The 1.1-Å Structure of the Spindle Checkpoint Protein Bub3p Reveals Functional Regions

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Bub3p is a protein that mediates the spindle checkpoint, a signaling pathway that ensures correct chromosome segregation in organisms ranging from yeast to mammals. It is known to function by co-localizing to at least two other proteins, Mad3p and the protein kinase Bub1p, to the kinetochore of chromosomes that are not properly attached to mitotic spindles, ultimately resulting in cell cycle arrest. Prior sequence analysis suggested that Bub3p was composed of three or four WD repeats (also known as WD40 and β-transducin repeats), short sequence motifs appearing in clusters of 4–16 found in many hundreds of eukaryotic proteins that fold into four-stranded blade-like sheets. We have determined the crystal structure of Bub3p from Saccharomyces cerevisiae at 1.1 Å and a crystallographic R-factor of 15.3%, revealing seven authentic repeats. In light of this, it appears that many of these repeats therefore remain hidden in sequences of other proteins. Analysis of random and site-directed mutants identifies the surface of Bub3p involved in checkpoint function through binding of Bub1p and Mad3p. Sequence alignments indicate that these surfaces are mostly conserved across Bub3 proteins from diverse species. A structural comparison with other proteins containing WD repeats suggests that these folds may bind partner proteins using similar surface areas on the top and sides of the propeller. The sequences composing these regions are the most divergent within the repeat across all WD repeat proteins and could potentially be modulated to provide specificity in partner protein binding without perturbation of the core structure.

The spindle checkpoint is a signal transduction pathway that blocks the progress of the cell cycle through mitosis in response to kinetochores that are not attached to the spindles responsible for chromosomal segregation (1). A thorough functional understanding of the proteins that mediate this signal transduction mechanism is key in elucidating the mechanism by which cells maintain the proper number of chromosomes after cell division. Errors in the segregation of chromosomes have been shown to result in aneuploidy and have also been associated with tumorigenesis (2). Defects in the spindle checkpoint provide one plausible explanation for this. Indeed, mutations have been found in at least one spindle checkpoint protein (Bub11, budding uninhibited by benomyl) in human cancers (3). Moreover, expression levels of these proteins have been found to be altered in cancer tissue and cell lines (4). Other cancers associated with checkpoint defects have also been discussed previously (5).

Bub3p is a 38-kDa WD repeat-containing protein and is conserved in organisms ranging from yeast to humans. Bub3p was initially discovered in a screen, searching for proteins involved in arresting the cell cycle in response to microtubule damage in budding yeast caused by the microtubule inhibitor benomyl (6). It functions in concert with several other proteins in yeast including Bub1p, Mad1p, Mad2p, and Mad3p, as well as Mps1p to mediate this pathway (7). The function of the protein is to bind to kinetochores that are not attached to opposing microtubules, thereby activating a cascade of incompletely understood biochemical events. The end effect of this pathway is the inhibition of the anaphase-promoting complex (APC), which is a ubiquitin ligase complex responsible for the degradation of protein, maintaining the cell in metaphase (8).

WD repeats, also known as β-transducin repeats and WD40 repeats, occur in tandem clusters, contain ~40 amino acids, and are widely distributed in many hundreds of eukaryotic proteins comprising a significant percentage of the genome. Approximately 2% of the proteins in Saccharomyces cerevisiae contain them, and it appears that similar values are likely in other organisms with sequenced genomes that are not as well annotated. The name is derived from a loosely conserved Trp–Asp dipeptide found at the C-terminal end of the consensus sequence. In general, these proteins function by specifically binding to one or more partner proteins. This is often done to present a protein substrate molecule to an enzyme such as a kinase or ubiquitin ligase. They also function as scaffolding proteins to aid in forming large complexes involved in processes such as mRNA splicing and translation initiation. Other roles for WD repeat proteins have been found in mediating signal transduction, cell cycle regulation, transcriptional regulation, vesicular trafficking, developmental regulation, mRNA processing, and numerous other physiologically relevant processes involving protein–protein interactions (9, 10).

The WD repeats in Bub3p have been shown to bind at least two other checkpoint proteins, Mad3p (11) and Bub1p, a serine/threonine kinase (12). In order to understand how Bub3p binds its partners to activate the checkpoint and to increase our general understanding of WD proteins, we have...
determined the crystal structure of the protein from S. cerevisiae at 1.1-Å resolution using multiple isomorphous replacement. Identification and analysis of site-directed and random mutants that fail to rescue BUB3-null mutants have allowed us to map residues on the surface of the protein that are responsible for checkpoint function.

EXPERIMENTAL PROCEDURES

Cloning, Purification, and Crystallization—The gene encoding Bub3p was amplified from S. cerevisiae genomic DNA using the primers 5'-CCATGGAGATACACTCCCTGGTGTCGAGATACACCTGGTTCTC-CATAGTCAAAATTAGG and 5'-CCCGGCGGTCTCCGCAATTTACATACCTGGTTCTCTATGGAATTCCAGATGCAAGAGCGGCTCCCGTCCATAGTCAAAATTAGG, which include extra sequences that enable cleavage by NdeI for the 5'-primer and SmaI for the 3'-primer (underlined). The resulting insert was ligated into the NdeI/SmaI site in the vector pTYB2 (New England Biolabs). The resulting construct was sequenced and used to transform the expression strain ER2566. Overexpressed protein was purified using a chitin affinity column. Secondary purification was carried out using an HQ anion exchange column mounted on a Perseptive Biosystems Perfusion Chromatography System at pH 6.0 using a 0–0.5 M NaCl gradient. Monodisperse protein was concentrated to 15 mg/ml, and the buffer was changed to 10 mM Hepes, pH 7.3. Crystals were grown using the hanging drop vapor diffusion method. Drops containing 1 μl of protein and 1 μl of well solution were suspended over a well containing 14% polyethylene glycol 8000, 200 mM calcium acetate, 0.1 M cacodylate, pH 6.5. Irregularly shaped crystals appeared in several days and were harvested into 25% (v/v) glycerol, 75% (v/v) well solution, and flash-cooled in a 100 K nitrogen stream for data collection.

Data Collection, Structure Determination, and Refinement—Native data were collected at beamline 5.0.2 at the Advanced Light Source to 1.1-Å resolution. Derivative data were collected on a R-AXIS IV mounted on a rotating anode (Table I). In both cases, the data were reduced using DENZO and SCALEPACK (13). This structure was initially refined at 1.37-Å resolution using CNS, which reduced Rmerge, and Rfree, to 21.8 and 24.1%, respectively. Subsequent refinement employed SHELX (17) using 1.1-Å data and anisotropic temperature factors. Addition of riding hydrogen atoms reduced the Rmerge and Rfree to the final values shown in Table I.

Yeast Strains and Media—All haploid yeast strains used in this work were derived from the CRY1 1401 strain. Plasmids containing the random mutants were transformed using the lithium acetate method. Yeast media, growth conditions, stock solutions, and molecular techniques were as previously described (18, 19).

To construct the bub3:pBub1-HA::3xHis, bub3::Δ:kan, ura3-1, can1-100, ade2-1, trpl-1, leu2-3, his3-11, tert-1, can1-100, ade2-1, trpl-1, leu2-3, his3-11, tert-1, can1-100, ade2-1, trpl-1, leu2-3, his3-11, tert-1, ura3-1, strain used for rescue experiments, three HA tags were introduced at the endogenous locus of the Bub1 gene as an in-frame C-terminal fusion protein with the coding sequence as described (20). Disruption of the BUB3 gene was performed as described (20). Correct modifications were confirmed by PCR and Western blot analysis.

The BUB3 construct containing the promoter and termination sequence used in rescue experiments was made using the primers 5'-ATCTGAGGAGCACAGCCCTTGGCATAATTGCTAC and 5'-AGT- CGACGTGAGGAATTTGGGAGCAGACTTTTGGC. These primers amplified approximately 200 base pairs flanking the BUB3 open reading frame from the CRY1 strain and allowed cloning of this product into a yeast single copy plasmid, the Escherichia coli/yeast vector YCPlac33 (21). PCR modification of the nucleotides directly upstream and downstream of the BUB3 open reading frame were used to remove the BUB3 coding region and replaced with an EcoRI and SmaI restriction sites and also incorporated thirteen copies of the Myc epitope (EQKLISEEDL) to yield pDC, the empty plasmid used in rescue experiments. The BUB3 gene was amplified and inserted into pDC to make the wild-type Myc-tagged expression construct (pBUB3) for immunoprecipitation. The pBUB3 was tested for its ability to rescue the ΔBUB3 strain phenotype and then sequenced.

Generation of Random and Site-directed Mutants—Random mutants were generated using the Diversify PCR random mutagenesis kit according to the manufacturer’s instructions (Clontech). Primers 5'-CTACTGTATATCTCGACAGAAGACAAACAGGACGAGAAGAGAGATACACCTGGTTCTC-CATAGTCAAAATTAGG and 5'-GGAGAGGAACGGGAAGAGAGACGAGATACACCTGGTTCTC-CATAGTCAAAATTAGG were used to amplify the BUB3 open reading frame in pBUB3.

The arginine to glutamate and serine/threonine to alanine site-directed mutants were generated using the mega primer PCR method (22). The primer sequences were designed to frame the mutation upstream and downstream of the in-frame codon. In all cases, the mutated inserts were placed into the vector pDC. These plasmids were used to transform a ΔBUB3 strain of CRY1 (Bub1-HA::3xHis, bub3Δ::kan, can1-100, ade2-1, trpl-1, leu2-3, his3-11, tert-1, can1-100, ade2-1, trpl-1, leu2-3, his3-11, tert-1, ura3-1). Transformants were plated on yeast synthetic drop out medium without uracil at 30 °C. Colonies were replica plated on to media supplemented with either 7.5 or 10 μg/ml of benimidazole.

In all cases, after 2 days at 30 °C, colonies with inhibited growth were identified and sequenced. After 2 days, clones that again failed to grow were identified and sequenced. Determined mutants were used to separate mutants from frame mutants and also incorporated thirteen copies of the Myc epitope (EQKLISEEDL) to yield pDC, the empty plasmid used in rescue experiments. The BUB3 gene was amplified and inserted into pDC to make the wild-type Myc-tagged expression construct (pBUB3) for immunoprecipitation. The pBUB3 was tested for its ability to rescue the ΔBUB3 strain phenotype and then sequenced.

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used at 0.5 μg/ml. All antibodies were diluted in 4% Blotto (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20, 4% nonfat powdered milk). The anti-Mad3p antibody was kindly provided by Kevin Hardwick, University of Edinburgh, UK. Blots were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse, at 1:5000 (NA 991, Amersham Biosciences) and HRP-conjugated anti-rabbit at a 1:2000 dilution in Blotto (sc-2004; Santa Cruz Biotechnology Inc.). After the final wash, blots were incubated in 10 mM Tris-HCl, pH 8.0, 150 mM, for 5 min and then developed with the Santa Cruz Biotechnology Western blotting luminol reagent according to the manufacturer’s instructions.

RESULTS AND DISCUSSION

Overall Structure—Bub3p is a member of a large superfamily of proteins that contains tandem sequence repeats termed WD repeats after the Trp-Asp dipeptide that is sometimes found at the C-terminal end of the repeat. Crystal structures of WD repeat proteins such as the transducin β-subunit (24, 25), budding yeast Tup1p (26), the p40 subunit of the Arp2/3 complex (27), and others have shown that the WD repeat effectively yields a four-stranded β-sheet. In almost all cases, these blade-like secondary structural modules pack into a seven-bladed propeller-like structure. By convention, these strands are labeled A through D starting with the innermost strand. Rather than forming a single blade, each repeat consists of the outside (D) strand of one blade then crosses over to create the inner three strands (A–C) of the next blade. The D strand from the first repeat is the outside strand for the last blade, creating an interlocking structure. Based on sequence analysis, Bub3 has been reported to have three or four WD repeats (28, 29). Despite this, threading techniques have suggested the existence of a seven-bladed propeller (28).

The refined model contains all Bub3p residues (1–341) and a C-terminal proline, which was appended from cloning. The high resolution structure clearly reveals that Bub3p possesses seven structural repeats (Fig. 1). These are arranged in a circular manner as seen in other WD repeat proteins with a completely hydrated central core. Using the conventional definition of the “top” of the protein as the side containing the loops connecting the D and A strands (the DA loop) and the loops connecting the B and C strands (the BC loops), the protein measures ~45 Å from top to bottom and 45 Å across the diameter. The top of the protein is characterized by the alternate stacking of DA and BC loops as opposed to the bottom, which has the AB and CD loops positioned roughly adjacent along the radius. There are no major structural excursions from the central core structure although somewhat elongated DA loops are found between blades 3 and 4 and blades 5 and 6. An elongated BC loop is also found in blade 7 (Fig. 1A).

In addition to 512 water molecules, four calcium ions were fit to regions of strong density with close (<2.5 Å) contacts to electronegative protein atoms or ordered water molecules. Because 100–150 mM calcium acetate was necessary for crystallization, these sites probably reflect the high concentration of calcium in the crystallization medium rather than any physiological role of calcium. The nature of the binding sites also suggests that they are low affinity. Each ion has only one, two, or three protein ligands. One of these is typically a carboxylate from a glutamate or aspartate. Two of these are present on the top of the protein, one associated with repeat 6 and the other facilitating a crystal packing contact near repeat 7. Another forms a packing contact on repeat 4, and the fourth is found in the central pore (Fig. 1). Interestingly, non-WD repeat β-propeller enzymes have been shown to bind cofactors, including calcium, in the central tunnel (30).

Bub3p Function—Various experiments have demonstrated that Bub1p and Mad3p bind directly to Bub3p during checkpoint function (11, 12, 31). Sequence alignments have shown that both of these proteins have two homologous segments dubbed region I and region II (11). In both proteins, region I has been shown to be involved in the interaction with Cdc20p, an activator of the anaphase promoting complex. Likewise, region II is responsible for Mad3p binding (11) and Bub1p binding to Bub3p (29). Sequence comparison between Bub1p and Mad3p show that 21 of 42 of the residues composing region II in Bub1p are identical, including many charged side chains (29). This high level of sequence conservation in the Bub3p binding domain would strongly suggest that they bind to the same surface of Bub3p.

To determine the location of surface residues interacting with partner proteins in Bub3p, the location of previously known mutations was examined. A deletion of residues 218–221 has been reported that corresponds to the removal of a major portion of strand C in WD repeat number 5 (29). Because these are internal residues, their deletion would severely destabilize or hamper the folding of the protein rather than specifically affect binding. Two surface mutants have also been constructed (W31G and W120G) yielding a protein that fails to bind to a complex of the checkpoint proteins Mad2p, Mad3p, and Cdc20p, possibly by disrupting a direct interaction with Mad3p (28). These are found at the C-terminal end of the B strands of repeats 1 and 3 which composes a part of the top of the propeller, a region implicated in partner protein binding in other WD repeat proteins (Fig. 2A).

To further define the protein binding surface of Bub3p, we have used error-prone PCR to produce mutant proteins that fail to rescue a BUB3-null strain when plated on selective media containing benomyl (Fig. 3). Several hundred colonies that gave reproducible phenotypes were further characterized. After identifying and discarding those with empty vectors, multiple mutations, frameshifts, and stop codons, there remained eight clones with single point mutations at positions 2, 188, 191, 192, 193, 226, 276, and 278. Mapping of these mutants to the protein structure revealed that most occur on the top and side of the propeller (Fig. 24), regions which have been implicated in protein binding in other WD proteins (Fig. 4) (24, 26, 27, 32, 33).
The 1.1-Å Structure of the Bub3 Checkpoint Protein

FIG. 2. Consolidation of random and site-directed mutations mapped onto the surface of Bub3p indicates the interaction surface is located on the top and side of the propeller. A, surface representation of Bub3p looking down upon the top of the protein in an orientation similar to Fig. 1A. Mutations in Bub3p impairing or abolishing checkpoint function are labeled and depicted in red. B, identical view of the protein identifying (in red) continuous patches of yeast Bub3p residues matching the residues conserved in four of five proteins aligned from human, mouse, Xenopus laevis, Drosophila melanogaster, and S. cerevisiae. C, discontinuous regions of conserved residues as described in Fig. 6 viewed looking down upon the bottom of the propeller. This does not participate in partner protein binding.

The effects of several of the mutations can be rationalized based upon structural results. The Q2L mutation may cause the formation of an unstable N terminus of Bub3p. The hydrophobic properties of leucine could affect the stability in the solvent-exposed surface of the D strand. This mutation is at the site of the “molecular velcro” of Bub3p where the N terminus forms the first D strand of the last blade, thereby securing the head to the tail end of the protein (Fig. 1A). This is a region that has been noted by others as important for protein stability. Despite this, the mutant appears to be stable within the cell as shown by Western blots (Fig. 5A). The mutant E185V removes the hydrogen bond of Glu-188 to the Trp-174 side chain, putatively perturbing its interactions with Arg-176, located 4 Å away. This arginine is solvent-accessible at positions 152, 162, 176, 193, 197, 217, 234, 235, 239, 242, 283, 284, 285, and 291. To investigate the roles of these positively charged residues in forming salt bridges with partner proteins mediating checkpoint function, we used site-directed mutagenesis to change arginine residues to glutamates at positions 162, 176, 217, 235, 239, and 242. These mutants were then assayed to determine which failed to rescue BUB3-null mutants using the benomyl plating assay used for the random mutant screen. Two of these, R217E and R242E failed to grow under these conditions. Taken together, all of these mutations (with the exception of W31G and W120G) map to a stripe along the side of the propeller involving blades four and five and to a lesser extent, blade six (Fig. 2A).

FIG. 3. Serial dilutions of exponentially growing cultures of wild-type (Bub3-Myc), pDC (empty vector), and mutants are denoted by their wild-type amino acid position and substitution. Transformants were spotted on ura synthetic media plates either lacking or containing benomyl and photographed after 2 days at 30 °C. Every clone is tested in a BUB1-(HA)3::HIS3, bub3-G418, ura3-, can1-100, ade2-1, trp1-1, leu2-3, −112, his3-11, −15, ura3-1 background strain.

FIG. 4. Conservation of protein binding surfaces in known structures of WD repeat proteins complexed with partner proteins. Functional surfaces of the WD repeat proteins Gβ and p40 indicate regions involved in partner protein binding and are similar to the Bub3p surface shown in Fig. 2, A and B. All models have an orientation similar to Fig. 1. Interaction surfaces: A, Gα binding to Gβ (35); B, phosphducin binding to Gβ (33); C, p16 binding to p40 (27); D, p20 binding to p40 (27) are shown in red. These figures were prepared using the program GRASP (45).

Another striking characteristic of this region is also the large number of conserved basic amino acid side chains which are solvent accessible at positions 152, 162, 176, 193, 197, 217, 234, 235, 239, 242, 283, 284, 285, and 291. To investigate the roles of these positively charged residues in forming salt bridges with partner proteins mediating checkpoint function, we used site-directed mutagenesis to change arginine residues to glutamates at positions 162, 176, 217, 235, 239, and 242. These mutants were then assayed to determine which failed to rescue BUB3-null mutants using the benomyl plating assay used for the random mutant screen. Two of these, R217E and R242E failed to grow under these conditions. Taken together, all of these mutations (with the exception of W31G and W120G) map to a stripe along the side of the propeller involving blades four and five and to a lesser extent, blade six (Fig. 2A).

In the past, locating conserved sequences on the surface of a WD repeat protein has been useful in identifying binding regions (26). Mapping of the 96 residues conserved between yeast Bub3p and four other species (Fig. 6) onto the surface of the

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protein reveals a large patch on the top and side of the propeller, again involving residues from blades 5 and 6 but also including some flanking the central pore (Fig. 2B). Smaller and non-continuous conserved surfaces are found on the bottom of the propellor underscoring the fact that it is the top and sides of the protein involved in partner binding (Fig. 2C). The conserved surface on the top of the propeller agrees well with the locations of the mutants previously identified at positions 31, 120, 176, 188, 191, 192, 193, 217, and 242 (Fig. 2, A and B).

Finally, it has been suggested earlier that Bub3p functions as a substrate for the Ser/Thr protein kinase Bub1p (12). To examine the possibility that a single phosphorylation plays a physiologically relevant role, we mutated all solvent accessible serines and threonines (at positions 68, 89, 144, 232, 233, 248, 315, 318, and 325) to alanines. These mutants were expressed in BUB3-null strains and their ability to grow on benomyl-containing media was assessed. All nine point mutants were able to restore checkpoint function (data not shown) indicating that phosphorylation of any one of these single sites on Bub3p is not physiologically relevant.

**Bub1p and Mad3p Binding**—To understand the biochemical effects of the BUB3 mutants that altered checkpoint function, immunoprecipitations from lysates were performed to determine whether binding of the known partner proteins, Bub1p or Mad3p, was affected. These mutants were all expressed in yeast tagged with a Myc13 epitope in a BUB3-HA3::HIS3, BUB1-HA3::G418, can1-100, ade2-1, trp1-1, leu2-3, −112, his3-11, −15, ura3-1 background strain. Fig. 5A shows that after developing immunoblots from immunoprecipitations, the levels of Bub3-Myc in all cases except for the negative control (pDC) were approximately constant, an indication that each version of Bub3p was properly folded and stable in the context of the cell.

When probed with anti-Mad3p antibodies, Mad3p binding was detected in E188V, G191R, L192E, K193T, and Q226L. Bub1-HA3 binding was detected in lysates of E188V, G191R, L192E, K193T, Q226L, and W278R (Fig. 5). The observation that binding of both Bub1p and Mad3p are found to be near wild-type levels in five of these mutants (E188V, G191R, L192E, K193T, and Q226L) suggests that one or more additional proteins may possibly bind directly to Bub3p. Furthermore, the overlap in the residues composing the partner protein binding sites demonstrates that Bub1p and Mad3p binding will be either competitive or possibly interdependent. Based on the fact that the Bub1p and Mad3p interaction with Bub3p relies on homologous regions on these proteins (discussed above), it seems likely that they are competitive binders.

**Implications for other WD Repeat Proteins**—Despite the fact that Bub3p contains only 12% sequence identity with the β-subunit of transducin, this protein was closest in terms of structural homology using a DALI search with an r.m.s. deviation between the homologous Ca of each protein of 2.6 Å (34). The repeats predicted by the sequence correspond to blades 1, 2, 3 and 6. Pairwise comparison of each repeat with repeat 1 gave an r.m.s. deviation of the Ca coordinates ranging from 0.61 to 1.21 Å. R.m.s. deviations of the unpredicted repeats were 1.04, 0.61, and 1.21 Å for repeats 4, 5, and 7, respectively indicating that all of these repeats are structurally authentic WD repeats (Fig. 7). Similar deviations are seen when the seven WD repeats of the β-subunit of the G protein are com-
pared (35). Slight structural excursions from the canonical fold are present, however. Insertions producing unusually long DA loops are found in repeats 4 and 6. A longer BC loop is noted in repeat 7. The loops in repeats 6 and 7 combine to form a protrusion on the top surface of the protein that is involved in partner protein binding (described above).

The unpredicted repeats 4, 5, and 7 are located in gaps composed of 40–50 amino acids distributed between the predicted repeats. A broader examination of other WD repeat proteins (9, 10) indicates that these gaps are present and could account for the presence of extra repeats in these other proteins. These hidden repeats have been experimentally found in several recent structures (32, 36–38). A detailed examination of these gaps in other representative proteins demonstrates plausible sequence homology with the WD consensus sequence (38). These alignments are particularly compelling in the context of the sequences derived from the newly found WD repeats from Bub3p, which demonstrate the divergence that can be found in the sequences. These sequences were compared for putative A, B, and C strands only because the D strands, which form the outside of the propeller, are ordinarily very divergent and therefore very difficult to align. In the majority of cases, these additional repeats would bring the total number to seven, indicating that the seven-bladed propeller is the general fold for a large number of WD repeat proteins or protein domains. Structurally, this is supported by the conserved nature of the packing of the core regions of the protein seen in Bub3p when compared with Gz, Hs, and the p40 subunit of the Arp2/3 complex. We note that a minority of these proteins such as Sec13p from S. cerevisiae must deviate from this rule, because there is not enough room in the sequence to accommodate seven repeats.

In the cases of protein sequences where more than seven repeats have been detected it has been proposed that the pro-

Fig. 6. Sequence alignments of Bub3 from various species. scBUB3, S. cerevisiae; hsBUB3, human; mmBUB3, mouse; xlBUB3, X. laevis; dmBUB3, Drosophila. Secondary structural elements are identified based on the yeast structure. Residues that are conserved among Bub3 homologs and shown in Fig. 2, B and C are shaded gray. Locations of mutants failing to rescue the BUB3-null mutant in this study are indicated by arrows.
tein might fold into either a large propeller incorporating all repeats or a two domain protein consisting of a seven-bladed propeller and a non seven-bladed structure (39). The structure of Aip1, one such protein involved in actin depolymerization, demonstrates that extra repeats can be found to bring the total number to multiples of seven: two seven-bladed domains (32, 36). Conservation of the number of secondary structural modules is not unprecedented and is probably best illustrated by the constant number of eight β α repeats observed in triosephosphate isomerase (TIM) barrels (40).

**WD Repeat Protein Function**—Similar structure often indicates similar function. To determine whether the proposed structural conservation of WD repeat proteins might lead to homologous binding of the partner proteins, a comparison of the WD repeat protein binding sites derived from structures of complexes was conducted. Four protein interfaces found in the structures of three different protein complexes were compared with reveal that there is a common region involved in partner protein binding (Fig. 4). In all cases, these interactions involve residues from the top of the molecule, primarily from the loops connecting the D and A strands and the B and C strands. Additionally, mutants of the WD repeat protein Tup1p that affect binding of Mata2 are all localized on the top of the propeller (41). This agrees well with sequence conservation data mapped onto the surface of the structure confirming that the top of the molecule is involved in binding its partner (26). Studies examining the location of mutations affecting function of the WD repeat protein Cdc4 also implicate the top surface in partner protein binding (42). There are no obvious sequence or structural motifs found in WD repeat binding proteins so partner proteins probably bind to the top of the propeller in a non-homologous manner. A similar consistency in the active site location is also seen in the example of the (βα)8 barrel. Despite an extremely diverse set of substrates, the catalytic site is always located at the C-terminal end of the internal β-barrel (40).

This model implies that the specificity of a particular WD repeat protein could be modulated by evolving or changing the properties of the DA and BC loops on the top of the protein or solvent-exposed residues on the D strand and retaining the stable core fold. When WD repeat sequences are aligned, the residues composing the DA loop and the D strand are indeed the most divergent, supporting this notion.

The broad implications resulting from this structure indicate that numerous WD repeats remain unidentified in sequence and that a seven-bladed propeller is likely to be the predominant fold for this class of proteins. This stable fold is able to form a scaffold for variable loops on the top of the protein to form a diverse protein-binding surface. This suggests that the WD sequence repeats themselves are more divergent than previously thought and many remain to be found. Furthermore, the location of this surface on WD repeat proteins and their binding partners is conserved among all structurally studied proteins, suggesting that this is a conserved feature.

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**Note Added in Proof**—A paper was recently published describing the crystallographic study of Bub3 (46). The overall structural results generally agree with those outlined here.

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