Saccharomyces cerevisiae RRM3, a 5’ to 3’ DNA Helicase, Physically Interacts with Proliferating Cell Nuclear Antigen*

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Proliferating cell nuclear antigen (PCNA) plays an essential role in eukaryotic DNA replication, and numerous DNA replication proteins have been found to interact with PCNA through a conserved eight-amino acid motif called the PIP-box. We have searched the genome of the yeast Saccharomyces cerevisiae for open reading frames that encode proteins with putative PIP-boxes and initiated testing of 135 novel candidates for their ability to interact with PCNA-conjugated agarose beads. The first new PCNA-binding protein identified in this manner is the 5’ to 3’ DNA helicase RRM3. Yeast two-hybrid tests show that N-terminal deletions of RRM3, which remove the PIP-box but leave the helicase motifs intact, abolish the interaction with PCNA. In addition, mutating the two phenylalanine residues in the PIP-box to alanine or aspartic acid reduces binding to PCNA, confirming that the PIP-box in RRM3 is responsible for interaction with PCNA. The results presented here suggest that the RRM3 helicase functions at the replication fork.

Proliferating cell nuclear antigen (PCNA) is an essential eukaryotic DNA replication factor. It functions as a homotrimer that forms a clamp that slides along double-stranded DNA serving as a processivity factor for DNA polymerases and as an attachment site for numerous other replication proteins (1). Similar processivity factors for DNA polymerases have been characterized in a wide spectrum of organisms and include gp45 of T4 bacteriophage (2) and the β-subunit of polymerase III of Escherichia coli (3). Despite sometimes low sequence similarity among members of this class of proteins, their three-dimensional structures are highly conserved, suggesting that their function has remained unchanged throughout evolution (4).

Our initial understanding of how PCNA interacts with other proteins comes from structural studies of the interaction between PCNA and p21WAF1/CIP1. The ability of human PCNA (hPCNA) to interact with DNA polymerases can be inhibited by p21WAF1/CIP1, an inhibitor of DNA replication that controls activity are revealed, new roles for PCNA beyond its primary function as a polymerase processivity factor may be identified. By searching the protein sequences encoded by more than 6000 open reading frames (ORFs) in the genome of Saccharomyces cerevisiae, we have identified 144 known or hypothetical proteins that contain the PIP-box consensus sequence. We have initiated a project with the aim to test all novel candidates for their ability to interact with PCNA of S. cerevisiae in vitro and in vivo methods. Here we present the examination of the first 26 candidates, among which we have identified RRM3 as a PCNA-interacting protein. RRM3 is a 5’-3’ DNA helicase of the PIF1 family (19, 20) and has been shown to be required for the stability of ribosomal DNA (rDNA) (21), for the inhibition of Ty1 transposition (22), and for telomere replication (20). We show that RRM3 interacts directly with PCNA in vitro and in vivo and that the interaction with PCNA is promoted by salt, suggesting a hydrophobic interaction. Using two-hybrid analysis we show that the interaction between RRM3 and PCNA depends on the presence of a canonical PIP-box motif at the N terminus of RRM3, and we propose that interaction between RRM3 and PCNA may be important for replication fork progression and for inhibition of site-specific hyper-recombination.

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The abbreviations used are: PCNA, proliferating cell nuclear antigen; hPCNA, human PCNA; ORF, open reading frame; SC media, synthetic complete media; rDNA, ribosomal DNA.

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45331
TABLE I

| Candidate | Plasmid | Function/cellular role | PIP-box position | Putative PIP box | PCNA interaction group No. |
|-----------|---------|------------------------|------------------|-----------------|--------------------------|
| BUD7      | pRDK1026| Required for bipolar budding pattern | 729/746 | QENLLNPF | 2 |
| CLN3      | pRDK1027| G3/S-specific cyclin, interacts with CDC28 to control START | 382/580 | QLKEFFY | 3 |
| Dpb2      | pRDK1029| DNA polymerase ε 80-kDa subunit | 216/892 | MQFLTRY | 3 |
| ECM12     | pRDK1030| Coll wall maintenance | 26/151 | QAIAMPF | 2 |
| ERP4      | pRDK1032| Vesicular transport | 191/207 | QALIQQF | 2 |
| EST1      | pRDK1033| Component of telomerase | 121/689 | QKMMQFF | 2 |
| GLG1      | pRDK1034| Initiator of glycosyn synthesis | 91/181 | QGLNQFF | 2 |
| GRH1      | pRDK1035| Spindle check point component | 77/372 | QFLQSSF | 2 |
| HSP90A1   | pRDK1036| U2 small nuclear RNP protein | 946/941 | QUAQPPPY | 2 |
| KRE28     | pRDK1037| Unknown | 102/355 | QTLDYFF | 2 |
| MDS3      | pRDK1038| Negative regulator of expression of early mitotic gene | 74/1487 | QKEILFY | 2 |
| NAT1      | pRDK1039| Protein N-acyetyltransferase | 84/604 | QNIELQY | 2 |
| OCT1      | pRDK1040| Mitochondrial peptidase | 21/733 | QNKLRFF | 3 |
| ORC1      | pRDK1041| Origin recognition complex subunit | 576/914 | QDIMYNFF | 2 |
| PHM8      | pRDK1042| Phosphate metabolism | 75/320 | QQSLSNFF | 2 |
| PTM1      | pRDK1043| Carbohydrate metabolism | 228/531 | QYLALLFF | 2 |
| RRM3      | pRDK1044| DNA helicase | 35/721 | QTQSSLFF | 1 |
| SED5      | pRDK1045| Vesicular transport | 305/340 | QRELHFF | 3 |
| SKN1      | pRDK1046| Glucan synthase subunit | 733/771 | QSHLNAYY | 2 |
| SKS1      | pRDK1047| Serine/threonine protein kinase | 68/502 | QTQLYHFF | 2 |
| YCR016W   | pRDK1048| Unknown | 250/289 | QELKKNFF | 2 |
| YDR117W   | pRDK1049| Unknown | 347/498 | QRVLTFPP | 2 |
| YHR153C   | pRDK1050| Unknown | 264/291 | QSLALYF | 2 |
| YMR115W   | pRDK1051| Unknown | 178/501 | QELLSRF | 3 |
| YOL078W   | pRDK1052| Unknown | 1141/1176 | QDDIKRY | 3 |
| YPL110C   | pRDK1053| Proteasome-interacting protein | 74/1223 | QKLASSF | 2 |

PCNA Pull-down Assay—Overexpressed PCNA of S. cerevisiae was purified from E. coli and bound to Affi-Gel-15 beads as previously described (10). Binding reactions containing 25 μl of in vitro translated protein, 15 μl of PCNA beads (or Mock beads without PCNA), and 460 μl of buffer A (25 mM Tris (pH 7.4), 200 mM NaCl, 1 mM EDTA, 0.01% Nonidet P-40, 10% glycerol) were incubated for 1 h at 4 °C. Beads were collected by centrifugation for 1 min at 5000 rpm and washed 3 times with 500 μl of ice-cold buffer A. Ten μl of the supernatant was saved for SDS-PAGE analysis (called unbound protein fraction, U). Bound protein was eluted from the beads by boiling for 5 min in 20 μl of SDS-PAGE sample buffer (called bound protein fraction, P). Samples were then analyzed on 4–15% gradient SDS-polyacrylamide Ready-gels (Bio-Rad), dried, and exposed overnight to a PhosphorImager screen (Molecular Dynamics). Images were developed using a PhosphorImager (Molecular Dynamics) and analyzed using ImageQuant version 1.2 (Molecular Dynamics). The same procedure was followed for the NaCl titration experiment except that the buffer A used for the incubation and wash steps contained different NaCl concentrations as indicated in individual experiments.

Yeast Two-hybrid Assay—Yeast strain RDKY2926 (strain EGY48 harboring reporter plasmid pSH18-34; Ref. 25; for review, see Ref. 26) was co-transformed with a lexA-fusion bait construct (in vector pEG202) and a B42-tagged prey construct (in vector pJG4-5*), and transformants were selected on synthetic complete (SC) medium plates lacking uracil, histidine, and tryptophan made using standard recipes and α-factor–induced E. coli. Single transformants were resuspended in 100 μl of sterile double-distilled H2O to spotted on SC-Ura-His-Trp plates, grown for 2 days at 30 °C, and then replica-plated onto SC-Ura-His-Trp plates containing 2% galactose to assess expression of the lacZ reporter gene. Plates were incubated overnight to test for reporter gene expression.

Verification of Bait and Prey Expression by Western Blot Analysis—Ten-ml cultures of the yeast strain RDKY2926 carrying pEG202-based expression constructs were grown overnight in liquid SC-Ura-His-Trp medium containing 2% glucose. Five-ml cultures of yeast strain RDKY2926 carrying pG4-5*-based expression constructs were grown for 2 days in liquid SC-Ura-His-Trp medium containing 2% galactose. Based on the Amax of each culture, an equal number of cells was harvested and then analyzed on 4–15% gradient SDS-polyacrylamide Ready-gels (Bio-Rad), dried, and exposed overnight to a PhosphorImager screen (Molecular Dynamics). Images were developed using a PhosphorImager (Molecular Dynamics) and analyzed using ImageQuant version 1.2 (Molecular Dynamics). The same procedure was followed for the NaCl titration experiment except that the buffer A used for the incubation and wash steps contained different NaCl concentrations as indicated in individual experiments.

EXPERIMENTAL PROCEDURES

Constructs for in Vitro Transcription/Translation—Complete ORFs of 26 candidate genes were amplified by PCR using genomic DNA from yeast strain RDKY3023 as a template. The sequence 5′-GCCGC-CACC-3′ was added to the forward primer sequence upstream of the ATG start codon to provide a eukaryotic ribosome binding site for in vitro translation (23). The PCR products were inserted into the E. coli expression vector pCRT7/CT-TOPO (Invitrogen) by TA-cloning to generate plasmids pRDK1026-BUD7 to pRDK1053-YPL110C (Table I). Primer sequences used to generate the plasmids used in the illustrated experiments are listed in Table II, and the remainder are available upon request.

Constructs for Yeast Two-hybrid Tests—The wild type POL30 gene and the pol30-104 allele (A251V) (24) were amplified by PCR from genomic DNA of yeast strains RDKY3023 and RDKY3921, respectively, using primer pair KHS-D304/KHS-D305 (Table II). The PCR products were digested with the restriction endonuclease XhoI and inserted into the single XhoI site of pGJ4–5* to yield pRDK1060-POL30 and pRDK1061-POL30-104, respectively. EST1 and RRM3 genes were amplified by PCR using primer pairs KHS-D312/KHS-D313 and KHS-D324/KHS-D325, respectively (Table II). The PCR products were digested with XhoI and inserted into the single XhoI site of pEG202 to generate pRDK1054-EST1 and pRDK1055-RRM3, respectively. Bait plasmids (pEG202) containing deletion mutations in the RRM3 gene (pRDK1056-RRM3-D54 and pRDK1057-RRM3-D230) were constructed by PCR using primer pairs KHS-D294/KHS-D289 and KHS-D329/KHS-D289, respectively (Table II). The PCR products were digested with XhoI and inserted into the XhoI site of pEG202. All constructs were confirmed by DNA sequencing.

Site-directed Mutagenesis—Amino acid substitutions in the putative PIP-box of RRM3 were introduced using the QuikChange method (Stratagene). The F41AF42A and F41DF42D mutations in RRM3 were generated using primer pairs KHS-D137/KHS-D188 and KHS-D442/KHS-D444 (Table II) and pRDK1055-RRM3 as template DNA to yield pRDK1058-RRM3-FPA and pRDK1059-RRM3-FDD, respectively. Both mutations were confirmed by DNA sequencing.

In Vitro Transcription and Translation—Reactions were performed in 50-μl volumes containing 0.5–1 μg of template DNA (pRDK1025-ASG7 to pRDK1053-YPL110C, 30 μl of 15N-labeled [35S]methionine (Amersham Biosciences) and 40 μl of rabbit reticulocyte lysate (TNT Quick Coupled Transcription/Translation System, Promega). Reactions were incubated for 1.5 h at 30 °C and used in the PCNA pull-down assay without further purification.
### Identification of Yeast ORFs Coding for Proteins with Putative PIP-box Motifs

To identify new PCNA-interacting proteins, we searched the yeast genome database for ORFs that encode proteins with the PIP-box consensus sequence (Q/MXQ/I/L/MXX/F/Y/F/Y/F). Although all known PIP-boxes in *S. cerevisiae* contain phenylalanine residues in positions seven and eight, we included the possibility of tyrosine residues in these positions since such PIP-boxes have been found in higher eukaryotes. We selected 144 potential PIP-box candidates and tested their ability to bind PCNA in vitro. We used an in vitro assay to identify new PCNA-interacting proteins. Using this assay, we were able to confirm interactions between PCNA and all four PIP-box containing proteins (MSH3, MSH6, UNG1, POL32) chosen as positive controls.

#### RESULTS

**Preparation of Whole Cell Extracts from Yeast Two-hybrid Strains and Quantification of β-Galactosidase Activity**

A single colony from a yeast two-hybrid strain of interest was inoculated into 10 ml of liquid SC-Ura-Trp-His medium containing 2% glucose and grown overnight at 30 °C. The cells were then pelleted by centrifugation for 2 min at 2,000 rpm. Each pellet was resuspended in the volume of liquid SC-Ura-Trp-His medium containing 2% glucose and grown overnight at 30 °C. 

**Preparation of Whole Cell Extracts from Yeast Two-hybrid Strains**

The cells were then pelleted and centrifuged in the presence of 100 μl of SDS-PAGE sample buffer, the solution was incubated at 100 °C for 5 min, and the proteins were separated by SDS-PAGE (4–15% Ready Gel, Bio-Rad) and transferred to a polyvinylidene difluoride membrane (Bio-Rad). The membranes were incubated with antibodies against the hemagglutinin tag (Sigma) to detect proteins expressed from pJd4–5 (PCNA and PCNA-104) and with antibodies against the lexA protein (Invitrogen) to detect proteins expressed from pEG202 (RRM3 wild type and RRM3 mutants) according to the manufacturer's instructions. The Enhanced Chemiluminescence Plus (ECL-Plus) kit from Amersham Biosciences was used for detecting the tagged proteins according to the manual provided by the manufacturer.

**PCNA Pull-down Assay**

We selected 26 known or hypothetical proteins (Table I) of the 135 novel PCNA-interaction candidates and tested their ability to bind PCNA *in vitro*. Candidate ORFs were *in vitro* translated in the presence of [35S]methionine and incubated with PCNA-Affi-Gel-15 beads or with Mock beads (without PCNA) in the presence of 200 mM NaCl, and the bound proteins were analyzed using SDS-PAGE. We found that the DNA helicase RRM3 bound to PCNA beads but not to Mock beads (Fig. 1A), whereas 16 of the candidates did not bind to either beads (Fig. 1B). The remaining nine candidates bound to both the PCNA beads and the Mock beads, suggesting that this assay is not suited for analysis of all candidates (Fig. 1C). This *in vitro* assay provided us with preliminary evidence that RRM3 is a PCNA-interacting protein. Using this assay, we were able to confirm interactions between PCNA and all four PIP-box containing proteins (MSH3, MSH6, UNG1, POL32) chosen as positive controls because they had been previously shown to interact with PCNA (data not shown).

### PCNA Complex Formation with RRM3 Is Promoted by Salt

*In vitro* co-precipitation experiments were carried out in the presence of 200 mM NaCl. However, when we tested the

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### Table II

**Table II**

Sequences of primers used in this study

| Sequence name | 5′-3′ sequence |
|---------------|----------------|
| MSH6-KHS-D115 | GCCGCCACCATGGCCAGCCAGCTAACCTAAAACCTT |
| MSH6-KHS-D116 | GAGATGTAATGCTCAATTTATAC |
| RRM3-KHS-D117 | GCCGCCACATGGCCAGCCAGCTAACCTAAAACCTT |
| RRM3-KHS-D118 | TCTTTTCTTTTGTGTTAACCC |
| YPL110C-KHS-D123 | GCCGCCACATGGCCAGCCAGCTAACCTAAAACCTT |
| YPL110C-KHS-D124 | TCTTTTCTTTTGTGTTAACCC |
| YMR115W-KHS-D135 | GCCGCCACATGGCCAGCCAGCTAACCTAAAACCTT |
| YMR115W-KHS-D136 | TCTTTTCTTTTGTGTTAACCC |
| POL32-KHS-D221 | GCCGCCACATGGCCAGCCAGCTAACCTAAAACCTT |
| POL32-KHS-D222 | TCTTTTCTTTTGTGTTAACCC |
| KHS-D187 | CAGGCAACAAAGGGCTTCGTCAGGCGCAATTGTTGGC |
| KHS-D188 | GCCGCCACATGGCCAGCCAGCTAACCTAAAACCTT |
| KHS-D189 | TCTTTTCTTTTGTGTTAACCC |
| KHS-D288 | GCTTTTCTTTTGTGTTAACCC |
| KHS-D305 | TCTTTTCTTTTGTGTTAACCC |
| KHS-D312 | GCCGCCACATGGCCAGCCAGCTAACCTAAAACCTT |
| KHS-D313 | TCTTTTCTTTTGTGTTAACCC |
| KHS-D318 | CAGGCAACAAAGGGCTTCGTCAGGCGCAATTGTTGGC |
| KHS-D322 | GCCGCCACATGGCCAGCCAGCTAACCTAAAACCTT |
| KHS-D324 | TCTTTTCTTTTGTGTTAACCC |
| KHS-D343 | GCCGCCACATGGCCAGCCAGCTAACCTAAAACCTT |
| KHS-D344 | TCTTTTCTTTTGTGTTAACCC |
ability of RRM3 to interact with PCNA in the presence of varying NaCl concentrations (50–700 mM) we found that RRM3/PCNA complex formation was reduced at the lower NaCl concentrations of 50 and 100 mM, whereas binding increased at higher NaCl concentrations (Fig. 2). Similar but less pronounced salt-induced binding was also observed for the known PCNA-binding proteins MSH6 and POL32 (Fig. 2). This salt-inducible complex formation is consistent with a hydrophobic interaction between two proteins, most likely between the hydrophobic pocket below the interdomain connector loop of PCNA and the PIP-box of the interacting protein.

**RRM3 Interacts with PCNA in Vivo**—The interaction between full-length wild-type RRM3 and PCNA proteins was tested in vivo using a lexA-based yeast two-hybrid system (Figs. 3 and 4). We also re-examined the possibility of an interaction between PCNA and the telomerase subunit EST1 (which contains a PIP-box but did not interact with PCNA beads in the pull-down assay) since yeast telomerase is a multi-protein complex in vivo and in our initial in vitro analysis EST1 was tested separately for its ability to interact with PCNA (Fig. 5). In these experiments, the POL30 gene was present in the bait vector pJG4–5* in-frame with a transcription activation domain and under control of a galactose-inducible promoter. The RRM3 and EST1 ORFs were present in the prey vector pEG202, fused to the lexA protein of E. coli. Expression of wild type RRM3 and PCNA proteins as well as mutant proteins was confirmed by Western blot analysis (Fig. 4, A and B). Complementation of the senescence phenotype of an est1/H9004 mutant confirmed that the EST1 gene in pDK1054-EST1 was expressed and that the lexA-EST1 fusion was a fully functional component of the telomerase complex (Fig. 5A). Interactions between bait and prey proteins were then detected by measuring the transcriptional activation of a lacZ reporter gene. As shown in Fig. 5C, RRM3 tested positive for induction of lacZ expression in the presence of galactose in the media (i.e. when
PCNA was expressed, Fig. 3C, left panel) but tested negative when glucose was added to the media (i.e. in the absence of PCNA, Fig. 3C, right panel). EST1 expression failed to activate the lacZ reporter gene (Fig. 5B). Testing combinations of bait and empty prey vector as well as of prey and empty bait vector ruled out the possibility of auto-activation of the reporter genes.

**Site-directed Mutagenesis Identifies PCNA Interaction Site on RRM3**—To localize the PCNA interaction domain on RRM3 we first generated 54- and 230-amino acid deletions of the N-terminus of RRM3 in the prey vector pEG202, (pRDK1056-RRM3-D54 and pRDK1057-RRM3-D230), both of which delete the putative PIP-box (35QQTLSSFF42) and leave the helicase motifs intact (Fig. 3B). Using a two-hybrid spot assay, we found that deletion of the N-terminal 230 amino acids eliminated the ability of RRM3 to interact with PCNA, whereas deletion of the N-terminal 54 amino acids significantly reduced the interaction (Fig. 3C). Quantification of the β-galactosidase activity in two-hybrid strains expressing RRM3-D54 or RRM3-D230 revealed that they contained 4% or less than 1%, respectively, of the β-galactosidase activity detected in a two-hybrid strain expressing wild type RRM3 and PCNA proteins, which was set to 100%. The average of β-galactosidase activity from at least two transformants is shown.

**Fig. 4. β-Galactosidase activity is reduced in two-hybrid strains expressing PIP-box mutants of RRM3.** Anti-lexA (A) and anti-hemagglutinin (HA) (B) Western blotting confirms the expression of bait proteins (RRM3, RRM3-FFAA, RRM3-FFDD, RRM3-D54, RRM3-D230) and prey proteins (PCNA, PCNA-104), respectively. C, β-galactosidase activity was measured in yeast two-hybrid strains expressing PCNA and RRM3 mutant alleles and compared with the two-hybrid strain expressing wild type RRM3 and PCNA proteins, which was set to 100%. The average of β-galactosidase activity from at least two transformants is shown.

To further localize the PCNA binding site on RRM3, the phenylalanine residues Phe-41 and Phe-42 in the putative PIP-box were changed to alanine using site-directed mutagenesis (Fig. 3B). Interaction between PCNA and this RRM3 mutant was detectable in the spot assay (Fig. 3C) but at a lower level than between the wild type proteins. Quantification of the β-galactosidase activity in whole cell extracts from this two-hybrid strain showed about 80% reduced β-galactosidase activity compared with the two-hybrid strain expressing wild type RRM3 and PCNA proteins, which was set to 100%. The lacZ reporter gene is not expressed in a EST1/PCNA two-hybrid strain, suggesting that EST1 and PCNA do not interact.

**Fig. 5. EST1 and PCNA do not interact in the yeast two-hybrid test.** A, EST1-lexA fusion expressed from pRDK1054-EST1 bait vector is fully functional as shown by complementation of the senescence phenotype of the est1Δ mutant RDKY3445 (31). RDKY3445 was transformed with pRDK1054-EST1 vector or empty pEG202 vector, streaked on SC-His media, and grown at 30°C for 2 days. B, yeast two-hybrid interaction was tested between PCNA (or pG4-5* vector) and EST1 in the presence of 2% galactose in the media (left panel) and in the presence of 2% glucose in the media as a negative control (right panel). The lacZ reporter gene is not expressed in a EST1/PCNA two-hybrid strain, suggesting that EST1 and PCNA do not interact.
RRM3 Helicase Interacts with PCNA of *S. cerevisiae*

104, which has an alanine to valine substitution at residue 251 (A251V). The crystal structure of PCNA indicates that this alanine residue is present in the same hydrophobic pocket that interacts with the PIP-box of p21 during the interaction between hPCNA and p21. Consistent with this, this amino acid substitution has been shown to disrupt the interaction between PCNA and MSH6 (10). However, Fig. 3C shows that PCNA-104 and wild type RRM3 interact strongly in the two-hybrid assay, similar to what was observed for the interaction between the wild-type proteins, suggesting that the pol30-104 mutation did not disrupt interaction with RRM3.

**DISCUSSION**

We have identified 144 ORFs in the genome of *S. cerevisiae* that encode proteins with the PIP-box consensus sequence, QXX(M/L)LXX(F/Y)(F/Y). Among them are nine proteins that have previously been shown to interact with PCNA (POL32, RAD27, MSH3, MSH6, UNG1, POL2, RFC1, CAC1, and DNA ligase). We have initiated a study with the aim to test all 135 new candidates for their ability to interact with PCNA. In the study presented here we have tested 26 candidates, most of which were chosen because the PIP-box was located near the N or C terminus of the protein or the protein was implicated in DNA metabolism, and we found that one of these candidates, the RRM3 helicase, interacts with PCNA. Fifteen of these 26 candidates did not interact with PCNA in the pull-down assay, suggesting that many PIP-box-containing proteins do not interact with PCNA. Consistent with this, most of these 15 proteins whose function is known do not have a function that suggests they might interact with PCNA (Table I). The remaining 109 candidates as well as the 9 candidates that nonspecifically precipitated with PCNA beads (Table I, group No. 3) will be tested for PCNA interaction using other assays including a yeast two-hybrid assay. Of these 118 candidates, 4 are known to function in DNA metabolism, making them the most logical candidates for testing. It is difficult to predict whether more PCNA-binding proteins will be identified among the remaining candidates since knowledge about their biological function and subcellular localization is often very limited.

The RRM3 gene (ribosomal DNA recombination mutation 3) was first identified in a screen for suppressors of recombination between naturally occurring tandem repeats such as the rDNA genes and the copper chelation genes *CUP1A* and *CUP1B* (21). The *RRM3* gene product is 38% identical to 485 amino acids spanning the helicase domain of PIF1, an ATP-dependent 5′-3′ DNA helicase that is involved in the maintenance of telomeric, ribosomal, and mitochondrial DNA (28). Like PIF1, RRM3 possesses 5′-3′ helicase activity (20), and its role in rDNA replication has been studied in great detail. Ivessa *et al.* (19) observe that in the absence of RRM3 activity replication pauses at a number of specific sites within rDNA repeats and that replication forks converging at the replication fork barrier are unusually persistent. The *rrm3* mutants exhibit RAD52-dependent accumulation of extrachromosomal rDNA circles and an increased presence of Holliday junctions (19). This suggested that pause sites are eventually converted into double-strand breaks that are then repaired by homologous recombination, which can result in the formation of rDNA circles. Interestingly, replication pause sites within the rDNA repeats coincided with sites that are bound by proteins, and it was therefore suggested that RRM3 might be required to replicate through such protein-rDNA complexes (19, 29). Furthermore, the report of telomere instability and destabilization of mitochondrial DNA in *rrm3* mutants may suggest that RRM3 also contributes to faithful replication by unwinding DNA secondary structures that are found at these sites (19).

In addition to its role in maintaining the stability of direct tandem repeats a recent study has identified RRM3 as a regulator of Ty1 transposition in yeast (22). A 110-fold increase in transposition events occurred in *rrm3* mutants despite the absence of increased Ty1 transcription. This suggests that RRM3 normally suppresses Ty1 transposition by inhibiting homologous recombination and/or integration at *de novo* sites. One possible explanation for the large increase in Ty1 transposition could be that in the absence of RRM3, increased DNA breakage occurs due to impaired replication fork progression, and this would result in a larger number of potential integration sites for Ty1 elements.

Taken together the observations discussed above suggest a role for RRM3 in genome replication although probably not as a replicative helicase but rather as an accessory helicase that facilitates progression of replication forks through obstructions such as bound proteins and possibly DNA secondary structure. The ability of RRM3 to directly interact with PCNA might help target RRM3 to these sites when the replication fork encounters them. Because the interaction between RRM3 and PCNA is mediated by the similar PIP-box motif found in many DNA replication proteins that bind PCNA, one can imagine a scenario where replication proteins occupy the interaction site(s) on the PCNA clamp during DNA replication but disengage upon encountering replication blocks, allowing “helper” proteins such as RRM3 to be brought to the stalled fork to deal with specific obstructions.

In contrast to a previous study that showed that the interaction between PCNA and the mismatch repair protein MSH6 could be disrupted by a pol30-104 mutation (10), we found that the pol30-104 mutation did not disrupt the interaction with RRM3. However, similar to our observation, Gomes and Burgers (17) find that the interaction between RAD27 and PCNA-90, which contains P252A and K253A amino acid substitutions, was similar to the interaction between the wild type proteins. Interestingly, the mutated residues in PCNA-90 are adjacent to the residue mutated in PCNA-104 (A251V), which is consistent with our observation that the interaction with RRM3 was unaffected by the PCNA-104 mutation. Although the contrasting findings on the interaction between PCNA and RRM3 or RAD27 (17) compared with the interaction between PCNA and MSH2-MSH6 (10) could be attributed to differences under “Experimental Procedures,” they could also be an indication that the various PIP-box proteins bind to PCNA with different affinities or include multiple sites despite the fact that they all interact via a conserved binding motif. To further elucidate this question we are currently investigating the ability of RRM3 to interact with various PCNA mutants.

It is interesting to note that the closely related PIF1 helicase may also interact with PCNA but indirectly through its ability to interact with the chromatin-assembly factor subunit CAC1, which is also a PCNA-interacting protein (13, 30). The N terminus of PIF1 contains the CAC1-interacting region, which is similar to the observation that the N terminus of RRM3 contains the PCNA-interaction region. This supports a previous suggestion that within the PIF1 family of DNA helicases functional specificity may be achieved by varying protein-protein interactions at the N terminus while the helicase motif region is highly conserved (29). One can speculate that the opposing roles of RRM3 and PIF1 in telomere elongation and rDNA replication (29) may be the result of competition between RRM3 and the PIF1-CAC1 complex for binding to PCNA, and this competition then regulates such processes as telomere elongation and rDNA replication.

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