Destabilized PCNA Trimers Suppress Defective Rfc1 Proteins in Vivo and in Vitro†

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ABSTRACT: Replication factor C (RFC) and the proliferating cell nuclear antigen (PCNA) are two essential DNA polymerase accessory proteins that are required for numerous aspects of DNA metabolism including DNA replication, DNA repair, and telomere metabolism. PCNA is a homotrimeric ring-shaped sliding DNA clamp that can facilitate DNA replication by tethering DNA polymerase δ or DNA polymerase ϵ to the DNA template. RFC is the 5-subunit multiprotein complex that loads PCNA onto DNA at primer-template junctions in an ATP-dependent reaction. All five of the RFC subunits share a set of related sequences (RFC boxes) that include nucleotide-binding consensus sequences. We report here that a mutation in the gene encoding the large subunit of yeast RFC gives rise to DNA metabolism defects that can be observed in vivo and in vitro. The *rfc1-1* substitution (D513N) lies within the widely conserved RFC box VIII consensus sequence and results in phenotypes including DNA replication defects, increased sensitivity to DNA damaging agents, and elongated telomeres. Mutant *Rfc1-1* complexes exhibit in vitro DNA replication defects that are sensitive to ATP concentrations, and these defects can be suppressed by mutant PCNA proteins which contain substitutions that destabilize the homotrimeric sliding DNA clamp.

The processes of DNA replication and DNA repair are vital for the stable duplication and maintenance of the genome and are critically dependent on the activities of the DNA polymerases and their associated proteins. Intense biochemical and genetic analysis has revealed that there are striking similarities between the DNA replication complexes found in prokaryotes and eukaryotes (*1*). These complexes include core polypeptides that, in association with numerous other proteins, are capable of catalyzing the addition of deoxyribonucleotides onto primer-template junctions. For example, in *Escherichia coli*, the catalytic polymerase of DNA polymerase III is contained in the α-subunit, but it associates with at least nine other protein subunits to form the processive DNA polymerase III holoenzyme (*2*, *3*). Similarly, in yeast and in higher eukaryotes, the replicative DNA polymerases δ (Polδ) and ϵ associate with accessory factors that serve to regulate and stimulate their activity (*4*−*7*). These accessory proteins are essential components of the DNA replication and DNA repair machineries, and significantly enhance polymerase processivity. Through structural and functional analysis, two classes of DNA polymerase accessory proteins can be identified in systems ranging from *E. coli* to yeast and humans: the sliding DNA clamps and the clamp loaders.

The sliding DNA clamps are homodimeric or homotrimeric, ring-shaped proteins that function to increase DNA polymerase processivity by tethering the polymerase to the DNA template. Members of this class of proteins have been identified in bacteriophage T4, *E. coli*, yeast, and higher eukaryotes (*1*). Although the amino acid and subunit composition of the clamps can vary considerably between organisms, the overall structure of this type of protein is highly similar. In *E. coli*, the ring-shaped sliding DNA clamp (the β-subunit of DNA polymerase III) is a homodimer formed by the association of two 45 kDa subunits (*8*). In yeast as in humans, the clamp [also known as the proliferating cell nuclear antigen (PCNA)][1] is a homotrimer of three 30 kDa subunits (*9*, *10*). The structure of the individual subunits is such that, when they multimerize to form the DNA clamp, they create a 6-fold symmetrical ring that contains a central cavity of roughly 35 Å diameter. These multisubunit rings are held together by protein−protein interactions at the interface regions of the individual subunits. These protein−protein interactions are likely to be critical for the function of the sliding clamps, since presumably it is these interactions that must be altered when the sliding clamps are assembled onto and off of duplex DNA. Support for this hypothesis comes from the observation that single mutations that map to the interface region of yeast PCNA (S115P) or human PCNA (Y114A) disrupt trimer formation which eliminates PCNA activity in vitro and compromises yeast cell growth (*11*, *12*).

The task of loading PCNA onto the DNA resides with the clamp loader, replication factor C (RFC). RFC has a DNA-dependent ATPase activity which is stimulated by primer-template junctions and PCNA, and ATP hydrolysis is required for efficient loading of PCNA (*5*, *13*, *14*). In

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[1] Abbreviations: RFC, replication factor C; PCNA, proliferating cell nuclear antigen; MMS, methylmethanesulfonate; SSB, *E. coli* single-stranded DNA-binding protein.

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Table 1: *S. cerevisiae* Strains Used in this Study

strain	alias	genotype[a]	ref
yMM10	CH1492	*MAT**a** ade2 leu2-3,112 ura3-52*	*24*
yMM16	CH1807	*MAT**a** his4-539 lys2-801 ura3-52 rfc1-1.URA3*[a]	*17*
yMM53	CH1974	*MAT**a** ade2 his4 lys2 ura3 rfc1-1.URA3*[a] *pol30-32*	*24*
yMM54	CH2021	*MAT**a** his4 lys2 ura3 rfc1-1.URA3*[a] *pol30-37*	*24*
yMM103		*MAT**a** his4 ura3-52 lys2-801 pol30-37*	this study
yMM119		*MAT*α *his3 pol30-32*	this study

[a] The *URA3*$^+$ marker in this strain is adjacent to but does not disrupt RFC1.

yeast, all five subunits of RFC are essential proteins, and each contains a central, conserved 225 amino acid domain (RFC boxes II−VIII) that includes a nucleotide-binding consensus sequence (*15*−*20*). In addition to this nucleotide-binding domain, the large subunit of RFC also contains an N-terminal DNA-binding domain (RFC box I) that exhibits similarity to sequences from prokaryotic DNA ligases (*17*, *21*). The C-terminal regions of the RFC proteins contain elements that are important for the formation of the entire 5-subunit RFC complex (*22*, *23*).

Previously, in an effort to determine the roles that the clamp loader and clamp proteins play in DNA metabolism, we characterized yeast strains bearing mutations in the large subunit of RFC (the *RFC1* or *CDC44* gene). We demonstrated that cold-sensitive *rfc1-1* mutants suffer defects in a number of aspects of DNA metabolism, including defects in DNA replication and DNA repair. Through genetic analysis, we isolated second-site suppressor mutations which suppressed the cold sensitivity of the *rfc1-1* allele. These suppressor mutations mapped to the *POL30* gene encoding PCNA (*24*). We have extended that analysis and report here that the suppressor *pol30* alleles can suppress multiple phenotypes associated with the *rfc1-1* mutation. The position of the *rfc1-1* substitution is predicted to lie within the putative nucleotide-binding domain of Rfc1p, and mutant Rfc1-1 complexes exhibit DNA replication defects in vitro that are sensitive to ATP concentrations. These defects can be alleviated by mutant PCNA proteins that are derived from the suppressor *pol30* alleles. Here, we report on the in vivo and in vitro activities of two strong suppressor alleles. They map close to the interface region of the PCNA homotrimer and destabilize the structure of the sliding DNA clamp.

MATERIALS AND METHODS

Strains and Media. The yeast strains used in this study are listed in Table 1. Standard yeast genetic techniques and media were used throughout (*25*).

Telomere Length Assay. Telomere length was determined essentially by the procedure reported previously (*26*). Briefly, strains were grown to saturation in YPD medium and genomic DNA was prepared by disrupting the cells with glass beads (*27*). The DNA was digested with *Xho*I, separated by agarose gel electrophoresis, and subjected to Southern analysis. Southern blots were probed with a *Xho*I−*Sal*I fragment of plasmid pJH345 [which contains telomere associated Y′ and poly(G_{1-3}T) sequences] and visualized by phosphorimaging analysis after an 18 h exposure.

UV and MMS Sensitivity Assays. Strains were grown in liquid YPD medium at 30 °C to early log phase, sonicated, and then diluted in water as necessary. The cells were then either exposed to 0.3% methyl methanesulfonate (MMS) and then plated or plated directly onto YPD plates and exposed to up to a maximum of 200 J/m^2 of 254 nm UV radiation from a model 2400 Stratalinker (Stratagene). Plates were scored for viability after incubation at 30 °C for 3 days. Plots reflect the mean viability derived from at least three experiments.

Purification of RFC and Rfc1-1. RFC was overproduced about 80-fold in yeast after induction of a strain containing all five *RFC* genes on a single plasmid (pBL420), with expression of each gene placed under control of the galactose-inducible *GAL1-10* promoter, and subsequently purified by Affigel Blue, PCNA-Agarose, and MonoS chromatography as described (*28*). The mutant RFC complex, Rfc1-1, containing the Rfc1-1 subunit, was similarly overproduced from plasmid pBL420-5 in strain PY63 which has both the plasmid and chromosomal *RFC1* genes replaced with the *rfc1-1* allele. Purification of the mutant complex proceeded similarly, with the exception that only about 10% of the Rfc1-1 activity bound to the PCNA-Agarose column, compared to about 90% of the wild-type RFC.

Construction of PCNA Expression Plasmids. For the expression of yeast PCNA proteins in *E. coli*, an expression plasmid (pMM115) was created in which the wild-type yeast PCNA gene was located downstream of an inducible T7 promoter. This plasmid was a derivative of plasmid pRSET A (Invitrogen) and contained an engineered *Nde*I site at the PCNA ATG translational initiation codon. Fragments bearing the *pol30*−*32* and *pol30*−*37* mutations were cloned into this plasmid (creating plasmids pMM117 and pMM125) to allow for the expression and purification of mutant PCNA proteins. To create the phosphotagged PCNA proteins, a short double-stranded DNA sequence coding for the phosphotag amino acids was cloned into the *Nde*I site of these plasmids. The resulting plasmids (pMM124, pMM136, and pMM138) contained a 16 amino acid N-terminal sequence (MRRASVGSMRRASVGS) which contained two protein kinase recognition motifs. Both wild-type and mutant yeast proteins were expressed in *E. coli* and purified by standard chromatographic methods as described previously (*11*). Typical yields were in the order of 15 mg/2 L of culture.

ATPase Assays. ATPase assays were performed as described previously (*14*). Briefly, 20 μg of either wild-type or mutant RFC was incubated in 15 μL reactions containing 40 mM Tris-HCl, pH 7.8, 1 mM $MgCl_2$, 0.2 mg/mL BSA, 1 mM DTT, 50 mM NaCl, 12 μM α^{32}P-ATP, 25 μg of of singly primed single stranded mp18 DNA, 150 μg of of *E. coli* single-stranded DNA-binding protein (SSB), and 10 μg PCNA as indicated for 10 min at 30 °C or 20 min at 20 °C. Reactions were stopped by the addition of 10 μL of 50 mM EDTA, 1% SDS, 25 mM ATP, and 25 mM ADP. Aliquots from the reactions were separated by thin-layer chromatography and then subjected to phosphor-

imaging analysis for 14 h to determine the proportions of ATP and ADP. The percent hydrolysis values correspond to the averages derived from two independent experiments.

Primer-Template Mobility Shift Assays. Primer-template mobility shift assays were based on the protocol described previously (*13*). Briefly, primer-template junction DNA was created by annealing a 25-mer (5′-TGAAAAGTTCTTCTC-CTTTACTGAA-3′) that was end labeled with [γ^{32}P]ATP with an excess of a complementary 50-mer (5′ATAGAC-CAGGAAAGAGTAGACGTGCCAGAAGATTCAGTAA-AGGAGAAGAACTTTTCA-3′). The primer-template DNA was electrophoresed on acrylamide gels and purified from unannealed primers. Five femtomoles of the primer-template DNA was incubated in 5 μL reactions containing 0−50 μg of RFC or Rfc1-1, 30 mM HEPES, pH 7.8, 7 mM $MgCl_2$, 100 mg/mL BSA, 0.5 mM DTT, and 0.4 mM ATP γ-S. Following a 15 min incubation at 25 °C, complexes were fixed with 1 μL of 5% glutaraldehyde, and they were then incubated for an additional 5 min at 25 °C. Samples were adjusted to 10% glycerol, loaded onto a pre-run 6% polyacrylamide gel, and separated for 3 h at 120 V in a 1/2× TBE system. Dried gels were then subjected to phosphorimaging analysis with an 18 h exposure time.

In Vitro DNA Replication Assays. *In vitro* DNA replication assays were performed in 30 μL aliquots that typically contained a final concentration of 20 mM Tris-HCl, pH 7.8, 0.2 mg/mL BSA, 1 mM DTT, 65 mM NaCl, 1 mM ATP, 100 μM each of dGTP, dTTP, dATP, and 25 μM dCTP (α ^{32}P, 5,000 cpm/pmol), 8 mM mg of acetate, 10 μg of PCNA, 10 μg of Polδ, 30 μg of RFC, 850 μg of *E. coli* single-stranded DNA-binding protein (SSB), and 100 μg of singly primed single-stranded M13mp18 DNA. Reactions were incubated at 30 °C with shaking for up to 20 min. For the determination of acid-insoluble radioactivity, reactions were stopped by the addition of 100 μL of 50 mM sodium pyrophosphate, 25 mM EDTA and 50 μg/mL calf thymus carrier DNA. Samples were precipitated in 10% trichloroacetic acid, filtered on Whatman glass fiber filters, and counted by liquid scintillation analysis. For electrophoretic analysis, reactions were stopped by the addition of DNA-loading buffer containing 50 mM EDTA. Samples were then subjected to alkaline electrophoresis on 1.2% agarose gels, dried, and subjected to phosphorimaging analysis for 18 h. Signal intensities between the RFC and Rfc1-1 reactions can be compared directly since the reactions were run at the same time. A λ *Hin*dIII digest was end labeled with [^{32}P]ATP and used for molecular weight markers.

Labeling of Phosphotagged PCNA Proteins. Phosphotagged PCNA preparations were labeled with [γ-^{32}P]ATP by the protein kinase catalytic subunit from bovine heart (Sigma). The 30 μL labeling reaction was incubated at 37 °C for 15 min and contained 20 mM Tris-HCl, pH 7.5, 12 mM magnesium−acetate, 100 mM NaCl, 0.1 mg/mL BSA, 10 units of protein kinase, 1 ug of PCNA and 2×10^6 cpm of [γ-^{32}P]ATP. After the initial 15 min of labeling, cold ATP was added to 1 μM and the reaction was incubated for another 15 min.

Gel Filtration Analysis. Either unlabeled PCNA or phospho-PCNA labeled with ^{32}P was diluted in 50 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, and 10 mM NaCl and incubated at 30 °C for 45 min. Samples were then subjected to gel filtration in the same buffer over a Superose 12HR 10/30 column (Pharmacia) at 0.3 mL/min flow rate with a Waters 650 Advanced Protein Purification System. Protein concentrations were detected either by UV absorbance for nontagged PCNA, or by collecting fractions and analyzing them by liquid scintillation counting. The analysis of molecular mass standards including carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), and alcohol dehydrogenase (150 kDa) revealed that a 90 kDa protein would be expected to elute at 12.5 mL, whereas 30 kDa proteins elute at about 14.5 mL (data not shown).

RESULTS

POL30 (PCNA) Mutations Can Suppress Multiple Defects of rfc1-1 Mutations in Vivo. Previous analysis of yeast strains bearing mutations in the gene encoding for the large subunit of RFC (the *RFC1* or *CDC44* gene) indicated that Rfc1p plays a role in DNA replication, DNA repair, and telomere metabolism in vivo (*17, 26, 29*). These results, in combination with the biochemical analysis of RFC and PCNA in vitro, suggest that these accessory proteins function to promote the polymerization of nucleotides at primer-template junctions. To further investigate the functional relationship between the RFC and PCNA proteins, we characterized a set of specific mutant *RFC1* and *POL30* alleles in vivo and in vitro. For this analysis, we have focused on the original cold-sensitive *rfc1-1* (*cdc44-1*) mutation and two of the *pol30* (PCNA) alleles that were isolated in a search for gene products that interact genetically with the Rfc1 protein (*24*). Cells bearing the *rfc1-1* allele are cold sensitive (Figure 1) and arrest in the cell cycle at the end of S phase when shifted to the restrictive temperature of 20 °C. This cold sensitivity can be suppressed by second site suppressor mutations in the gene that encodes for PCNA (i.e., *pol30−32* or *pol30−37*). This suppression is specific to certain *pol30* alleles, and they cannot suppress the lethal phenotype of a *rfc1* deletion (data not shown). When combined with a wild-type *RFC1* allele, the *pol30* suppressor alleles do not give rise to a temperature-sensitive defect of their own.

Since *rfc1-1* mutants display a wide range of DNA metabolism-related phenotypes in vivo and the RFC complex potentially interacts with a number of DNA replication factors, we sought to determine to what extent the *rfc1-1*-mediated defects were related to the interaction of RFC with PCNA. RFC is known to be involved in DNA repair, and *rfc1-1* mutants are sensitive to DNA damaging agents. To determine if this phenotype is related to the ability of RFC to interact with PCNA, we assayed our set of strains for sensitivity to the DNA-damaging agent methyl methanesulfonate (MMS) and to UV irradiation. Strains were exposed to either UV irradiation or to MMS, plated onto YPD plates at 30 °C, and then scored for growth after 3 days. We observed that the *rfc1-1* mutants were sensitive to both DNA damaging agents, and that this sensitivity was partially suppressed by the suppressor *pol30* alleles (Figure 1B). Strains bearing only the *pol30* mutant alleles were as sensitive as wild-type strains to MMS, but did show an increased sensitivity to UV irradiation. Thus, the DNA repair defect of the *rfc1-1* mutants appears related to the ability of RFC to interact with PCNA.

Recently, it was reported that, in addition to exhibiting defects in DNA replication and DNA repair, *rfc1-1* mutants

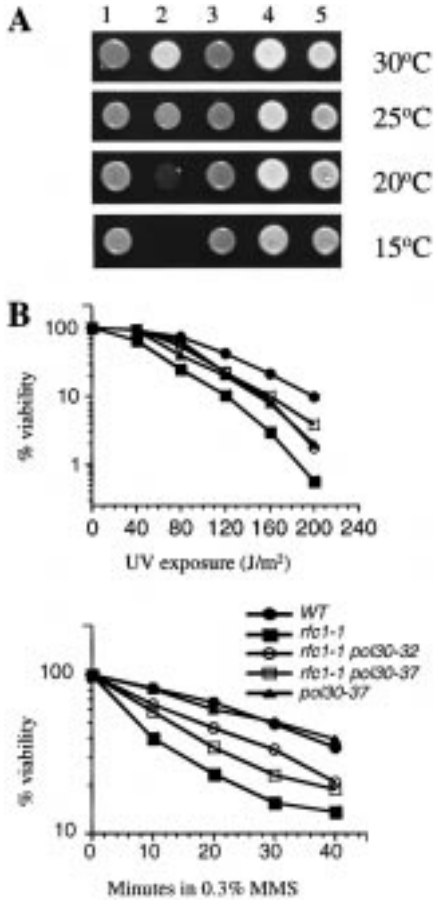

FIGURE 1: Phenotypes of *rfc1* and *pol30* mutants. (A) *pol30* mutations can suppress the cold-sensitive phenotype of *rfc1-1* mutants. Strains bearing either wild-type or mutant *RFC1* and *POL30* alleles were inoculated onto YPD plates and incubated at 30, 25, 20, and 15 °C for 2−4 days. Lanes: 1 strain yMM10 (*RFC1 POL30*); 2, strain yMM16 (*rfc1-1 POL30*); 3, strain yMM53 (*rfc1-1 pol30-32*); 4, strain yMM54 (*rfc1-1 pol30-37*); 5, strain yMM103 (*RFC1 pol30-37*). (B) *pol30* mutations can suppress the DNA-damaging agent sensitivity phenotype of *rfc1-1* mutants. The same strains from panel A were grown to early log phase and exposed to the DNA damaging agent as indicated. The cells were then diluted, plated onto YPD plates, and incubated at 30 °C for 3 days before scoring for viable colonies.

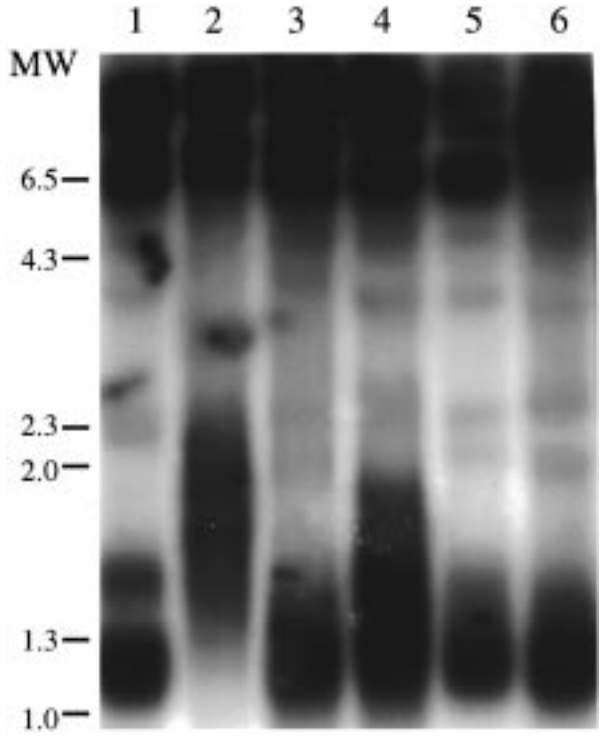

FIGURE 2: *pol30* mutations can suppress the telomere elongation phenotype of *rfc1-1* mutants. Genomic DNA was prepared from strains that were grown to late log phase. The DNA was digested with *Xho*I, electrophoresed on an agarose gel, and then Southern blotted. The blot was hybridized with a telomere sequence containing probe and visualized by phosphor-imaging analysis. Under these conditions, telomere-specific bands migrate at positions between 1.0 and 2.0 kbp. Lanes: 1 strain yMM10 (*RFC1 POL30*); 2, strain yMM16 (*rfc1-1 POL30*); 3, strain yMM53 (*rfc1-1 pol30-32*); 4, strain yMM54 (*rfc1-1 pol30-37*); 5, strain yMM103 (*RFC1 pol30-37*); lane 6 strain yMM119 (*RFC1 pol30-32*).

also have abnormally elongated telomeres (*26*). Although the mechanism of this telomere elongation is not well understood, it may be that inefficient DNA replication by the replicative polymerases at telomeres perturbs telomere length regulation. To determine whether the *rfc1-1*-mediated telomere defect was related to the interaction of RFC with PCNA, we assayed for telomere length in strains bearing combinations of alleles in the *RFC1* and *POL30* genes. Telomere length was assayed by probing Southern blots of genomic DNA with a probe containing telomeric repeat sequences. In this assay, *Xho*I-digested telomeric fragments from wild-type strains normally migrate at a position between 1.0 and 1.5 kb in length (Figure 2). In contrast, *rfc1-1* mutants exhibit elongated telomeres that migrate at positions between 1.5 and 2.0 kb. Strains that contained *pol30−32* or *pol30−37* suppressor alleles in addition to *rfc1* mutations had telomeres that reverted to near wild-type lengths (the *pol30−32* allele being a stronger suppressor than the *pol30−37* allele). Strains that contained only the suppressor *pol30* alleles had near normal-length telomeres, suggesting that the *pol30* alleles did not cause telomere shortening on their own. Therefore, as with the cold-sensitive and DNA-damaging agent sensitivity phenotypes, the telomere length phenotype of the *rfc1-1* mutants appears related to the interaction of RFC with PCNA.

Purification of Wild-Type and Mutant RFC and PCNA Proteins. To gain a more detailed understanding of how the *rfc1-1* and *pol30* alleles give rise to the range of DNA metabolism-related phenotypes observed in vivo, we characterized the wild-type and mutant RFC and PCNA proteins in vitro. We sought to determine whether the mutant Rfc1-1 protein exhibits defects in in vitro assays, and if so, if those defects could be suppressed by the same PCNAs that suppress the *rfc1-1* defect in vivo. To this end, we purified the entire 5-subunit RFC complex from a plasmid containing yeast strain overproducing all five subunits (*28*). In a similar fashion, the mutant Rfc1-1 complex was purified from a *rfc1-1* strain containing a plasmid overproducing the mutant Rfc1-1 subunit together with the wild-type Rfc2, Rfc3, Rfc4, and Rfc5 subunits (see Materials and Methods). This purification included a PCNA-affinity chromatography step (*28*). Additionally, we expressed recombinant wild-type and mutant yeast PCNA in *E. coli* cells and purified the PCNA proteins by conventional chromatographic methods. Aliquots of the final fractions were run on SDS−PAGE to assess the composition and purity of the preparations (data not shown).

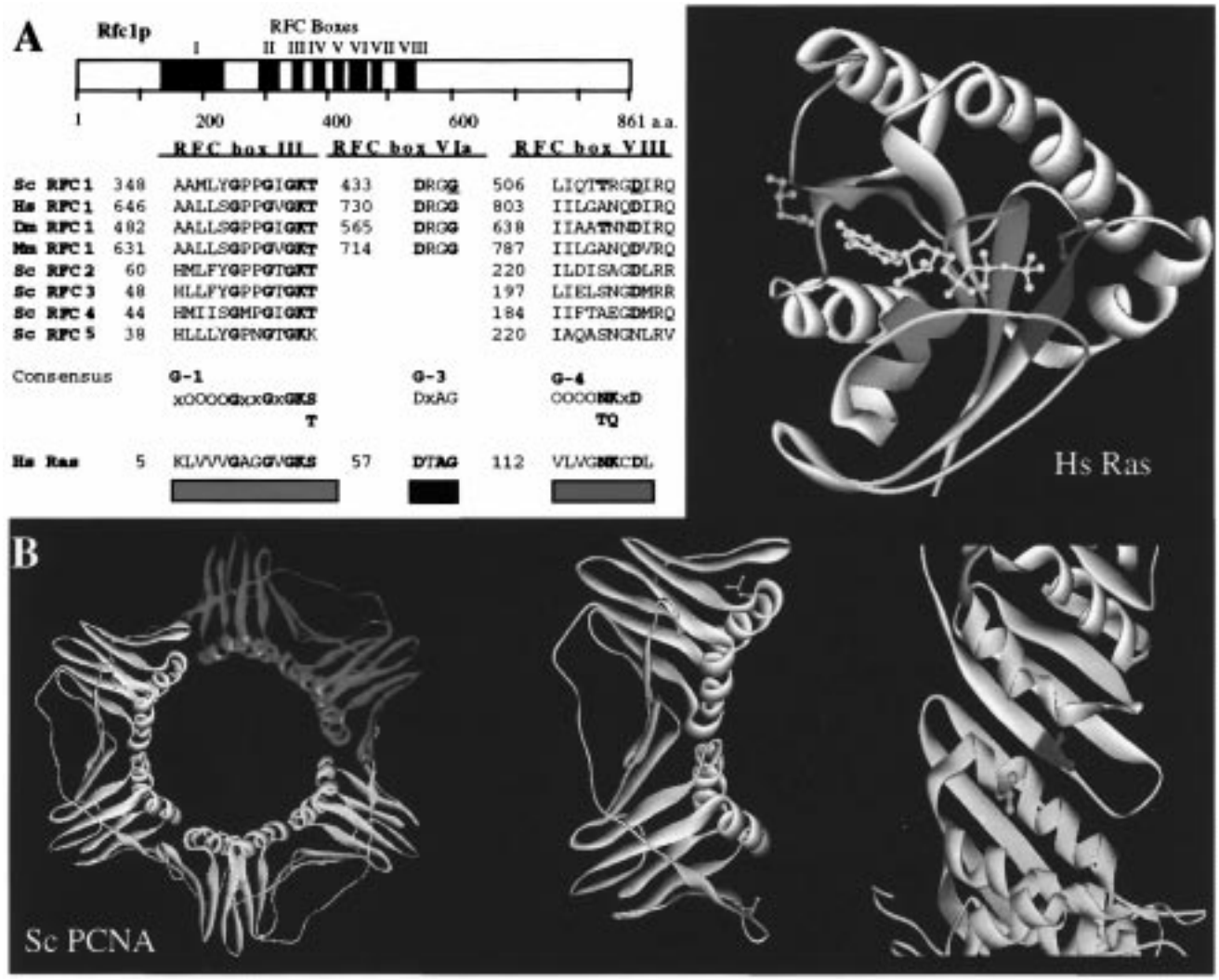

FIGURE 3: Nature of the *rfc1* and *pol30* mutations (A). At left, a schematic of the Rfc1 protein is shown with the relative positions of the RFC boxes I through VIII. An amino acid alignment is indicated which includes sequences from RFC boxes III, VIa, and VIII for the 5 yeast RFC subunits, the human Rfc1 protein, the nucleotide-binding protein G-1, G-3, and G-4 consensus sequences (*30*), and Hs p21Ras. Residues in bold are those that match the consensus sequences. Underlined residues correspond to the positions of the *rfc1-1* mutation D513N, and *rfc1−4* mutation G436R. At the right, the positions of the G-1, G-3, and G-4 consensus sequences are shown on a ribbon diagram of the crystal structure of Hs p21Ras bound to GppNp (shown in yellow) (*32*). The positions of the residues equivalent to D513 and G436 are indicated in ball-and-stick format. (B) Positions of the *pol30* mutations on the crystal structure of yeast PCNA. A ribbon diagram of the homotrimeric PCNA clamp protein is shown with the three identical subunits depicted in red, white, and yellow (left) (*10*). The position of the *pol30* suppressor substitutions are indicated on the structure of a single PCNA monomer (center). The colors of the mutant positions are green, A112; pink, L151. A close-up view of the interface region reveals that the A112T and L151S substitutions map directly at the subunit interface (right).

For the mutant Rfc1-1 complex, the large subunit contained the D513N *rfc1-1* substitution. Since as yet there are no crystal structures solved for any of the RFC subunits, we used sequence similarities and the structures of other related proteins to estimate the position and potential effect of this change. Comparisons between the amino acid sequences of the human, mouse, and yeast RFC subunits has revealed that all of these proteins share a set of related sequences that have been called RFC boxes (Figure 3A) (*15*). RFC boxes II−VIII are widely conserved between prokaryotes and eukaryotes, and are found within all of the subunits of the clamp loaders from *E. coli*, yeast, and human cells. Several of these RFC boxes also overlap with consensus sequences that are common to nucleotide-binding proteins. For example, within RFC box III, there exists a match to the G-1 consensus sequence as has been defined for GTP-binding proteins (*30*). This consensus sequence, also known as the "Walker-A motif", is also found in ATP-binding proteins (*31*). This motif forms a distinctive phosphate-binding segment or "P-loop" that interacts with the phosphate tail of nucleotides. Additionally, the large subunit of yeast RFC contains sequences within RFC boxes VIa and VIII that match in content and spacing to the G-3 and G-4 consensus sequences. These residues are likely to be important for RFC function, since they are highly conserved within the large subunits from yeast, drosophila, mouse, and humans.

We used this sequence similarity between Rfc1p and known nucleotide-binding proteins as a basis to predict the position of the *rfc1-1* substitution. Although biochemical analysis suggests that RFC is an ATPase rather than a GTPase, the presence of the G1 to G4 consensus sequences suggests that Rfc1p may contain a nucleotide-binding pocket that is similar to that found in GTP-binding proteins. The crystal structure of human p21Ras bound to the GTP

analogue GppNp has been solved, and it was used as a model on which to map the position of these consensus sequences (Figure 3A) (*32*). In p21 Ras, the G-1/RFC box III motif forms the P-loop (in blue), the G-3/RFC box VIa motif (in green) corresponds to sequences that lie close to the base end of the bound GTP (yellow), and the G-4/RFC box VIII motif (in pink) lies near the terminal phosphates. Given this alignment, the relative position of the D513N substitution is predicted to lie within the putative nucleotide-binding pocket of Rfc1p. In p21Ras, the equivalent amino acid (D119) corresponds to the highly conserved last position of the G-4 consensus sequence. This amino acid is important for forming hydrogen bonds with groups on the guanine ring, and the equivalent *rfc1-1* substitution of aspartic acid to asparagine at this residue lowers GTP binding by 100-fold (*33*).

This alignment can also be used to estimate the position of the *rfc1-4/cdc44-4* mutation. This G436R substitution corresponds to the highly conserved glycine within the G-3 consensus sequence. In p21Ras, this residue is important for forming a hydrogen bond with the terminal phosphate (*32*). Interestingly, the *rfc1-4* mutation gives rise to a cold-sensitive phenotype that, like the phenotype of the *rfc1-1* allele, can be compensated by suppressor PCNA mutations (*24*). Since the phenotypes of other *rfc1* alleles cannot be suppressed by these *pol30* mutations, the *rfc1-1* and *rfc1-4* mutations may disrupt a similar function of the Rfc1p. Although the exact positioning of these residues will require solving the structure of the protein, these similarities suggest that these *rfc1* substitutions may disrupt the putative nucleotide binding pocket of Rfc1p.

The suppressor *pol30* alleles considered in this study each result in a single amino acid substitution in the PCNA protein. The *pol30*−*32* allele substitutes a threonine for an alanine at position 112, and the *pol30*−*37* allele encodes for a substitution of serine for leucine at position 151. These substitutions map to opposite ends of the monomeric PCNA polypeptide, and in each case, a nonpolar side chain is replaced with a polar one (Figure 3B). These residues are located at the interface regions of the PCNA molecule that provide the protein−protein interactions that keep the three monomeric subunits of PCNA together to form the doughnut-shaped, homotrimeric sliding DNA clamp. Positions 112 and 151 lie directly within this interface region and are in close contact with other nonpolar residues from the neighboring subunits. Due to the nature and position of these substitutions it would be expected that they could alter the stability of the trimeric sliding clamp (see below).

Mutant Rfc1-1 Complexes Can Hydrolyze ATP and Bind DNA in Vitro. In the effort to characterize the defects associated with the *rfc1-1* mutation, we assayed the wild-type and mutant RFC complexes in vitro. The five subunit RFC complex is known to contain an ATPase activity that is stimulated by the addition of DNA and PCNA (*5*). Although each of the individual RFC subunits contains nucleotide-binding consensus sequences, it is not clear to what extent these individual subunits contribute toward the ATPase activity observed for the entire RFC complex. To determine whether the *rfc1-1* mutation affected this activity, we conducted ATPase assays on the wild-type and mutant RFC complexes at the permissive and semirestrictive temperatures of 30 and 20 °C, respectively (Figure 4A). We

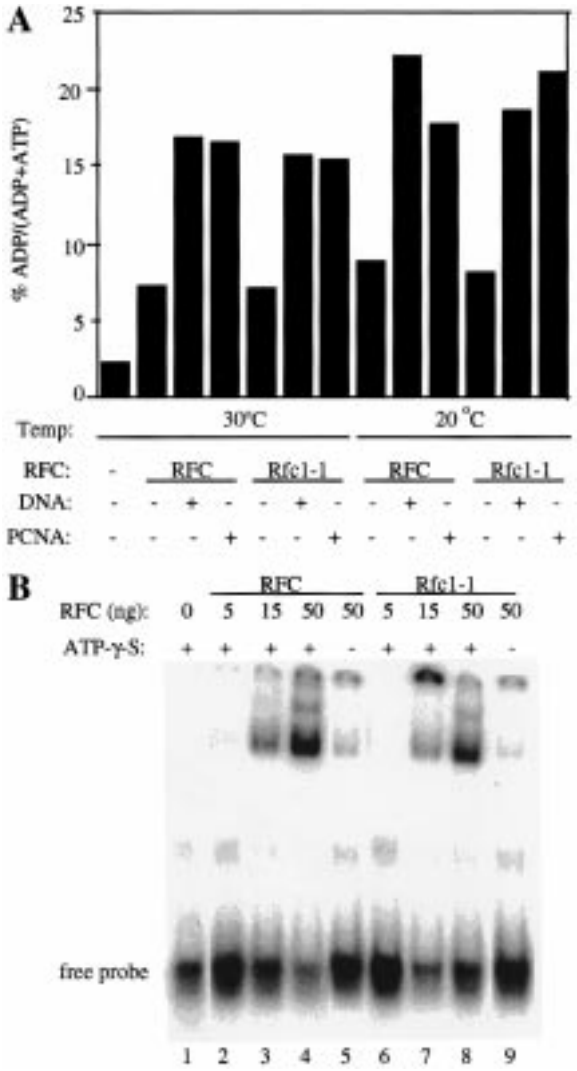

FIGURE 4: ATPase and DNA binding activities of wild-type and mutant RF−C complexes. (A) Wild-type and mutant RFC complexes were incubated in reactions containing 12 μM [α^{32}P]ATP, DNA, and PCNA as indicated for 10 min at 30 °C or 20 min at 20 °C. Reactions were stopped, separated by thin-layer chromatography and subjected to phosphorimaging analysis. (B) Wild-type or mutant RF−C complexes were incubated with P^{32} labeled primer-template DNA, ATPγS, and glutaraldehyde as indicated. Reactions were separated by acrylamide gel electrophoresis, dried, and subjected to phosphorimaging analysis.

found that both complexes exhibited similar ATPase activities, and that these activities were stimulated by the addition of DNA and PCNA. Thus, the *rfc1-1* substitution does not significantly affect the ATPase activity of entire RFC complex as measured by this assay. Either the large subunit of RFC does not contribute significantly to the total ATPase activity of RFC under these conditions, or if it does contribute, it is not significantly affected by the *rfc1-1* substitution.

The RFC complex is also known to contain a DNA-binding activity (*13*, *14*), and in humans as in drosophila, this activity has been mapped to an N-terminal domain of Rfc1p (*21*, *34*). Although this DNA-binding activity maps outside of RFC box VIII, it was possible that the D513N change could alter the conformation of the Rfc1 protein in such a way as to disturb its interaction with DNA. Also, there is evidence for a C-terminal DNA-binding domain in

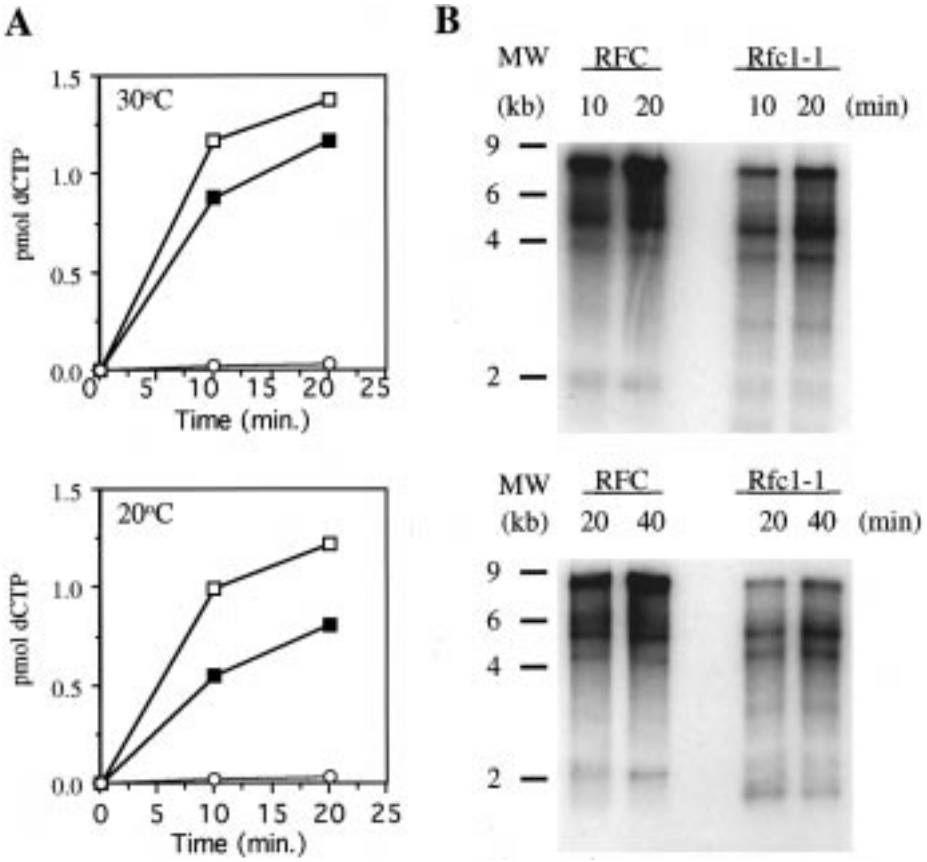

FIGURE 5: In vitro DNA replication assays at permissive and restrictive temperatures. DNA replication assays were performed with primed Mp18 single-stranded circular DNA, PCNA, DNA polymerase delta, *E. coli* SSB, dNTP's, [α^{32}P]dCTP, ATP, and either wild-type RFC (open squares), mutant Rfc1-1 (closed squares), or no RFC (open circles) at 20 or 30 °C for up to 20 min. The extent of DNA replication was monitored by either (A) precipitating the labeled DNA replication products in acid, followed by filtration through glass fiber filters, or (B) by alkaline agarose gel electrophoresis and phoshor-imaging analysis.

human Rfc1p (*22*). To determine whether the *rfc1-1* mutation affected DNA-binding activity, we assayed mutant and wild-type RFC complexes for their ability to bind primer-template junction sequences by a gel mobility shift assay (*13*). It has previously been demonstrated that the binding of RFC to primer-template junctions is enhanced by the addition of the nonhydrolyzable ATP analogue ATPγS. We assayed both wild-type and mutant RFC complexes and found that they exhibited similar DNA-binding activities (Figure 4B). Both complexes could recognize the primer-template DNA in a reaction that was stimulated by ATPγS.

Mutant RFC Complexes Are Defective in in Vitro DNA Replication Assays. To further characterize the activity of the wild-type and mutant RFC proteins, we investigated their ability to stimulate the polymerization of nucleotides in an in vitro DNA replication assay. Due to the poor processivity of the DNA polymerase alone, singly primed, single-stranded M13mp18 DNA, coated with single-stranded DNA binding protein, is only efficiently replicated by Polδ when PCNA, RFC, and ATP are also present (*14*). We monitored DNA replication efficiency in reactions that contained either wild-type or mutant RFC complexes, PCNA, Polδ, *E. coli* SSB, ATP, ^{32}P labeled dNTPs, and a primed circular M13mp18 DNA template.

Since the original *rfc1-1* mutation gave rise to a cold-sensitive phenotype, we performed the DNA replication assays at the permissive temperature of 30 °C and the semi-permissive temperature of 20 °C. DNA replication in this assay is highly dependent on RFC activity, since without RFC, very little incorporation of nucleotides is observed. Comparable levels of RFC and Rfc1-1 were required to saturate the replication assays (data not shown). However, at 30 °C the total incorporation of nucleotides in the mutant Rfc1-1 reaction was reproducibly about 15% lower than the incorporation levels for the wild-type RFC complex (Figure 5). When the reactions were incubated at 20 °C, the relative activity of the mutant complex dropped to about 55% of wild-type levels. When the replication products from these reactions were separated by alkaline gel electrophoresis, the mutant Rfc1-1 reactions yielded considerably fewer full-length replication products than the wild-type reactions (Figure 5B). The M13mp18 plasmid DNA used in this assay is 7.2 kb in length, and by 20 min at 30 °C or by 40 min at 20 °C, the wild-type RFC reactions yielded mostly full-length products. The presence of discrete bands that migrate below the size of the full-length products, suggests that there are sites on the plasmid (perhaps due to secondary structures), where DNA polymerase preferentially stops incorporating nucleotides (*35*). For the mutant Rfc1-1 reactions, much of the incorporation corresponded to products that were less than full-length. This failure to achieve full-length synthesis was most pronounced in the mutant reactions that were incubated at 20 °C.

Given that the *rfc1-1* mutation is predicted to map close to the putative nucleotide-binding domain in Rfc1p, we tested whether these RFC- and Rfc1-1 dependent DNA synthesis

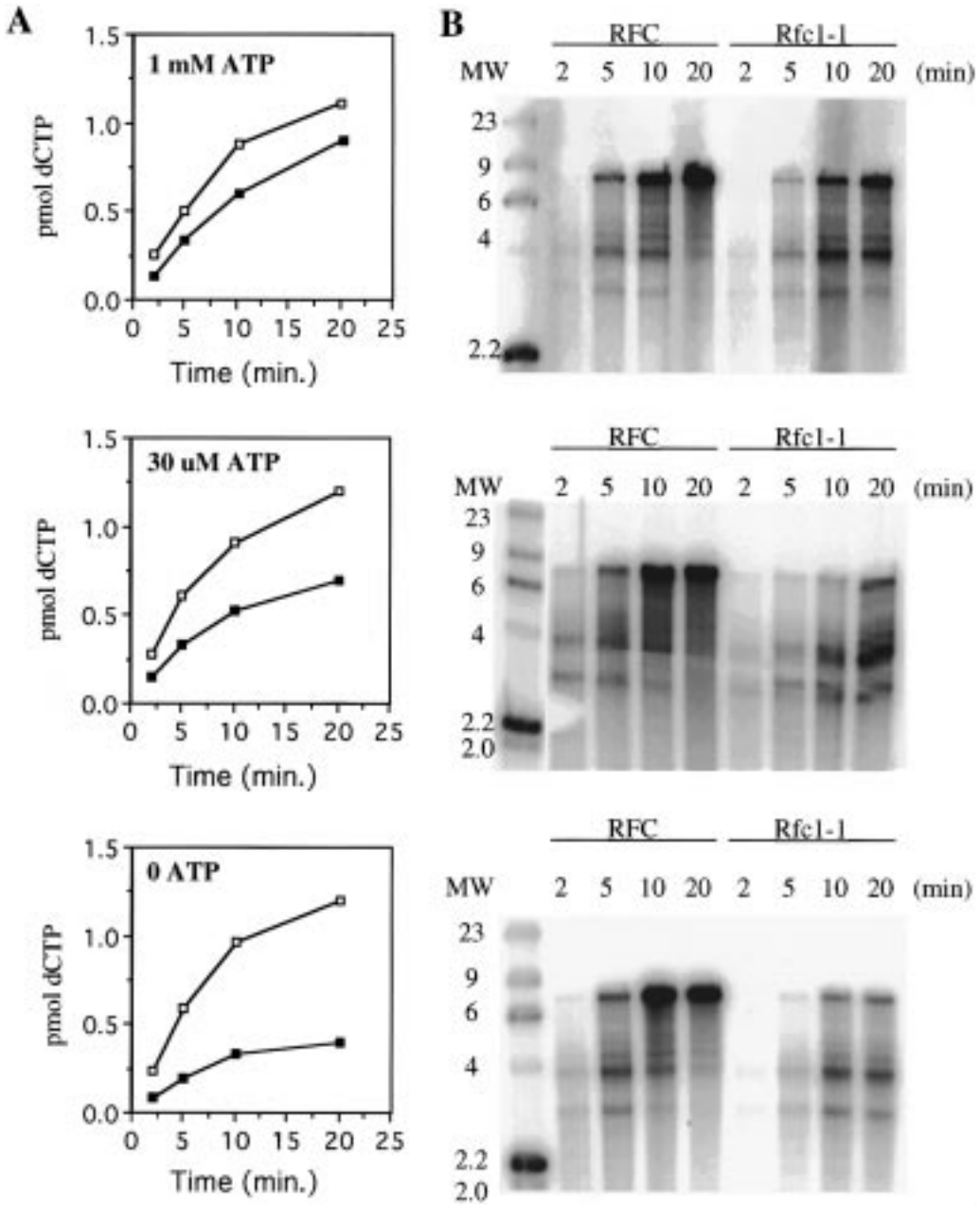

FIGURE 6: Rfc1-1 complexes are defective in in vitro DNA replication assays at low ATP concentrations. DNA replication assays containing either wild-type RFC (open squares) or mutant Rfc1-1 (closed squares) complexes were performed at 30 °C in the presence of either 1 mM, 30 μM, or 0 μM ATP. The extent of DNA replication was monitored by (A) acid precipitation, or (B) by alkaline gel electrophoresis.

reactions exhibited different requirements for ATP. In standard replication assays, 1 mM ATP is included to allow for the loading of PCNA onto the DNA template by the RFC complex, and 100 μM dATP together with the other dNTPs is included to allow for the incorporation of dNTPs during DNA synthesis. It has previously been shown that wild-type RFC is able to use either ATP or dATP hydrolysis to load PCNA onto DNA (*14*, *36*, *37*). To investigate the potentially altered nucleotide requirements of the Rfc1-1 complex, we conducted the replication assays at 30 °C in the presence of 1 mM, 30 μM, or 0 μM ATP. Again, at high levels of ATP (i.e., 1 mM), the mutant and wild-type reactions yielded similar profiles of total nucleotide incorporation (Figure 6A). At low levels of ATP, however, the mutant Rfc1-1 complexes were severely restricted in their ability to promote DNA synthesis. After 20 min in the absence of ATP, the level of incorporation in the mutant reactions was less than one-third of the incorporation of the wild-type reactions. The small amount of synthesis that was observed in these reactions, again corresponded to less than full-length replication products (Figure 6B). It is not clear whether the accumulation of shorter replication products reflects a decrease in polymerase processivity or a requirement to reload PCNA at sites of DNA secondary structure. It appears that although the wild-type RFC complex can promote DNA synthesis effectively in the absence of ATP when dATP is present, the mutant Rfc1-1 complex substitutes dATP much less efficiently for ATP. These results indicate that the *rfc1-1* substitution does indeed alter the nucleotide binding or hydrolysis activities of the entire RFC complex.

Mutant PCNA Proteins Can Suppress Rfc1-1 Defects in Vitro. Having established that Rfc1-1 complexes exhibit a

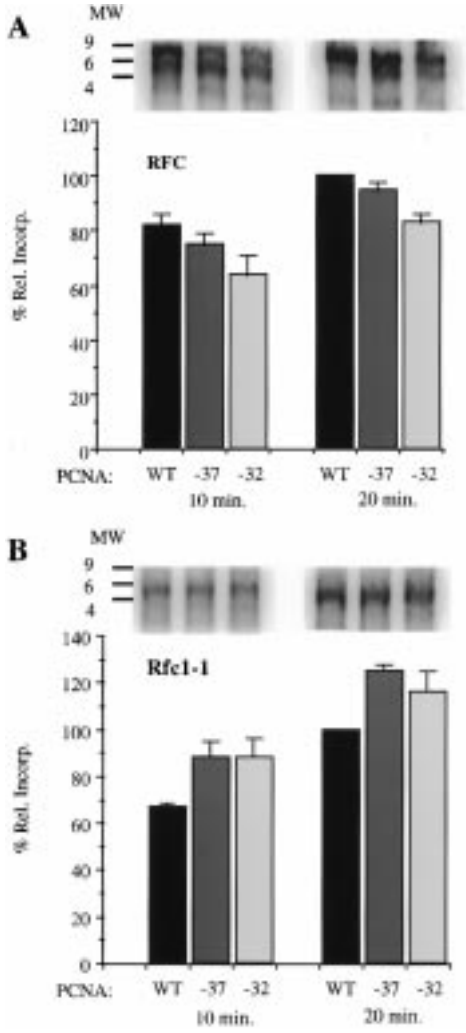

FIGURE 7: Mutant PCNA proteins can suppress Rfc1-1 DNA replication defects in vitro. DNA replication assays containing wild-type or mutant RF−C and PCNA proteins were performed at 30 °C for 10 or 20 min in the presence of 30 μM ATP. For each set of reactions, the level of incorporation from the 20 min reaction containing wild-type PCNA was defined as 100%. Histograms represent the mean and standard deviations derived from three independent experiments. Representative samples of the DNA replication products are shown above the histograms, with the position of the full-length products indicated with an arrow. (A) DNA replication assays containing wild-type RFC with wild-type or mutant PCNA proteins. (B) DNA replication assays containing mutant Rfc1-1 with wild-type or mutant PCNA proteins.

DNA synthesis defect in vitro, we were interested to determine if this defect could be alleviated by mutant PCNA proteins that were derived from the suppressor *pol30* alleles. To test this hypothesis, we set up DNA replication assays containing either wild-type or mutant RFC and PCNA proteins. One set of replication assays combined wild-type RFC with the different PCNA proteins (Figure 7A), and the other set combined mutant Rfc1-1 complexes with the different PCNA proteins (Figure 7B). Nucleotide incorporation was determined after 10 and 20 min at 30 °C, and to facilitate comparisons between multiple experiments, the level of incorporation observed for each 20 min reaction containing wild-type PCNA was defined as 100%. The relative levels of incorporation for the other reactions containing mutant PCNAs were calculated from this value. We observed that when combined with wild-type RFC, the mutant PCNA proteins were less active than wild-typePCNA. There was a consistent 10−20% drop in replication activity in the mutant PCNA reactions, with pcna-32 being less active than pcna-37 (Figure 7A). This decrease in incorporation was associated with fewer full-length DNA replication products than were present in the wild-type RFC/PCNA reactions. However, when the mutant PCNA proteins were combined with Rfc1-1 complexes, replication activity increased over wild-type PCNA levels by 15−25% (Figure 7B). Whereas the mutant PCNAs reproducibly decreased replication activity in combination with wild-type RFC, they enhanced replication activity when combined with mutant Rfc1-1 complexes. Gel analysis indicates that the increased incorporation resulted in less than full-length DNA replication products. On average, the two mutant PCNAs increased the levels of incorporation in the Rfc1-1 reactions to nearly 80% of the levels seen with the wild-type RFC complexes. Similar levels of suppression were obtained in reactions containing 30 μM and no ATP (data not shown).

The Suppressor PCNA Mutations Alter the Stability of the Trimeric Sliding DNA Clamp. We were interested in determining how the *pol30* suppressor mutations affected the structure and function of PCNA so as to compensate for the Rfc1-1 defects observed in vivo and in vitro. Presumably, the *rfc1-1* substitution induces a structural defect in Rfc1p that can be compensated for by alterations in the structure of PCNA. Different models can be proposed as to how these two substitutions combine to restore RFC activity. The D513N substitution may perturb a region of RFC that is important for forming specific protein−protein interactions between RFC and PCNA. If so, one could imagine that specific compensatory mutations at a particular region of PCNA may be able to restore the protein−protein interactions between the mutant Rfc1-1 and PCNA proteins. Under this model, it could be reasonably expected that there would be only few substitutions in PCNA that could compensate for the D513N alteration. In an alternative model, the Rfc1-1 complex may be compromised in its function to open up the PCNA trimer and load it onto DNA. In this case, suppression may be achieved by destabilizing the protein−protein interactions that keep the trimer together. Potentially, there are many more possible PCNA mutations that could have this affect, and these changes need not be tightly clustered on the surface of the PCNA protein. In support of this model, the mutations considered in this study lie at opposite ends of the monomeric PCNA protein, and both residues form part of the interface region where the PCNA monomers interact to form the complete homotrimeric clamp. Substitutions at these positions might disturb the protein−protein interactions that hold the PCNA trimer together and shift the equilibrium from trimer toward monomer.

To test this hypothesis, we assayed both wild-type and mutant PCNA proteins for their oligomeric status by gel-filtration chromatography on a Superose 12 HR column (Figure 8). Previous analysis has indicated that wild-type yeast PCNA is stable as a trimer at concentrations down to the micromolar range (*11*). If the mutant PCNA proteins are in fact less stable as trimers than wild-type PCNA, then at low concentrations, one would expect to be able to detect monomeric mutant PCNA, but not monomeric wild-type PCNA. To address this question, wild-type and mutant PCNA proteins were diluted to the appropriate concentrations

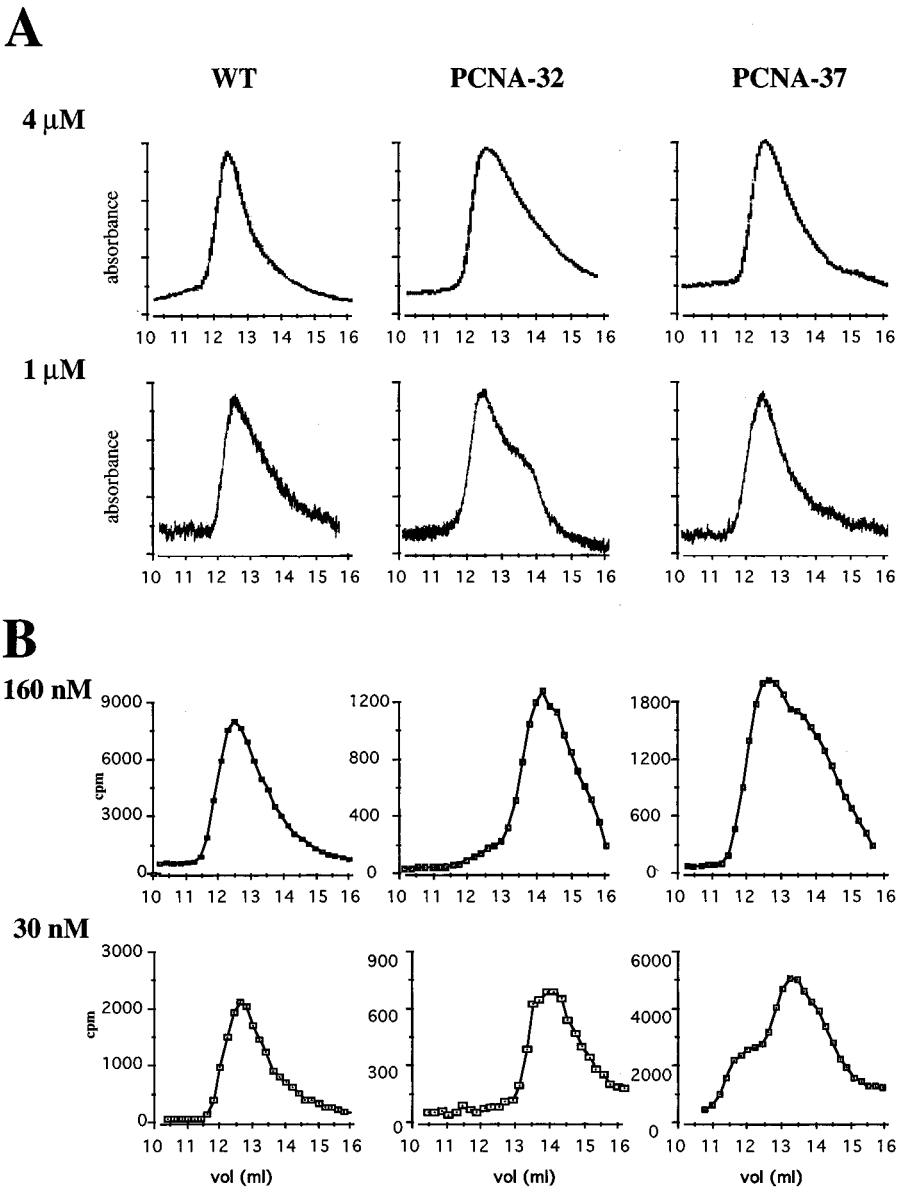

FIGURE 8: Gel filtration analysis of wild-type and mutant PCNA proteins. Samples of PCNA proteins at 4 μM, 1 μM, 160 nM, and 30 nM were subjected to gel filtration on a Superose 12 HR column at 22 °C in 50 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, and 10 mM NaCl. (A) At higher concentrations (i.e., 1 and 4 μM) protein levels were detected by UV absorption. (B) To detect concentrations of protein below the sensitivity of the UV detector (i.e., 30 and 160 nM), phospho-tagged PCNA proteins were produced and labeled with [γ-^{32}P]ATP. The labeled PCNA proteins were applied to the column, fractions were collected, and counted. Under these conditions, proteins of 90 and 30 kDa molecular mass elute at 12.5 and 14.5 mL, respectively.

and subjected to gel filtration. The resulting protein elution profiles were detected by UV absorbance and compared to the elution values of known molecular weight standards (Figure 8A). We observed that at concentrations of 4 μM, the peaks of the wild-type and mutant PCNA protein profiles eluted at a volume consistent with the proteins being in the trimeric form (i.e., 12.5 mL). At 1 μM, however, we could detect a significant population of the pcna-32 sample that eluted at a volume later than 12.5 mL. This elution profile indicates that at this concentration, in addition to trimeric molecules, the pcna-32 sample also contains dimeric and or monomeric forms of PCNA. This result indicates that the *pol30−32* substitution does indeed shift the trimer to monomer equilibrium of the mutant protein toward the monomeric form. To confirm that the protein preparations were not being degraded during this analysis, we verified that the monomeric subunits were intact after gel filtration by SDS−PAGE.

Since the limitations of the UV detector prevented us from assaying PCNA protein levels below a concentration of 1 μM, we took advantage of a technique that has been used to label PCNA proteins to high specific activity with ^{32}P (*38*). This labeling technique allowed us to follow the chromatographic properties of PCNA to the nanomolar levels typically used in in vitro DNA replication assays. For these experiments, we produced wild-type and mutant PCNA proteins that also contained a short N-terminal amino acid sequence (phosphotag) bearing a protein kinase substrate motif. These phosphotagged PCNA proteins were purified by the standard PCNA purification protocol, and to verify that this phosphotag did not significantly alter the function of the PCNA proteins, we confirmed that all of the preparations were active

in our in vitro DNA replication assays (data not shown). We labeled the phosphotagged PCNA preparations with ^{32}P, diluted them to 160 and 30 nM, and subjected them to gel filtration chromotography (Figure 8B). As we had observed at the micromolar concentrations, the wild-type PCNA protein was primarily in the trimeric form at 160 nM. At this concentration, however, we could begin to detect a mixture of trimers and dimers for the pcna-37 sample, suggestive of a shift in the trimer/monomer equilibrium. The pcna-32 preparation remained predominately monomeric at 160 nM. At 30 nM, only the wild-type PCNA protein retained its trimeric structure, with the peaks of the two mutant preparations eluting later than 12.5 mL. These data indicate that indeed, both mutant PCNA proteins are less stable as trimers than wild-type PCNA, with the A112T substitution destabilizing the PCNA trimer more than the L151S substitution. Suppression of the *rfc1-1* mutation is, therefore, associated with substitutions in the PCNA protein that lower the stability of the homotrimeric sliding DNA clamp.

DISCUSSION

The genetic approach of identifying *pol30* alleles that can alleviate *rfc1-1* mediated defects in vivo offers the opportunity to uncover interactions between RFC and PCNA that are physiologically relevant. The subsequent analysis of the mutant RFC and PCNA proteins in vitro can provide valuable insights concerning how these proteins function in various aspects of DNA metabolism. In this study, we demonstrate that the telomere elongation and DNA-damaging-agent sensitivity phenotypes associated with the *rfc1-1* mutation are related to the ability of RFC to interact with PCNA. These results, in conjunction with our previous studies on the cell cycle and DNA replication phenotypes associated with the *rfc1-1* mutation, suggest that the appropriate interaction between RFC and PCNA is critical for numerous aspects of DNA metabolism in vivo (*29*). In vitro, RFC and PCNA can be shown to promote the association between DNA polymerases and primer-template junctions. Since DNA structural motifs similar to primer-template junctions are found at sites of DNA replication (including the sequences adjacent to telomeres) and DNA repair, it is likely that RFC and PCNA serve a similar function in these aspects of DNA metabolism in vivo. The presumed inability of *rfc1-1* mutants to process these DNA structures effectively is predicted to lead to the observed defects in telomere metabolism, DNA replication, and DNA repair.

Although, as yet no high-resolution structural information is available for any of the eukaryotic RFC subunits, we suggest that the *rfc1-1* D513N substitution maps to the putative nucleotide-binding pocket of Rfc1p. Rfc1p contains matches to the G-1 to G-4 consensus sequences that were originally defined for GTP binding (*30*) and these short motifs are likely to be critical for Rfc1p function since they are highly conserved among the large subunits of RFC from humans, mouse, drosophila, and yeast. In GTP-binding proteins, these motifs contribute to forming the nucleotide-binding pocket (*32*), and the positions equivalent to the *rfc1-1* and *rfc1-4* substitutions participate in forming hydrogen bonds with the bound nucleotide. Although the exact position of the D513 amino acid will require further structural analysis, support for the prediction that it may lie close to the putative nucleotide-binding pocket of Rfc1p comes from recent work on the structure of one of the subunits of the clamp loader from *E. coli*. The δ'-subunit of the γ complex also contains RFC box sequences, and structural analysis reveals that they do indeed adopt a nucleotide-binding-like fold (*39*). Like the G60 position in p21Ras, the equivalent position to the Rfc1p D513 residue in δ' (S197) also maps toward the base end of the nucleotide-binding pocket. Nucleotide-binding pockets may be important structural motifs in all the subunits of the clamp loaders, and they could be used to couple the conformational changes induced by the binding of nucleotides to the steps of clamp loading and unloading.

The original suppressor *pol30* alleles that were identified were spontaneous mutations that permitted *rfc1-1* mutants to escape cell cycle arrest and grow at the restrictive temperature of 20 °C (*24*). Since this was a strong selective approach (only the rare mutants that compensated for Rfc1-1p defects would survive), the *pol30* alleles that it identified could have revealed changes in the structure of PCNA that restored specific protein−protein interactions with the mutant Rfc1-1 complex. The five suppressor mutations that we identified consist of single amino acid substitutions that map toward either end of the monomeric PCNA subunit. Since the substituted amino acid positions do not colocalize to a single well-defined surface of the PCNA protein, they are unlikely to define a specific contact point between PCNA and the D513 position of Rfc1p. Also, in a charged amino acid to alanine scanning mutational analysis of PCNA, two mutant *pol30* alleles were identified (out of a total of 22) that, in addition to exhibiting DNA metabolism defects, could also efficiently suppress *rfc1-1* defects (*11*). Neither of these two alleles (*pol30−6*, DD41 and 42AA, and *pol30−16*, EK143,146AA) map directly to the subunit interface, and both show replication defects in vitro (*11*, *40*). Possibly, these and the other *pol30* suppressor alleles that do not map directly to the subunit interface destabilize the PCNA structure through long-range effects. Of course, other mechanisms for suppression are also possible.

Although clamp-loading reactions can be reconstituted in vitro, the exact mechanism whereby RFC uses ATP to load PCNA onto DNA is not well understood. It is clear, however, that RFC undergoes conformational changes upon binding to ATP, DNA, or PCNA and that these changes are likely critical for its function as a clamp loader. Insights into these conformational changes can be gained through the analysis of a variety of in vitro assays. For example, when RFC is incubated with ATP, it exhibits a greater affinity for both DNA and for PCNA (*5*, *13*, *14*, *28*). Likewise, both PCNA and DNA can stimulate the ATPase activity of RFC. Therefore, to understand how RFC functions, it will be necessary to further define which subunits of RFC interact with ATP, DNA, and PCNA, and how these interactions alter the conformation of the whole RFC complex. Studies on the large subunit of human RFC have revealed that it contains an N-terminal domain that can bind to DNA, and a central domain that can interact with PCNA (*21*, *34*). Since this central domain contains the conserved RFC boxes II−IV and portions of the nucleotide-binding consensus sequences, the PCNA binding and putative nucleotide-binding activities of Rfc1p may be related. Although direct evidence for the binding of ATP to Rfc1p has not been demonstrated, the

inability of the mutant Rfc1-1 complexes to effectively use dATP to stimulate DNA replication supports the prediction that the putative nucleotide-binding domain of Rfc1p is important for RFC activity. One possibility is that the large subunit of RFC functions to open up the PCNA trimer during the clamp-loading or -unloading reaction. This activity may involve conformational changes that are induced in Rfc1p by the binding or hydrolysis of ATP. Rfc1p substitutions could disrupt this activity, and these defects could potentially be compensated by substitutions in PCNA that destabilize the homotrimer. Alternatively, the Rfc1p substitutions could interfere with the ability of other RFC subunits to bind to and hydrolyze ATP during the loading or unloading reaction. Further analysis is required to distinguish between these possibilities and to determine the roles that the individual RFC subunits play in RFC function in vivo and in vitro.

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