds many individual TPRs. Coomassie blue-stained SDS gels show the results of passing bacterial extracts containing overexpressed $\alpha 2$ over columns bearing GST fusions to two or fewer TPRs: (A) GST:1-2; (B) GST:3; (C) GST:6; (D) GST:7; (E) GST:8; and (F) GST:9. $\alpha 2$ is indicated by an arrow. Flowthrough fractions represent every other fraction collected and are loaded sequentially from left to right. The eluate lane represents a pool of all the elution fractions containing protein. The lane designated Col. Bed represents a sample of the column bed boiled in Laemmli buffer after the elution step.

protein-protein interactions. The first model proposes that distinct repeat units are each responsible for binding particular target proteins; this may be the way another repeated, degenerate sequence motif—the LIM domain—functions (Schmeichel and Beckerle 1994). An alterna-

Figure 5. $\alpha 2$ does not bind a TPRs from Pas10. The Coomassie blue-stained SDS gel shows the results from passing bacterial extracts overexpressing $\alpha 2$ over a column bearing the GST:Pas10-TPR5 fusion. $\alpha 2$ is indicated by an arrow. Flowthrough fractions are loaded sequentially from left to right. The eluate lane represents a pool of all the elution fractions containing protein. The lane designated Col. Bed represents a sample of the column bed boiled in Laemmli buffer after the elution step.

tive model suggests that most or all of the TPRs in a protein are capable of interacting weakly with target proteins, creating a large surface on which the interaction can occur. According to this model the reiteration of TPRs would increase the probability that a productive collision will occur between the TPR protein and its target and would result in an increase in the K_D of the interaction. Because Ssn6 is complexed with Tup1 in the cell and because $\alpha 2$ interacts with both Ssn6 and Tup1, it is difficult to predict the increase in affinity contributed by Ssn6 having 10 TPRs rather than a single TPR. A nominal calculation indicates that the affinity of an $\alpha 2$ monomer is 10-fold higher for a string of 10 TPRs of equal binding strength than for a single TPR. This prediction is roughly consistent with the results of the column-binding experiments, although many features of the column chromatography (e.g., the stoichiometry of $\alpha 2$ to TPRs) are not accurately known. These experiments show that the ability of a TPR-containing fragment to bind $\alpha 2$ increases with the number of TPRs present in that construct [cf. the number of flowthrough fractions depleted of $\alpha 2$ in fusions of 9, 6, 3, or 1 TPRs in length (Figs. 2C, 3C, 3B, and 4B, respectively)]. Thus, the TPRs of Ssn6 are crudely analogous to the repeats of the Tau protein that function in binding microtubules. Each

Figure 6. $\alpha 2$ interacts with the TPRs through its homeo domain. Coomassie blue-stained SDS gels show the results of passing: (A) purified $\alpha 2$ treated with chymotrypsin over a GST:1–9 column. Arrows indicate the amino- and carboxy-terminal fragments of $\alpha 2$. (B) Purified $\alpha 2$ homeo domain over a GST:1–9 column. The homeo domain is indicated by an arrow. (C) A mixture containing the carboxy-terminal fragment of $\alpha 2$ (128–210, homeo domain and tail) and the en homeo domain over a GST:1–9 column. The $\alpha 2$ and en fragments are indicated by arrows. In A–C flowthrough fractions are loaded sequentially from left to right. The lane designated Col. Bed represents a sample of the column bed boiled in Laemmli buffer after the elution step.

of the four Tau repeats is capable of binding microtubules on its own, and the affinity of a Tau fragment for a microtubule increases as the number of repeats in the fragment increases (Butner and Kirschner 1991). In addition, Terlecky et al. (1995) have shown that the TPRs of Pas8, the *Pichia pastoris* homolog of Pas10, interact with the –SKL peptide and that the affinity of this interaction increases with the number of TPRs present in the construct used to test binding.

Ssn6 has been proposed to interact with five proteins in addition to $\alpha 2$, and many of these additional interac-

tions appear to be mediated by the TPRs (Tzamarias and Struhl 1995). A prediction based on the work with $\alpha 2$ is that at least some (and possibly all) of the TPRs must interact with several different target proteins. In support of this idea, a fragment containing Ssn6 TPRs 1–3 has been shown to interact with both $\alpha 2$ (this work) and Tup1 (Tzamarias and Struhl 1995).

What is the advantage of having an array of weak sites capable of interacting with many different target proteins?

The presence of an array of weak sites, each capable of interacting with a number of target proteins, enhances the specificity of binding of the Ssn6/Tup1 complex to DNA-binding proteins (for review, see Frankel and Kim 1991) and, in addition, generates a great deal of flexibility in the possible arrangements of the Ssn6/Tup1 complex with respect to DNA-binding proteins. The regulatory regions of different genes have very different architectures; for example, the distance from an operator (a negatively acting DNA sequence) to the TATA box varies considerably from gene to gene, and because Ssn6 and Tup1 are thought also to interact with the general transcription machinery they must be able to accommodate these differences. A distributed array of weak sites such as those found within both the TPRs of Ssn6 and another repeated amino acid motif, the WD, repeats of Tup1 could provide a solution to this problem by permitting the Ssn6/Tup1 complex to assemble in different orientations with respect to the DNA-bound proteins (Fig. 7). Although DNA and protein conformational flexibility are often invoked to explain the ability of transcriptional regulators to act at a distance, the model for flexibility in assembly proposed here may provide an additional way to span variable distances in DNA.

Figure 7. A schematic diagram showing how the Ssn6/Tup1 complex may accommodate differences in promoter architecture. A represents a regulatory protein (such as $\alpha 2$) bound to DNA; B represents the components of the general transcriptional machinery that are thought to be targets of Ssn6 and Tup1.

Materials and methods

Construction of expression plasmids

Desired TPR sequences from *SSN6* and *PAS10* were amplified with PCR using *Taq* polymerase (Boehringer Mannheim) and primers that contained a 5' *Bam*HI and 3' *Eco*RI site to facilitate cloning into pGEX-1 (Smith and Johnson 1988). PCR was conducted using reduced nucleotide concentrations (50 μ M), which have been shown to reduce substantially the error frequency of *Taq* (Innis et al. 1988). The following constructs were sequenced: GST:TPR6, GST:TPR7, GST:TPR8, and GST:Pas10-TPR5.

GST:Ssn6 contains residues 1–443, GST:1–9 contains residues 39–365, GST:1–7 contains residues 39–295, GST:3–9 contains residues 114–365, GST:1–3 contains residues 39–156, GST:6–8 contains residues 220–328, GST:1–2 contains residues 39–112, GST:3 contains residues 114–156, GST:6 contains residues 220–259, GST:7 contains residues 258–296, GST:8 contains residues 292–328, GST:9 contains residues 328–365, and GST:Pas10-TPR5 contains Pas10 residues 416–459.

Preparation of affinity resin

GST–fusion expression vectors were transformed into *Escherichia coli* DH5 α and were grown to saturation in 100 ml of 2 $\times$ YT containing 100 μ g/ml of ampicillin. This culture (5–10 ml) was then used to inoculate 3 liters of 1/2 $\times$ LB, 1 $\times$ YT containing 100 μ g/ml of ampicillin. Cells were grown at 37°C, shaking, to an optical density of $\sim$ 0.6. The cultures were then shifted to room temperature (23–26°C) and isopropyl- β -D-thiogalactoside (IPTG, Ambion) was added to a concentration of 60 μ M. Cells were grown for an hour at room temperature, and harvested by centrifugation. Cells were washed once in PBS (140 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 138 mM NaCl, 2.7 mM KCl) and were then frozen at –20°C. Cells were lysed by the addition of PBS containing 2 mM DTT, 1 mM EGTA, 1 mM EDTA, 2 mM PMSF, 2 mM benzamidinium-HCl, 200 μ g/ml of lysozyme, 0.8 M NaCl, 10% glycerol, and 0.5% NP-40 (lysis buffer). The resuspended pellet was sonicated and the extract was centrifuged at 87,000g for 60 min at 4°C. Batch binding to 5 ml of glutathione agarose (Sigma) was carried out at 4°C for 1 hr. The resin–extract slurry was spun for 15 min at 1000 rpm and the excess supernatant was removed. The remaining resin–extract slurry was poured into a column and was washed with five column volumes of lysis buffer. The resin was washed with an additional 15 column volumes of wash buffer (PBS containing 0.8 M NaCl, 2 mM DTT, 2 mM PMSF, and 2 mM benzamidinium-HCl). The prepared resin was stored in wash buffer containing 0.2% NaN₃ at 4°C.

α 2 extracts

Escherichia coli extracts containing α 2 were prepared as described in Komachi et al. (1994).

Chymotryptic digests of α 2

Purified α 2 (14.4 nmoles) diluted to a salt concentration of 50 mM NaCl in binding buffer (BB: 100 mM HEPES at pH 7.5, 2 mM EGTA, 2 mM EDTA, 2 mM DTT, 2 mM PMSF) was digested with 1 μ g/ml of α -chymotrypsin (Worthington Biochemical, NJ) overnight at room temperature (see Sauer et al. 1988 for additional details). The reaction was stopped by the addition of 0.1 volume of PMSF (50 mM in EtOH).

Affinity chromatography

Prepared resin was mixed with unconjugated glutathione agarose to produce a 0.5-ml column with $\sim$ 100 μ g of bound GST fusion. The column was equilibrated by washing with 5 ml of binding buffer with 40 mM NaCl (BB + 40: 100 mM at HEPES pH 7.5, 2 mM EGTA, 2 mM EDTA, 2 mM DTT, 2 mM PMSF, 40 mM NaCl), 5 ml of elution buffer (100 mM HEPES at pH 7.5, 2 mM EGTA, 2 mM EDTA, 2 mM DTT, 2 mM PMSF, 1 M NaCl), 1 ml of BB, followed by 3 $\times$ 2.5 ml of BB + 40. The conductivity of the equilibrated resin was checked to ensure that binding would occur at the NaCl concentration of 40 mM.

Approximately 1.5 nmole of protein (α 2, α 2 homeo domain, α 2 digested by chymotrypsin, or en homeo domain) was diluted in BB to 40 mM NaCl. Protein was diluted further to a final volume of 5 ml in BB + 40 and was then spun at 81,000g, 4°C, for 45 min. Samples were loaded over the columns at 1.5 ml/hr, and 0.5-ml fractions were collected. Columns were washed with 2.5 ml of BB + 40, then eluted with 1.5 ml of elution buffer. In both the wash and elution steps the first fraction collected was 0.5 ml, with the subsequent fractions being 1 ml. After discarding the void volume of the flowthrough fractions, every other fraction was pooled, unless noted otherwise in the figure legends. All fractions were precipitated with 10% trichloroacetic acid (TCA). Subsequent to the elution step, the column bed was resuspended in 0.5 ml of BB, and 200 μ l of the 1:1 column bed slurry was removed and the resin pelleted. Protein and resin pellets were resuspended in SDS sample buffer, and electrophoresed through 15% SDS–polyacrylamide gels and stained with Coomassie blue.

Note that in the mixed α 2/en homeo domain experiment, we used the carboxy-terminal fragment of α 2 (128–210, homeo domain and tail) to distinguish the two fragments (α 2 and en) by gel electrophoresis. As discussed in the text, the carboxy-terminal tail is not required for binding the TPRs.

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