The Brf1 and Bdp1 Subunits of Transcription Factor TFIIIB Bind to Overlapping Sites in the Tetratricopeptide Repeats of Tfc4*

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The TPR motifs, as the name implies, are typically 34 amino acids in length and fold into two antiparallel α-helices (designated A and B, Ref. 15). Although no position within the motif is invariant, a pattern of small and large hydrophobic residues defines the loose TPR consensus and provides stacking interactions between adjacent repeats (15–17). Multiple adjacent repeats form a right-handed superhelix in which the inner channel (formed by the A-helices) provides a ligand binding interface (18–20). The TPRs in Tfc4 are organized into two arrays in the N terminus, TPR1–5 and TPR6–9 (with five and four repeats, respectively) that are separated by a region of minimal sequence conservation, and two solo repeats in the C terminus (depicted in Fig. 1 and Refs. 13, 14, and 21). Conservation of the number of TPRs and their organization in Tfc4 orthologues suggests that the function of the protein is based on the preservation of a common TPR-based tertiary structure (21).

The TPR motifs of Tfc4 contain multiple independent binding sites for Brf1 (3, 22, 23) although the step(s) in complex assembly in which these sites are used is not known. The N-terminal region of Tfc4 in combination with the first repeat (Nt-TPR1) can support Brf1 binding, as detected by a two-hybrid interaction (1), although the first array (TPRs1–5) is minimally sufficient in the absence of the Nt-region (23). The second TPR array (TPRs6–9) contributes an additional binding site for Brf1 (3, 23). The ligand binding activity of TPRs10 and 11 is unclear since the C-terminal region is neither required for nor independently binds Brf1 in a two-hybrid assay (1) yet binds Brf1 in a pull-down assay (22). The N-terminal half of Tfc4 (Nt-TPR9) forms a structure that is largely stable to limited proteolysis and binds Brf1 in solution, in Far-Western and two-hybrid assays (1, 24). Yet the apparent Brf1-binding affinity of the Nt-TPR9 region is lower than that of either smaller Brf1-binding region (Nt-TPR5 and TPR6–9) (23). These data suggest that the Brf1 binding sites are masked in the context of the Nt-TPR9 region (23) and indeed the entire Tfc4 protein (1). A study of the human homolog of Tfc4 (hT- 

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‡ The abbreviations used are: pol III, RNA polymerase III; TPR, tetratricopeptide repeat; NTA, nitritoltriacetic acid.

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in and around TPR2 and increase the recruitment of Brf1 to TFIIC-DNA (6). A structural model of TPRs1–3 showed that the conserved residues and sites of the gain of function mutations cluster into two potential binding sites: one that traverses the ligand binding channel and another that lies across the back side of the array (29). Biochemical studies of the T167l (PCF1-2) gain of function mutation show that the channel formed by TPRs1–3 binds Brf1 directly. In contrast, characterization of the H190Y (PCF1-1) mutation shows that Brf1 does not interact on the back side of the TPR5–9 array. This latter mutation is proposed to affect an intramolecular interaction that indirectly influences Brf1 binding, leading to relief of autoinhibition (23).

Unlike the first TPR array, the conserved non-structural residues in TPRs6–9 map predominantly to the A-helices (23). Thus, the ability of TPRs6–9 to bind Brf1 in vitro suggests that these conserved residues form a binding site for Brf1. This was confirmed in the current study by in vivo and in vitro analysis of mutations at specific sites in the second TPR array. We show that conserved residues in TPRs6–9 cluster into two groups; one in TPR9 that affects Brf1 binding and the other in TPRs7 and 8 that generates a more complex effect on ligand binding. Biochemical studies on one mutant, L469K in TPR7, reveals effects on two steps in complex assembly; Brf1 and Bdp1 incorporation into TFIIB-TFIIC-DNA complexes. The minimal region of Tfc4 required for detectable Bdp1 binding in our assays was mapped to TPRs1–9, a region previously defined as containing two binding sites for Brf1. We discuss the implications of two ligands, Brf1 and Bdp1, apparently competing for binding to Tfc4, in TFIIC-directed TFIIB complex assembly.

EXPERIMENTAL PROCEDURES

Mutagenesis of Tfc4—The TransformerTM Mutagenesis kit (Clonetech) was used to introduce amino acid substitutions at selected positions in TPR7–9 in Tfc4. The following mutagenic primers were used to introduce mutations in the plasmid pRS13/PCF1-1° (the underlined nucleotides mark the mutation site(s); L469K, 5'-GAGCITTCGGAAGAATATTGAGGTCTGC-3'; E472K, 5'-GTTCCGAGTTATATTTTIXAGGCTGCCAC-3'; E498K, 5'-GCGGTTGTTACCTTTAAGAGGTG-3'; 5'-AGGAAAAGGAAAGTTATGAAACG-3'; 5'-AGGCTGCAAC-3'; I515K, 5'-GCTACAAGGAAGAAAAAGTTGGATAACG-3'; D537R, 5'-CCGATGATTTTAGAGATTCTGCTATC-3'; 5'-AGATCTGTGTTAATTTTGCCAGGAGTT-3'; L542G, 5'-GATCTCTGTGTTACCTTTGCGAGAAGATT-3' and E694K, 5'-GCTCGCTGAAGAAGAGGACCAACCC-3'). The selection primer 5'-GATCCCTGCTGACAGCTGCGGACCCGG-3' was used to identify successfully mutated plasmids through loss of XhoI and gain of BamHI (underlined) restriction sites in vector sequence. Candidate clones were retransformed into DE53 and sequenced to confirm the identity of the amino acid substitution. Mutant PCF1 alleles on pRS113 were transformed into the yeast strain supAC1+ that contained a wild-type PCF1+ gene and the pol III reporter gene sup9-e A19-supS1 (14) and the rescuing wild-type plasmid (pRS13/PCF1-1°) was evicted on 5-FOA-containing media. Single colonies were obtained and assayed for growth and suppression phenotypes at 16, 30 or 37 °C. Strains were grown to early log phase (OD 1.0) in synthetic complete media before dilution and spotting onto minimally complete or selective media. Each suppressor defective strain was transformed with plasmids that overexpressed either Brf1 (Yep24TD84, Ref. 30), TBP (pDE31-7, a gift from Greg Prelich) or Bdp1 (pRS269B90, recombined from pRS113, Ref. 7).

Transcription and Complex Assembly—Extracts from wild-type and L469K mutant strains were fractionated on Biorex 70 and DEAE-A25 resins before sequential gradient purification on hepaparin-agarose and mono Q resins (as previously described in Ref. 24). Recombinant wild-type and L469K fragments that encompass the TPR6–9 array were expressed and purified under denaturing conditions and refolded as described previously (25). The calculated extinction coefficients were used to determine protein concentration at 280 nm. Purity (77–80%) was confirmed by SDS-PAGE analysis using direct staining with Coo-
mutations in TPRs weren’t evaluated for growth at several temperatures on synthetic complete medium. The specific activity of TFIIIC fractions prepared from wild-type and L469K extracts were analyzed in parallel for DNA binding and transcription activity (see “Experimental Procedures”). TFIIIC preparations from L469K and wild-type strains were indistinguishable in protein composition (as determined by the abundance of TFIIIC subunits; Tfc4, Tfc1, and Tfc3, data not shown) and yielded comparable TFIIIC-DNA binding activity (data not shown). TFIIIC-DNA complexes were assembled based on the empirically determined DNA binding activity and assayed for the ability to support transcription in a reconstituted system. These transcription reactions contain TFIIIC, RNA polymerase III, and the TFIIIB factors (recombinant TBP and Brf1 and yeast-purified Bdp1). Transcription of the tRNA<sup>40S</sup> template (shown in Fig. 3) is dependent on TFIIIC (Fig. 3, lane 1) and generates highly reproducible amounts of transcript (duplicate reactions generate standard deviations of less than 10%, data not shown). Quantitation of the transcription products showed a linear dependence on Brf1 levels as reported previously (6) and regression analysis of the data in

either the loss of an inhibitory interaction or the acquisition of a positive interaction. However, 5 of 10 mutations (including the L469K mutation) exhibited significantly lower suppressor activity than wild-type (Fig. 2A, middle panel). Whereas wild-type growth was apparent on selective medium after 5 days, the five defective strains did not show significant growth even after 14 days. Consistent with the idea that ligand binding in the second TPR array is important for Tfc4 function in vivo, all of the defective mutations mapped to residues in the A-helices of the repeat (Fig. 1).

Previous work has established that transcription of the sup9-e A19-supS1 reporter gene in vivo is sensitive to the level of Brf1 as well as to the Brf1 binding activity of Tfc4 (5–7, 33). Thus, it was interesting to find that only two of the mutations (S541I and L542G in TPR9, Fig. 2B) showed increased sup9-e A19-supS1 activity in the presence of a multicopy plasmid containing Brf1. The interpretation of multicopy suppression data for presumed loss of function mutations in TFIIIC subunits is not unambiguous in the absence of supporting biochemical studies. The increase in reporter gene expression could indicate that these two mutations have a defect in Brf1 binding that is rescued by elevated Brf1 protein levels. Alternatively, these mutations may cause a defect in a step that precedes Brf1-binding (e.g. TFIIIC-DNA binding). In contrast, sup9-e A19-supS1 expression conferred by the mutations L469K, E472K, and V504K was not increased by overexpression of Brf1 (Fig. 2B) or either of the other two TFIIIB components (see Fig. 2C for L469K and data not shown). The different pattern of multicopy suppression seen for the S541I and L542G mutations in TPR9 versus the L469K, E472K, and V504K mutations, suggests that TPR9 is functionally distinct from TPRs7 and 8 in its ligand interactions. The latter group is most likely affected at more than a single step in transcription complex assembly or function.

The presence of non-conserved proline residues in the B-helix of TPR7 and in the A-helix of TPR8 in S. cerevisiae Tfc4 (23) complicates the construction of a molecular model for the entire TPR6–9 array. However, the structures of TPR6 and 7A and TPRs8B and 9 have been successfully modeled and show that residues Leu<sup>469</sup> and Glu<sup>472</sup> in TPR7, Val<sup>504</sup> in TPR8, and Leu<sup>469</sup> in the TPR channel together with the cold-sensitive growth phenotype and the pol III transcription defect of the L469K mutation led us to choose this mutant for biochemical analysis.

The L469K Mutation in Tfc4 Affects Brf1 Binding in Vitro—

The specific activity of TFIIIC fractions prepared from wild-type and L469K extracts were analyzed in parallel for DNA binding and transcription activity (see “Experimental Procedures”). TFIIIC preparations from L469K and wild-type strains were indistinguishable in protein composition (as determined by the abundance of TFIIIC subunits; Tfc4, Tfc1, and Tfc3, data not shown) and yielded comparable TFIIIC-DNA binding activity (data not shown). TFIIIC-DNA complexes were assembled based on the empirically determined DNA binding activity and assayed for the ability to support transcription in a reconstituted system. These transcription reactions contain TFIIIC, RNA polymerase III, and the TFIIIB factors (recombinant TBP and Brf1 and yeast-purified Bdp1). Transcription of the tRNA<sup>40S</sup> template (shown in Fig. 3) is dependent on TFIIIC (Fig. 3, lane 1) and generates highly reproducible amounts of transcript (duplicate reactions generate standard deviations of less than 10%, data not shown). Quantitation of the transcription products showed a linear dependence on Brf1 levels as reported previously (6) and regression analysis of the data in...
Previously documented interactions between Brf1 and the TPR6 (data not shown), TPR6 translated in vitro various labeled Tfc4 fragments that had been transcribed and translated under each lane. However, the L469K mutation caused a decrease in the ability of the TPR6–9 array (Fig. 4, middle panels). The two-hybrid interaction between Brf1 and TPR6–9 fragments was affected in a comparable manner by the L469K mutation. The Nt-TPR9 and TPR1–9 fragments of Tfc4 both showed the negative effect of the L469K mutation on Brf1 binding whereas no effect was apparent with the isolated TPR6–9 fragment or in the context of full-length Tfc4 (Fig. 6).

Previous studies have demonstrated that the TPR6–9 array of Tfc4 binds Brf1 in solution and can prevent its incorporation into TFIIB-TFIIC-DNA complexes (23). Since Brf1-TFIIC-DNA complexes can be resolved from the faster migrating TFIIC-DNA complexes by native gel electrophoresis (Fig. 3, compare lanes 2–4 to 5–7).

The previously documented interaction between the TPR6–9 array and Brf1 (23) suggested that the L469K mutation likely affected the interaction between Tfc4 and Brf1 and generated a defect in the assembly of TFIIB complexes. Although Brf1 binding by TFIIC-DNA complexes can be measured directly by native gel electrophoresis, the anticipated loss of function effect of the L469K mutation prompted the use of an assay in which higher-order (TFIIB-TFIIC-DNA) complex formation provided the readout for Brf1 binding. TFIIB-TFIIC-DNA was assembled under equilibrium conditions using equal numbers of TFIIC-DNA complexes over a range of Brf1 concentrations. The amounts of TBP and Bdp1 were equal and the Bdp1 concentration was empirically determined to be saturating for both mutant and wild-type TFIIC complex assembly. As shown in Fig. 4A, TFIIB-TFIIC-DNA complexes are well resolved from TFIIC-DNA complexes demonstrating an efficient conversion of the intermediate species (Brf1-TFIIC-DNA and TBP-Brf1-TFIIC-DNA) into the higher order complex. Mutant TFIIC was less effective in forming TFIIB-TFIIC-DNA complexes at all concentrations of Brf1. Complex formation was quantified, scaled and fitted (see “Experimental Procedures”) to generate the Brf1 binding isotherms shown in Fig. 4B. The lower apparent binding affinity of mutant TFIIC for Brf1 (12.4 ± 0.8 nM), compared with that obtained for wild-type TFIIC (4.3 ± 0.2 nM), indicates a defect in Brf1 recruitment caused by the L469K mutation.

The effect of the L469K mutation on Brf1 binding was assessed in two other assays; by pull-down and yeast two-hybrid interactions. Recombinant Brf1 was immobilized via its C-terminal His6 tag to Ni2+ -NTA-agarose and incubated with various labeled Tfc4 fragments that had been transcribed and translated in vitro (see “Experimental Procedures”). The previously documented interactions between Brf1 and the TPR1–5 (data not shown), TPR6–9, TPR1–9, and Nt-TPR9 fragments of Tfc4 (Fig. 4C, left panels) were readily detected in this manner. Given the semi-quantitative nature of pull-down assays small differences in binding affinity may not be readily apparent. Indeed, the L469K mutation did not obviously affect the interaction with Brf1 to the isolated TPR6–9 array (Fig. 4C, top right panel) under these or lower stringency binding and washing conditions. However, the L469K mutation caused a decrease in the ability of the TPR1–9 and Nt-TPR9 fragments (70 and 85%, respectively) to bind Brf1 (Fig. 4C, middle and lower right panels). The two-hybrid interaction between Brf1 and Tfc4 fragments was affected in a comparable manner by the L469K mutation. The Nt-TPR9 and TPR1–9 fragments of Tfc4 both showed the negative effect of the L469K mutation on Brf1 binding whereas no effect was apparent with the isolated TPR6–9 fragment or in the context of full-length Tfc4 (Fig. 6).
Bdp1 and Brf1 Bind to TPRs1–9 in Tfc4

The recruitment of Brf1 by TFIIIC-DNA complexes constitutes the first step in the sequential assembly of the transcription initiation factor TFIIIB. This step is limiting for complex assembly in yeast (5–7) and is targeted for regulation in mammalian systems. Importantly, the association of Bdp1 with either the TPR1–9 or Nt-TPR9 fragment of Tfc4 was significantly decreased by the L469K mutation (Fig. 5, lower right panels). Binding of the mutant TPR1–9 and Nt-TPR9 proteins to the Bdp1-containing resin was reduced 98 and 83%, respectively, relative to the wild-type proteins.

The negative effect of the L469K mutation on the interaction between Tfc4 and Bdp1 was confirmed by the two-hybrid assay. Although this assay generates only a low level of β-galactosidase activity for full-length Tfc4 binding to Bdp1 (26, 33), the L469K mutation significantly decreased the interaction (Fig. 6). In contrast, other pol III defective mutations in TPR6–9 (E472K, V504K, and L542G) showed no significant effect on Bdp1 binding (data not shown). The two-hybrid assay was therefore used to delimit the region(s) in Tfc4 responsible for binding Bdp1. Despite previous reports that the Nt-TPR9 region of Tfc4 does not bind Bdp1 in this assay (21, 26), the Nt-TPR9 fusion generated a robust signal with Bdp1 (Fig. 6) and confirmed the positive pull-down interaction between these proteins (Fig. 5C). The TPR1–9 fragment, containing both TPR arrays and the sequences that separate them (IVR), was found to be minimally sufficient to bind Bdp1. Significantly, the Nt-TPR9 and TPR1–9 fragments both show the negative effect of the L469K mutation on Bdp1 binding (Fig. 6, top row). The TPR1–5 and TPR6–9 arrays by themselves or with the IVR sequence (TPR1-IVR and IVR-TPR9) did not bind to Bdp1 (Fig. 6, third row). The high affinity binding site previously defined for Brf1 binding to Tfc4 (Nt-TPR5) (23) also did not bind Bdp1. Together these interactions infer that the tertiary structure of Tfc4 fragments containing both arrays is critical for the detectable association with Bdp1. Moreover, since the L469K mutation in TPR7 negatively affects Bdp1 binding, the channel formed by the TPR6–9 array contributes to the Bdp1 binding site in Tfc4.

The effect of the L469K mutation on the interaction with Brf1 was also evaluated in the two-hybrid system. Although the full-length Tfc4 interaction with Brf1 generates significant β-galactosidase activity (1, 3), no effect of the L469K, E472K, V504K, and L542G mutations was discernable (data not shown and Fig. 6, second row). Nonetheless, the Nt-TPR9 and TPR1–9 fragment interactions with Brf1 were decreased by the L469K mutation (Fig. 6, second row). The known interactions between Tfc4 and Brf1 (Nt-TPR5, TPRs1–5, TPRs6–9 and Nt-TPR9, (23, 24) were confirmed in this assay (Fig. 6, second and fourth rows). Interestingly the IVR sequence decreased Brf1 binding to both the TPR1–5 and TPR6–9 arrays, possibly contributing to the previously reported autoinhibition of Brf1 binding by Tfc4 (23). The TPR arrays in Tfc4 therefore provide a binding site for Bdp1 that differs from but overlaps those previously defined for Brf1.

DISCUSSION

The recruitment of Brf1 by TFIIIC-DNA complexes constitutes the first step in the sequential assembly of the transcription initiation factor TFIIIB. This step is limiting for complex assembly in yeast (5–7) and is targeted for regulation in mammalian systems.
with Bdp1-bound or control Ni²⁺ prepared by coupled in vitro fragments of Tfc4, TPR1, and TPR6, mutation on Tfc4 binding to Bdp1. Wild-type and L469K-containing symbols recruit into TFIIIB-TFIIIC-DNA complexes derived from the analysis of multiple data sets (generated in four independent experiments) are summarized as follows: – background level of activity (<0.5); ++, +++, +++++, and ++++++ indicate <5, 5–20, 20–50, 50–200, and >200 Miller units per mg of protein, respectively. Representative colony lifts are shown, overexposed for the Brf1 interactions with Nt-TPR5 and Nt-TPR9, in order to present the weaker Bdp1 interactions. IVR (interacting region) represents the sequence that lies between the two TPR arrays, TPR1–5 and TPR6–9.

Fig. 5. The L469K mutation in TPR7 directly affects Bdp1 recruitment to TFIIIC-DNA. A. TFIIIB complex assembly by wild-type and L469K TFC4. TFIIIC-DNA complexes were preassembled on a 32P-labeled tRNA-containing DNA probe prior to the addition of Bdp1 (1.5, 3, 5, 7.5, 15, 30, 50, 75, 150, 300, 500, and 750 fmol) at saturating levels of TBP (250 fmol) and Brf1 (6 pmol). TFIIIB-TFIIIC-DNA complexes were incubated for 20 min at 20°C and resolved from Brf1-TBP-TFIIIC-DNA complexes by native gel electrophoresis. Representative gels for wild-type TFIIIC (upper panel) and L469K TFIIIC (lower panel) are shown. Note the equal Brf1-TBP-TFIIIC-DNA complexes, the substrate for Bdp1, in lane 1. B, binding isotherms for Bdp1, recruitment into TFIIIB-TFIIIC-DNA complexes, derived from the analysis of multiple data sets (such as in panel A) for wild-type TFIIIC (solid symbols) and L469K TFIIIC (hollow symbols). C, effect of the L469K mutation on Tfc4 binding to Bdp1. Wild-type and L469K-containing fragments of Tfc4, TPR1–5, TPR6–9, TPR3–9, and Nt-TPR9, were prepared by coupled in vitro transcription and translated with Bdp1-bound or control Ni²⁺-NTA-resin. Bound Tfc4 protein was detected by direct autoradiography after SDS-PAGE. Input lanes contain 10% of the binding reaction. Note that a low level of both mutant and wild-type TPR6–9 protein binds nonspecifically to the resin.

Mammalian cells (reviewed in Ref. 35). For example, an important aspect of cell cycle regulation of pol III transcription in mammals involves the binding of Brf1 by the tumor suppressor protein RB and the subsequent inhibition of Brf1 recruitment by TFIIIC (36). The Brf1-TFIIIC interaction is a complex one that is mediated primarily by TFC4 (reviewed in Refs. 29, 37). TFC4 contains multiple binding sites for Brf1 and intramolecular interactions in both proteins mask the interacting regions (1, 22, 23). The ligand binding channel formed by the first TPR array in TFC4 (TPR1–5) binds Brf1 in vitro and dominant gain of function mutations in the second repeat, TPR2, can directly or indirectly increase the interaction with Brf1 both in vivo and in vitro (6, 33). Although the second TPR array, TPR6–9, has also been shown to bind Brf1 in vitro (23), the biological significance of ligand binding in this array has not been demonstrated until now. A phylogenetic analysis of TPRs6–9 in Tfc4 orthologs noted four absolutely conserved residues, one in each TPR, which potentially defined a ligand interaction site in the TPR channel (23). Mutagenesis of these and other highly conserved residues in TPRs6–9 uncovered five positions that disrupted Pol III transcription. The wild-type residues at these positions are predicted to project into the TPR channel and thus, the mutations were expected to interfere with ligand binding. One such mutation, L469K, was characterized in detail to identify the affected step(s) in Pol III transcription complex assembly. Using quantitative biochemical approaches we showed that the L469K mutation decreases the apparent binding affinity of Brf1 for incorporation into TFIIIB-TFIIIC-DNA complexes (Fig. 4). The binary interaction between specific TFC4 fragments and Brf1, as reported by pull-down and two-hybrid assays, was also decreased by the L469K mutation (Figs. 4C and 6). These findings were further supported by the decreased ability of the mutant TPR6–9 fragment to bind Brf1 in solution and inhibit its incorporation into TFIIIC-DNA complexes (Fig. 4D). Thus, the L469K mutation directly affects Brf1 binding in the TPR6–9 channel. In agreement with genetic data showing that the decreased function of the L469K mutant could not be suppressed by overexpression of Brf1 (Fig. 2, B and C), a second step in complex assembly was found to be compromised: the mutation decreased the apparent binding affinity of Bdp1 in the final step of TFIIIB complex assembly (Fig. 5). As for the studies with Brf1, the effect of the L469K mutation on Bdp1 binding was shown to be direct by pull-down and two-hybrid assays. Thus, mutation of a surface accessible residue in the ligand binding channel formed by TPRs6–9 affects TF4 interactions directly, and in the context of TFIIIC-DNA complexes, with two TFIIIB subunits, Brf1 and Bdp1. The minimal region
of Tfc4 that supported a binary interaction with Bdp1 was mapped and shown to require both TPR arrays (Fig. 6). Unlike the case for Brf1, Bdp1 did not to bind to either individual TPR array nor did it bind to the Nt-TPR5 region defined as a high affinity binding site for Brf1. The binding requirements of Bdp1 and Brf1 in Tfc4 are therefore distinct but overlapping.

Targeting conserved residues in the TPR6–9 array successfully generated mutations that decreased pol III activity at some but not all sites. The S15K mutation (in TPR8 at position 14) caused a slight increase in the suppression phenotype (Fig. 2). The location of S15K in the turn between the A- and B-helices of TPR5 predicts that the substitution would affect interactions at the edge of the TPR channel. Including L469, mutations were generated at 3 of 4 absolutely conserved residues in the TPR6–9 array. The Cys511 site (in TPR 8 at position 10) is the only cysteine in the A-helices of the TPRs in Tfc4. Since the C511A mutation did not cause a growth or suppression phenotype, we conclude that disulfide bond formation in TPR8 does not contribute in an important way to Tfc4 function. A recent global propensity analysis at each position in the TPR motif found cysteine and alanine to be the two most common residues at position 10 (5.7 and 2.8%, respectively, Ref. 17). Thus, the function of the amino acid at position 511 in Tfc4 has yet to be rigorously tested. The two other absolutely conserved amino acids noted in TPRs6 contribute to the packing of the hydrophobic core of the TPR array is necessary for Brf1 binding in the TPR channel. These two mutations suggest that correct packing of TPR9 in the array is necessary for Brf1 binding in the TPR channel.

The identification of a Bdp1 binding site in Tfc4 that requires both TPR arrays suggests that Bdp1 binds in the first array, TPRs1–5, as well as to TPRs6–9. That TPRs1–5 contribute to Bdp1 binding is supported by studies of a deletion mutant, tfc4ΔTPR2, that uncovered a negative role for TPR2 in Bdp1 binding (21, 26) and the synthetic lethal phenotype of a gain of function mutation in TPR2, PCFI1–1 (27). Together these data suggested that gain of function mutations that map to TPR2 might negatively affect the interaction between Tfc4 and Bdp1. However, a comparison of wild-type TFIIIC to PCFI1–1 and PCFI1–2 mutant TFIIICs showed that neither mutation affected Bdp1 recruitment or the two-hybrid interaction between Tfc4 and Bdp1 (33). A structural comparison of an idealized TPR array to several naturally occurring TPRs found that small differences in repeat sequence cause changes in inter-repeat stacking that alter overall TPR structure (17). The deletion of any single TPR in a multi-TPR structure would clearly reposition individual repeats relative to one another, alter the stacking interactions between helices and in the case of Tfc4, affect the TPR1–9 tertiary structure. Thus, while the requirement for the TPR1–5 array is clear, residues in the TPR channel that interact with Bdp1 have yet to be identified and differ in at least one position (defined by the PCFI1–2 mutation) from those that interact directly with Brf1.

As described above, the importance of the ligand binding channel formed by TPRs6–9 for interactions with both Brf1 and Bdp1 is demonstrated by structural mapping of Tfc4 residues (Leu469, Glu472, Val504, and Ser541) onto TPR protein structures (29) and by the effect of the L469K mutation in a variety of assays (Figs. 2, 4C, 5C, and 6). Additionally, mutation of an adjacent residue (D468N) is known to cause a significant defect in the two-hybrid interaction between full-length Bdp1 and Tfc4 (28). However, only one assay, the coupled equilibrium binding assay (Fig. 4D), was sufficiently sensitive to show that the L469K mutation affected Brf1 binding directly to the isolated TPR6–9 array. An equivalent demonstration for Bdp1 will require the development of an assay capable of detecting significantly weaker interactions.

The relative disposition of the two TPR arrays in Tfc4 is unknown and only one structure has been solved to date for a protein containing multiple TPR arrays, namely the peroxisomal importor PEX5 with bound PTS1 peptide (18). The requirement for both TPR arrays of Tfc4 for Bdp1 binding can readily be interpreted in terms of a PEX5-like structure for the TPR1–9 region; i.e. the two arrays, oriented anti-parallel to one another and separated by the IVR region, form a bipartite binding site in which both TPR channels surround the ligand (18, 29). This structural organization would also position TPR2 and TPR9 (known2 and putative (Fig. 2B) Brf1 binding regions, respectively) in relatively close proximity to one another and to the N terminus of Tfc4, which makes a positive but Brf1-specific contribution to ligand binding (Fig. 6, Ref. 23). In an alternative model for the organization of multiple TPRs, adjacent repeats align to form a continuous extended superhelical structure (15). Notably, this second model places the ligand binding sites in the groove of TPRs1–3 and in TPRs6–9 at distal locations (reviewed in Ref. 29). Regardless of the organization of the TPRs in Tfc4, the binary interaction between Bdp1 and TPR1–9 (but not Brf1) is diminished in the context of the full-length Tfc4 protein (Fig. 6) and more significantly, Bdp1 does not detectably bind to TFIIIC-DNA or Brf1-TFIIIC-DNA complexes (8). Yet, the affinity of Bdp1 for Brf1-TBP-TFIIIC-DNA complexes is approximately two orders of magnitude higher than that for the interaction of Brf1 with TFIIIC-DNA (33). Together these data suggest that the accessibility of the Bdp1 binding site in Tfc4 is altered dramatically during complex assembly. Given the ordered nature of the binding reaction (8) and the evidence presented here for distinct but overlapping sites in Tfc4 for Brf1 and Bdp1, it appears that TFIIIC-dependent assembly of TFIIIB involves dynamic protein-protein interactions that will likely include the repositioning of Brf1 to facilitate the recruitment of Bdp1.

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