Monomeric Yeast PCNA Mutants Are Defective in Interacting with and Stimulating the ATPase Activity of RFC†

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ABSTRACT: Yeast PCNA is a homo-trimeric, ring-shaped DNA polymerase accessory protein that can encircle duplex DNA. The integrity of this multimeric sliding DNA clamp is maintained through the protein−protein interactions at the interfaces of adjacent subunits. To investigate the importance of trimer stability for PCNA function, we introduced single amino acid substitutions at residues (A112T, S135F) that map to opposite ends of the monomeric protein. Recombinant wild-type and mutant PCNAs were purified from *E. coli*, and they were tested for their properties in vitro. Unlike the stable wild-type PCNA trimers, the mutant PCNA proteins behaved as monomers when diluted to low nanomolar concentrations. In contrast to what has been reported for a monomeric form of the β clamp in *E. coli*, the monomeric PCNAs were compromised in their ability to interact with their associated clamp loader, replication factor C (RFC). Similarly, monomeric PCNAs were not effective in stimulating the ATPase activity of RFC. The mutant PCNAs were able to form mixed trimers with wild-type subunits, although these mixed trimers were unstable when loaded onto DNA. They were able to function as weak DNA polymerase δ processivity factors in vitro, and when the monomeric PCNA-41 (A112T, S135F double mutant) allele was introduced as the sole source of PCNA in vivo, the cells were viable and healthy. These *pol30−41* mutants were, however, sensitive to UV irradiation and to the DNA damaging agent methylmethane sulfonate, implying that DNA repair pathways have a distinct requirement for stable DNA clamps.

The DNA polymerases and their associated proteins are the central components of the DNA replication and DNA repair machineries. Among the more important and widely conserved classes of DNA polymerase accessory proteins are the sliding DNA clamps and the clamp loaders (reviewed in refs *1* and *2*). Sliding DNA clamps are homo-multimeric, ring-shaped proteins that can encircle duplex DNA, thereby tethering their associated polymerases to the DNA template. Examples of this type of protein have been identified in bacteriophage T4 (gp45), *E. coli* (the β subunit of DNA polymerase III), yeast, and humans (PCNA). Structural analysis has revealed that each of these proteins adopts a 6-fold symmetrical ring-like structure that contains a central cavity that is large enough to accommodate a DNA duplex (reviewed in ref *3*). In *E. coli*, the clamp is formed via the head-to-tail assembly of two β subunits, each of which contains three structurally similar subdomains. In T4, yeast, and humans, the clamps are homo-trimers, with each subunit containing two structurally similar subdomains.

The task of assembling sliding clamps around the DNA molecules lies with the clamp loaders. In *E. coli*, this activity resides with the γ complex, a protein having five different subunits (the $\gamma\delta\delta'\chi\psi$ subunits) that can load β rings onto DNA in an ATP dependent reaction (*4*). Three of these subunits are essential for the loading reaction ($\gamma\delta\delta'$). The δ subunit can bind directly with β, but this interaction can be blocked by other components of the γ complex (*5*, *6*). During the loading reaction, the δ subunit is freed to interact with and open up one of the two interfaces of the β dimer (*7*). The opened β dimer is then released onto the DNA at a primer template junction (*8*). The conformational changes associated with this reaction are presumed to be coordinated, in part, by the binding and hydrolysis of ATP by the γ subunits. Multiple subunits of the γ complex contact the β clamp, including δ, γ, χ, and possibly δ', but not ψ (*9*). Detailed analysis of these subunits reveals the specific roles they play in the loading reaction.

In eukaryotes, the clamp loading RFC complex also contains five subunits, but in this case, all of the subunits are distinct. Each of the subunits contains ATP-binding consensus sequences, and like the case for *E. coli*, four of them (Rfc1p, Rfc2, Rfc3p, and Rfc4p) have been reported to bind to PCNA (*10−13*). Detailed analysis of the loading reaction suggests that there is an ordered series of steps in the pathway that are associated with the sequential binding of four ATP molecules (*14*, *15*). A model has been suggested whereby RFC first binds two ATP molecules and then forms a complex with PCNA. This results in a conformational change, allowing the binding of a third ATP molecule. Similarly, the binding of DNA and the loading of the PCNA clamp are thought to be coupled to the binding of the fourth molecule of ATP. The absence of detailed structural information concerning the assembly of the RFC complex and its interaction with PCNA has made it difficult to further define the individual steps of this reaction.

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[1] Abbreviations: RFC, replication factor C, PCNA, proliferating cell nuclear antigen, MMS, methyl-methanesulfonate.

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Although the prokaryotic and eukaryotic clamp loaders share many properties, it is not yet known whether they function by the same mechanism. For example, it is not clear whether the different subunit compositions of the respective clamps (the dimeric β vs the trimeric PCNA) impacts how they interact with their clamp loaders, or in how the clamps are opened up and loaded onto DNA. Recently, a monomeric form of the β subunit was reported, and it was found to interact with the δ subunit and the entire γ complex clamp loader with significantly higher affinity (*5*−*9*) than the native β dimer (*7*). Also, recent crystallographic evidence indicates that δ interacts with only one subunit of β (*8*). On the basis of these data, the authors proposed that the binding energy of δ to β is needed for the opening of one interface in the β dimmer. It is not clear whether the eukaryotic loader functions in a similar fashion or whether it interacts differently with the trimeric PCNA clamp for clamp opening.

In addition to interacting with RFC and DNA polymerases (*16*), eukaryotic PCNAs have been shown to interact with a number of other proteins involved in DNA replication, DNA repair, and cell cycle control (*17*). This list includes DNA ligase I (*18*), FEN-1 (*19*), the DNA repair proteins XPG (*20*), UNG2 (*21*), MLH1 and MSH2 (*22*), the MCMT methyltransferase (*23*), and the cell cycle related proteins cyclin D (*24*), Gadd45 (*25*), and p21 (*26*). Many of these proteins interact with residues within the PCNA protein that map to the interdomain connecting loop, or near the carboxy terminus (*17*, *27*). Extensive mutagenesis of the PCNA protein has been reported, and mutants have been described that reveal distinct roles for PCNA in DNA replication and DNA repair (*28*). Similarly, specific mutations have been reported that disrupt interactions between PCNA and other proteins. Where investigated, the overwhelming majority of the mutants that have been described have retained their trimeric structure in vitro (*16*, *28*−*31*). Thus, it remains to be determined to what extent PCNA trimer formation is required for these interactions. For example, specific, stable protein−protein interactions may involve interactions between either a single, or multiple PCNA subunits. In the case of single subunit interactions, a monomeric form of PCNA may be sufficient.

In this study, we generated *pol30* (PCNA) mutants that were designed to interfere with the protein−protein interactions that hold the trimeric PCNA ring together. One such mutant protein (PCNA-41) was greatly impaired in its ability to form homo-trimers in vitro, but it could form mixed trimers with wild-type subunits. This mutant monomer was found to interact poorly with the clamp loading RFC complex compared to wild-type PCNA. The mutant PCNA-41 protein was functional in vitro and in vivo, although the resulting mutant PCNA strain was sensitive to DNA damaging agents.

EXPERIMENTAL PROCEDURES

Strains and Media. The yeast strains used in this study are listed in Table 1. Standard yeast genetic techniques and media were used throughout (*32*).

Construction of PCNA Expression Plasmids and the Purification of PCNA. For the expression of recombinant yeast PCNA in *E. coli*, an expression plasmid (pMM119) was created in which the wild-type yeast PCNA gene was located downstream of an inducible T7 promoter. This plasmid was a derivative of pRSET A (from Invitrogen) and contained an engineered *Nde1* site at the ATG translation initiation codon. Fragments bearing the *pol30*−*32* and *pol30*−*41* mutations were cloned into this plasmid (creating plasmids pMM127 and pMM338, respectively) to allow for the expression and purification of mutant PCNA proteins. To create the phospho-tagged PCNA proteins (Ph-PCNA), a short double stranded DNA sequence coding for the phosphotag amino acids (MRRASVGSMRRASVGS) was cloned into the *Nde1* site of these plasmids. The resulting plasmids pMM123, pMM135, and pMM254 contained *POL30*, *pol30*−*32*, and *pol30*−*41* alleles, respectively. Both wild-type and mutant yeast proteins were expressed in *E. coli*, and purified by standard chromatographic methods as described previously (*33*).

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

strain	relevant genotype	ref source
yMM122	*MAT a lys2 his3 ade2 ura3*−*52 pol30*−*32*	this lab
yMM357	*MAT a ura3*−*52 trp1*Δ*63 pol30::LEU2* pMM263 (*URA3 POL30*) pMM323 (*pol30*−*41, TRP1*)	this lab
yMM358	*MAT a ura3*−*52 trp1*Δ*63 pol30::LEU2* pMM321(*POL30, TRP1*)	this lab
yMM359	*MAT a ura3*−*52 trp1*Δ*63 pol30::LEU2* pMM323 (*pol30*−*41, TRP1*)	this lab

Native Gel Analysis. Protein samples (8 μg each) were subjected to nondenaturing continuous gel electrophoresis in 200 mM Tris-Cl (pH 8) at acrylamide concentrations of 5%, 10%, 7.5%, and 12.5% (*34*). Gels were run at 10 mA for 6−10 h and stained with Coomasie blue. Ferguson plots having the log of the relative mobility of the proteins ($LogR_F$) plotted versus acrylamide concentration were generated. Linear regression analysis was used to determine the retardation coefficient (K_r) for each band (protein species) by plotting the slope of the Ferguson plot against the molecular weight of the proteins. The K_r of the proteins bovine serum albumin and carbonic anhydrase (and their isoforms) were used to define a K_r versus molecular weight standard curve (*35*).

Labeling of Phospho-Tagged PCNA Proteins. Ph-PCNA preparations were labeled with γ-^{32}P-ATP by the protein kinase catalytic subunit from bovine heart (Sigma). Thirty microliter labeling reactions containing 20 mM Tris-HCl, pH 7.5, 12 mM Mg−acetate, 100 mM NaCl, 0.1 mg/mL BSA, 10 U of protein kinase, 8 μg of PCNA, and 2×10^6 cpm of γ-^{32}P-ATP were incubated at 37 °C for 15 min. After the initial 15 min of labeling, cold ATP was added to 1 mM, and the reaction was incubated for another 15 min. Labeled Ph-PCNA proteins were separated from free γ-^{32}P-ATP by gel filtration.

Gel Filtration Analysis. ^{32}P-labeled Ph-PCNA and unlabeled PCNA molecules were combined as indicated and incubated in 50 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 10 mM NaCl. The proteins were then subjected to gel filtration in the same buffer over a Superose 12HR 10/30 column (Pharmacia) at 4 °C at 0.3 mL/minute flow rate. Proteins were detected by collecting fractions and analyzing them by liquid scintillation counting. The proteins carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), and alcohol dehydrogenase (150 kDa) were used as size markers (data not shown).

Purification of Recombinant Yeast RFC. Recombinant yeast RFC was overproduced in an *E. coli* strain harboring a plasmid bearing all five of the yeast RFC genes under inducible promoters. Ten liters of the *E. coli* strain was grown up, and the RFC was purified by Affigel Blue, PCNA−agarose, and MonoS chromatography as described previously (*36*).

PCNA Loading Assays. PCNA was assembled onto DNA by incubating 50 nM ^{32}P-PCNA, 1.5 μg of double-stranded nicked DNA (ΦX174 RF II from New England BioLabs), 50 nM RFC in 60 μL reactions containing 0.5 mM ATP, 20 mM Tris-Cl, pH 7.5, 0.1 mg/mL BSA, 0.5 mM DTT, 5 mM $MgCl_2$, and 2% glycerol, at 37 °C for 10 min. In the case of the mixed PCNA-41 reactions, 50 nM ^{32}P-PCNA-41 was preincubated with a 30-fold excess of cold wild-type PCNA at 30 °C for 90 min. The reactions were gel filtered on Bio-Gel A15m (5 mL bed) at 4 °C in elution buffer containing 20 mM Tris-Cl pH 7.5, 8 mM $MgCl_2$, 4% glycerol, 0.5 mM EDTA, 2 mM DTT, 0.1 mg/mL BSA, and 50 mM NaCl. The peak fractions containing ^{32}P -PCNA−DNA complexes were collected, incubated as desired, and subjected to a second round of gel filtration.

Agarose Gel Band Shift Assay. Gel mobility shift assay were performed with 50 nM ^{32}P-PCNA and different concentrations of RFC (30nM, 50nM, 75nM), as indicated, in 20 μL reactions containing 150 μM ATP, 30 nM HEPES, pH 7.5, 5 mM $MgCl_2$, 100 mM NaCl, and 5% glycerol. Reactions were incubated for 10 min at room temperature before being loaded onto a 2% agarose gel. Gels were electrophoresed at 80V for 4h, fixed in 5% acetic acid for 10 min, dried on 3MM CHR Whatman paper, and analyzed by PhosphorImager analysis.

RFC ATPase Assays. RFC ATPase assays were performed at 30 °C in 25 μL reactions containing 40 mM Tris-Cl, pH 7.8, 50 mM NaCl, 8 mM $MgCl_2$, 0,2 mg/mL bovine serum albumin, 1 mM DTT, 500 μM ATP + α^{32}-P-ATP (1 μCi), 0.1 μM RFC, and a range of PCNA concentrations. Aliquots of 5 μL were removed at 5, 15, 30, and 60 min and quenched with 5 μL of 0.5 M EDTA. Aliquots (1 μL) from the quenched reactions were separated by thin-layer chromatography using a 0.6M KH_2PO_4 pH 3.4 buffer, and then subjected to phosphorimaging analysis for 14 h to determine the proportions of ATP and ADP. By plotting the molar amount of ADP produced in each reaction against the reaction time, we obtained the velocity for reactions with different PCNA concentrations. The k_{cat} was calculated by dividing the velocity (μmol/s) by the RFC concentration. The k_{cat} (s^{-1}) was plotted versus PCNA concentration using and yielded the Michaelis−Menten constant K_m.

DNA Replication Assay. In vitro DNA replication assays were performed in 10 μL reactions containing 20 mM Tris-HCl (pH 7.8); 65 mM NaCl; 0.2 mg/mL bovine serum albumin; 8 mM $MgAc_2$; 1 mM dithiothreitol; 100 μM each dTTP, dCTP, dGTP; 25 μM α-^{32}P-dATP (5000 cpm/pmol); 1 mM ATP; 100 ng singly primed single-stranded M13mp18 DNA (from New England BioLabs); 850 ng SSB (from Stratagene); 30 ng RFC; 10 ng Polδ; and 20 or 200 ng PCNA as indicated. Reactions were incubated at 30 °C, withdrawn at 5, 10, and 15 min, and stopped by addition of 5 μL of 100 mM EDTA-1% SDS. Samples were subjected to alkaline electrophoresis on 1.2% agarose gel, dried, and exposed to phosphoimager screen for 16 h. A λ HindIII digest was end labeled with ^{32}P-ATP and used for molecular weight marker. The DNA polymerase δ was a kind gift of Peter Burgers.

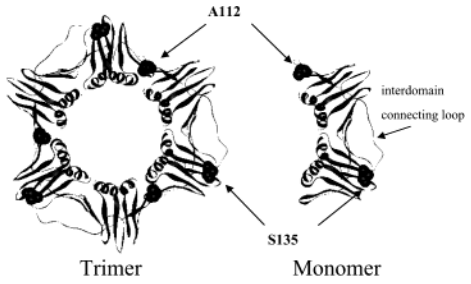

FIGURE 1: Mapping of the amino acid substitutions onto the crystal structure of yeast PCNA. The positions of the PCNA-32 (A112T) and PCNA-41 (A112T, S135F) substitutions are indicated with respect to the structure of the trimeric and monomeric forms of yeast PCNA. Also indicated is the location of the interdomain connecting loop.

UV and MMS Sensitivity Assays. Strains were grown in liquid YPD medium to early log phase at 30 °C; they were sonicated, and then diluted as necessary in water. Cells were then either exposed to 1% methyl methanesulfonate (MMS) and then plated, or else the cells were 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 for 3 days at 30 °C. Plots reflect the mean viability derived from at least three experiments.

RESULTS

One of the most obvious features that is conserved among the sliding DNA clamps is their ring-shaped structure. In yeast, the PCNA clamp is formed via the head to tail assembly of three PCNA monomers. The protein−protein interactions at the subunit interfaces are sufficiently strong such that wild-type PCNA forms a stable trimer even at concentrations into the low nanomolar range. To investigate how important this overall ring shape is for PCNA function, we investigated *pol30* mutations that were specifically chosen to interfere with PCNA subunit assembly. To do this, we took advantage of previously characterized single-site amino acid substitutions that were shown to partially destabilize the PCNA clamp (*33*). Since each PCNA monomer normally interacts with its adjacent subunits at two distinct interfaces (*37*), we chose from our collection of *pol30* mutants two alleles that encoded for substitutions near opposite ends of the molecule. One allele (*pol30−32*) encodes for the A112T substitution which maps to one subunit-subunit interface, and the other allele, *pol30−33*, encodes for a substitution (S135F) that is located near the opposite interface (Figure 1). These two mutations were combined within the same gene to create the double mutant *pol30−41* allele.

To investigate the properties of the various mutant PCNA proteins in vitro, the *pol30* alleles were engineered into appropriate plasmids for expression and purification from *E. coli*. We also created PCNA expression constructs bearing an N-terminal phospho-tag sequence (Ph-PCNA) that can be specifically recognized and phosphorylated by bovine heart protein kinase. The untagged and tagged PCNA, PCNA-32, and PCNA-41 proteins were expressed in *E. coli* and purified by conventional chromatographic methods (Figure 2A). The phospho-tagged PCNA molecules could

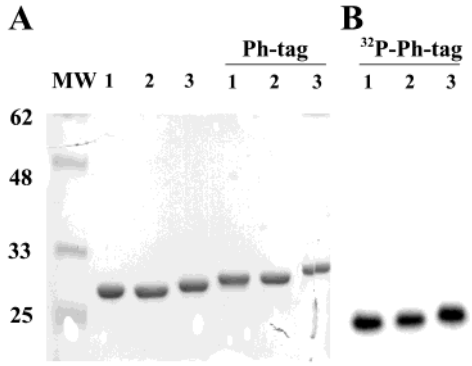

FIGURE 2: Purification and labeling of recombinant PCNA proteins. (A) PCNA proteins with or without an N-terminal phospho-tag (Ph-tag) were overexpressed in *E. coli*, purified by column chromatography, and separated by SDS−PAGE. (1) wild-type PCNA, (2) PCNA-32, and (3) PCNA-41. (B) The Ph-PCNA proteins were labeled with ^{32}P by incubation with bovine heart protein kinase and $\gamma-^{32}$P-ATP, subjected to SDS−PAGE, and visualized by phosphorimage analysis.

be readily radio-labeled with ^{32}P phosphate upon incubation with protein kinase and γ-^{32}P-ATP (Figure 2 B).

All six of the purified PCNA proteins were soluble throughout their purification, and each exhibited the expected molecular mass of around 30 kDa under denaturing SDS−PAGE. To investigate the extent to which the various PCNA molecules multimerized in vitro, they were subjected to native polyacrylamide gel electrophoresis (Figure 3). With this technique, one can estimate the molecular weight of a protein by measuring its migration through native gels of various concentrations (see Materials and Methods). Wild-type Ph-PCNA behaved as a uniform species, consistent with an expected trimeric molecular weight of 93 kDa (lane 3). For the Ph-PCNA-32 sample (lane 4), we could detect species with apparent molecular weights of 40 kDa, 75 kDa and 100 kDa, presumably corresponding to monomers, dimers, and trimers, respectively. For the Ph-PCNA-41 sample (lane 5), we could detect similar monomer, dimer and trimers species, but we could also detect progressively higher molecular weight Ph-PCNA-41 species on the 12.5% gel. Since no species higher than trimers were seen with wild-type PCNA, the multimerization properties of the Ph-PCNA-41 proteins were unlikely to involve the same type of subunit−subunit interactions associated with wild-type Ph-PCNA. Similar results were obtained for the migration patterns of PCNA molecules that did not contain the phospho-tag (data not shown).

In an independent approach, the apparent molecular weights of the various Ph-PCNA proteins were determined by gel filtration analysis. To detect lower concentrations of proteins than were possible in the native gel analysis, the Ph-PCNA proteins were first labeled with ^{32}P. At low nanomolar concentrations, we could detect the monomeric forms for all three proteins. At increasing concentrations we could detect proportionally more trimeric species, more so with the wild type than the Ph-PCNA-32 samples. At a concentration of 2.4 μM, the wild-type and Ph-PCNA-32 proteins were principally trimeric, but the Ph-PCNA-41 profile was consistent with a mixture of monomers, dimers and trimers (Figure 4A).

One potential reason the mutant PCNA-41 molecules behaved as monomers could be that the point substitutions caused a general misfolding of the proteins, precluding their assembly into a ring-shaped trimer. To test whether these PCNA proteins had a generally distorted structure, we investigated whether they could form mixed trimers with wild-type PCNA subunits. Radiolabeled Ph-PCNA-32 and Ph-PCNA-41 molecules were incubated with a 30-fold excess of unlabeled wild-type PCNA, and the reaction mixtures were incubated for up to 135 min at 30 °C before gel filtration. Ph-PCNA-32 and Ph-PCNA-41 molecules could form mixed trimers with wild-type PCNA subunits, and in both cases, the exchange equilibrium was reached within 90 min (Figure 4B). Consistent with the gel filtration profiles of the unmixed samples, the Ph-PCNA-32 reactions contained a higher concentration of mixed trimers than did the Ph-PCNA-41 mixtures. We also tested for mixed timer formation when mutant Ph-PCNA samples where incubated with increasing concentrations of wild-type PCNA for 15 min. We could observe appreciable trimer formation with both Ph-PCNA-32 and Ph-PCNA-41 samples in just 15 min if the concentration of wild-type PCNA was in excess (i.e., 55-fold). Again, under these conditions, over half of the Ph-PCNA-41 sample remained monomeric (data not shown)

Having demonstrated that the mutant PCNA proteins could be incorporated into mixed trimer molecules, we then sought to determine whether these molecules could be loaded onto DNA. To do this, we first purified the yeast RFC clamp loader complex from an *E. coli* overexpression strain (Figure 5) (see Materials and Methods). This strain harbors a single plasmid, which contains all five of the yeast RFC genes under control of inducible promoters. The gene encoding the large subunit of RFC (*RFC1*) contained a deletion that removed the N-terminal 273 amino acids from the Rfc1 protein. This domain of Rfc1p contains a DNA binding motif that is neither essential in vivo, nor required for clamp loading activity in vitro.

PCNA loading assays were then performed by mixing ^{32}P-Ph-PCNA, RFC, ATP, and nicked double-stranded plasmid DNA at 37 °C for 10 min (Figure 6). The reaction mixtures were subjected to gel filtration at 4 °C to separate the free ^{32}P-Ph-PCNAs from the loaded molecules. Wild-type ^{32}P-Ph-PCNA could be loaded onto DNA in a reaction that was dependent on RFC and ATP. ^{32}P-Ph-PCNA-32 was less effective as a loading substrate, and the monomeric ^{32}P-Ph-PCNA-41 could either not be effectively assembled onto DNA, or if it was loaded, it was not stable. ^{32}P-Ph-PCNA-41 molecules could be stably loaded onto DNA, however, if they were preincubated with wild-type unlabeled PCNA, presumably as mixed wild-type and mutant trimers (Figure 6A).

Once loaded, the PCNA−DNA complexes could be recovered from the gel filtration fractions, manipulated, and then resubjected to gel filtration to assess the stability of the complex. Loaded Ph-PCNA molecules could be released from the plasmid DNA by incubating the mixture with a restriction enzyme that would cleave the circular DNA template (data not shown). Loaded Ph-PCNA clamps would also dissociate from the circular DNA molecules over time in the absence of enzyme treatment (Figure 6B). The stability of the loaded complexes varied depending on the incubation temperature, and the approximate half-lifes for the Ph-PCNA was found to be 25 min, 2 h, and over 6 h at 37, 25, and 4 °C, respectively. The loaded Ph-PCNA-32 and Ph-PCNA-

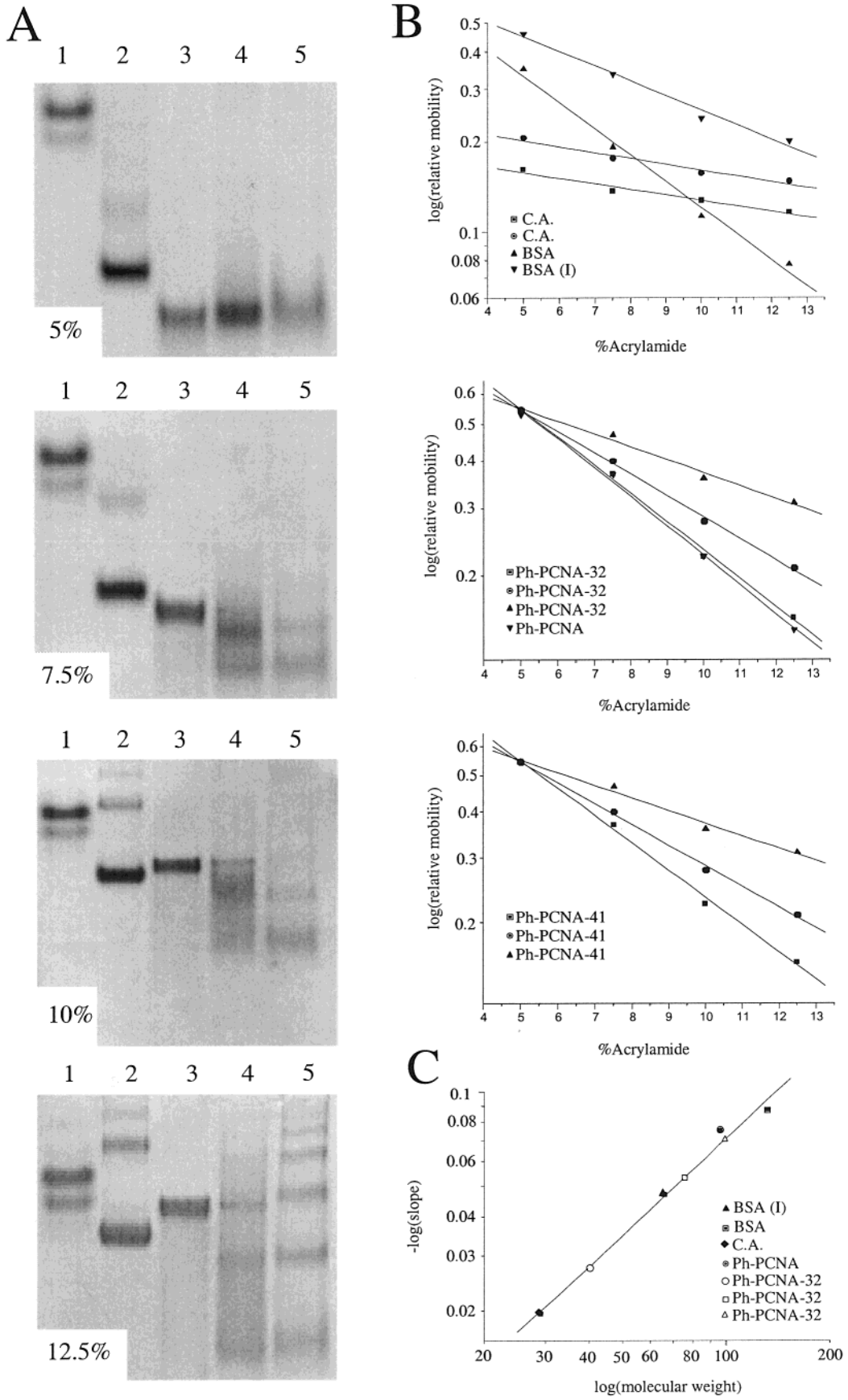

FIGURE 3: Native gel analysis of Ph-PCNA proteins. (A) Carbonic anhydrase, BSA and Ph-PCNA proteins were separated on native gels containing from 5% to 12.5% acylamide. (1) Carbonic anhydrase, (2) BSA, (3) Ph-PCNA, (4) Ph-PCNA-32, (5) Ph-PCNA-41. (B) The relative mobilities of the various protein species (i.e., distance-migrated) were calculated and plotted with respect to the acrylamide percentage. (C) The slopes of the migration plots from panel B were calculated, and used to generate a Ferguson protein migration-molecular weight standard plot. BSA and carbonic anhydrase were used as standards.

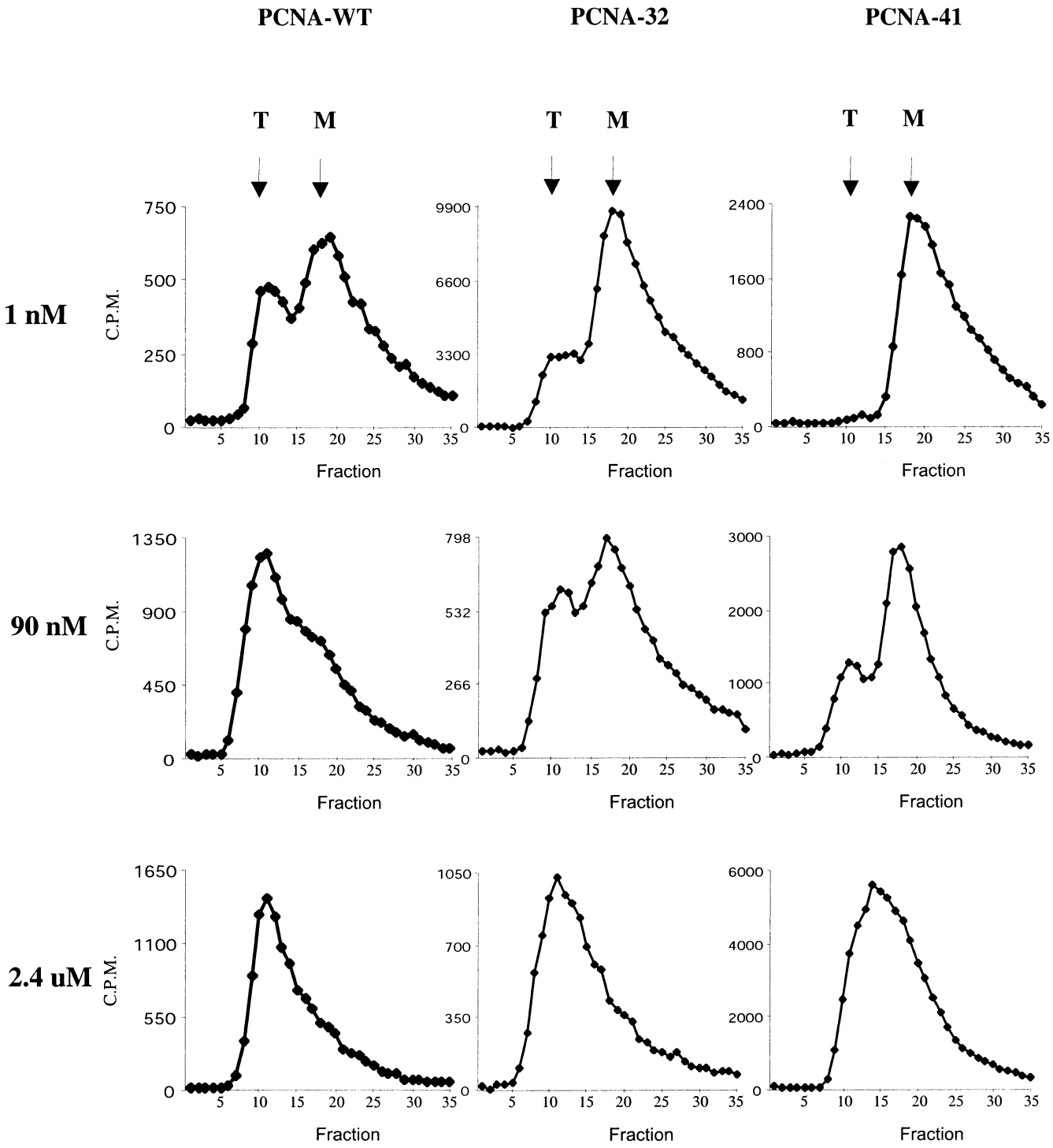

FIGURE 4: Gel filtration analysis of Ph-PCNA proteins. (A) ^{32}P-Ph-PCNA, ^{32}P-Ph-PCNA-32, and ^{32}P-Ph-PCNA-41 proteins were subjected to gel filtration on a Superose 12HR 10/30 column.

41/PCNA mixed trimer molecules were much less stable than wild-type PCNA and exhibited half-lifes of under 60 min at 4 °C.

One potential reason the PCNA-41 protein could not be stably loaded onto DNA is that monomeric PCNA subunits may not interact well with RFC. Alternatively, the monomeric PCNAs may interact with RFC, albeit in a nonfunctional complex. To discern these possibilities, we investigated PCNA/ RFC interactions by a mobility shift assay. ^{32}P-labeled Ph-PCNAs were incubated in reaction buffers either with or without RFC and then subjected to nondenaturing agarose gel electrophoresis. PCNA could be seen to form multiple gel shift complexes with RFC, and the formation of these species was sensitive to the nucleotide included in the reaction mixture. The highest levels of RFC−PCNA interactions were observed in the presence of γ-S-ATP in comparison to those observed with dATP, ADP, or ATP (data not shown). Ph-PCNA-32 interacted with RFC to a lesser extent than did wild-type Ph-PCNA, while Ph-PCNA-41 interacted very poorly (Figure 7). These results indicate that the monomeric PCNA-41 proteins are severely compromised in their ability to interact with RFC.

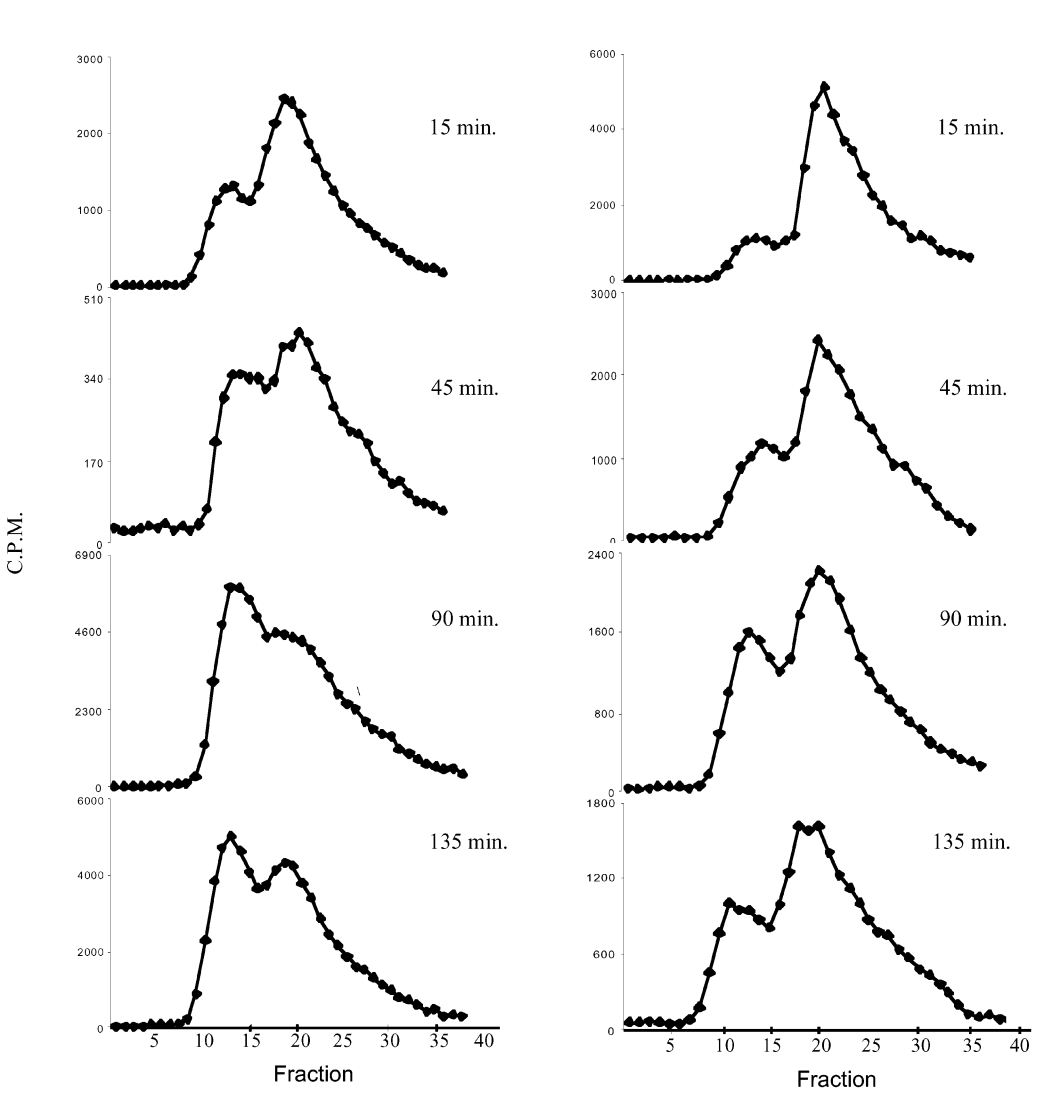

FIGURE 4: (B) ^{32}P-Ph-PCNA-32 and ^{32}P-Ph-PCNA-41 proteins were incubated at 30 °C with a 30-fold excess of wild-type PCNA for the indicated times, and then the mixture was subjected to gel filtration. In each case, fractions were collected and aliquots were subjected to liquid scintillation counting. The expected elution positions for 90 and 30 kDa proteins are fraction numbers 11 and 19, respectively.

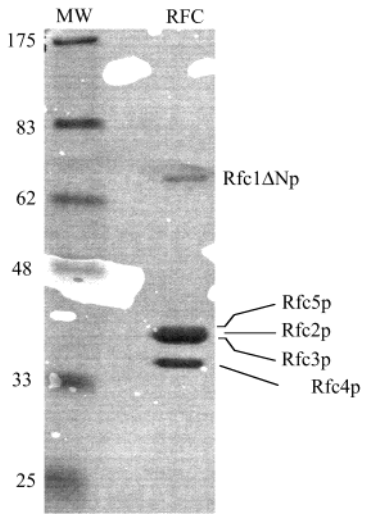

FIGURE 5: RFC purification. Recombinant yeast RFC was purified from *E. coli*, subjected to SDS-PAGE, and stained with Coomasie blue.

We then tested the mutant PCNA proteins for their ability to stimulate the ATPase activity of RFC (Figure 8). RFC-dependent ATPase assays were performed for up to 60 min at 30 °C in the presence of nicked DNA and increasing amounts of PCNA, PCNA-32, or PCNA-41 proteins. For each PCNA concentration, at least three independent experiments were used to determine the reaction rates. The resulting PCNA dependent ATPase reaction rates were then plotted versus PCNA concentrations. Although all three PCNAs were able to stimulate the RFC-dependent ATPase reactions to comparable levels (K_{cat} ranging from 1.2−1.7 s^{-1}), PCNA-41 was found to be much less effective at the lower concentrations. The calculated K_m values for wild-type PCNA, PCNA-32, and PCNA-41 were found to be 26, 105, and 755 nM, respectively. This result is consistent with the hypothesis that monomeric PCNA interacts poorly with RFC. Interestingly, RFC ATPase activity in the presence of high concentrations of PCNA-41 reaches nearly the same Vmax as in the presence of wild-type PCNA. At this concentration PCNA-41 is expected to form some trimers (see Figure 4). Thus, these data also suggest that the maximal stimulation of the RFC ATPase requires interaction with a trimeric (or potentially dimeric) PCNA.

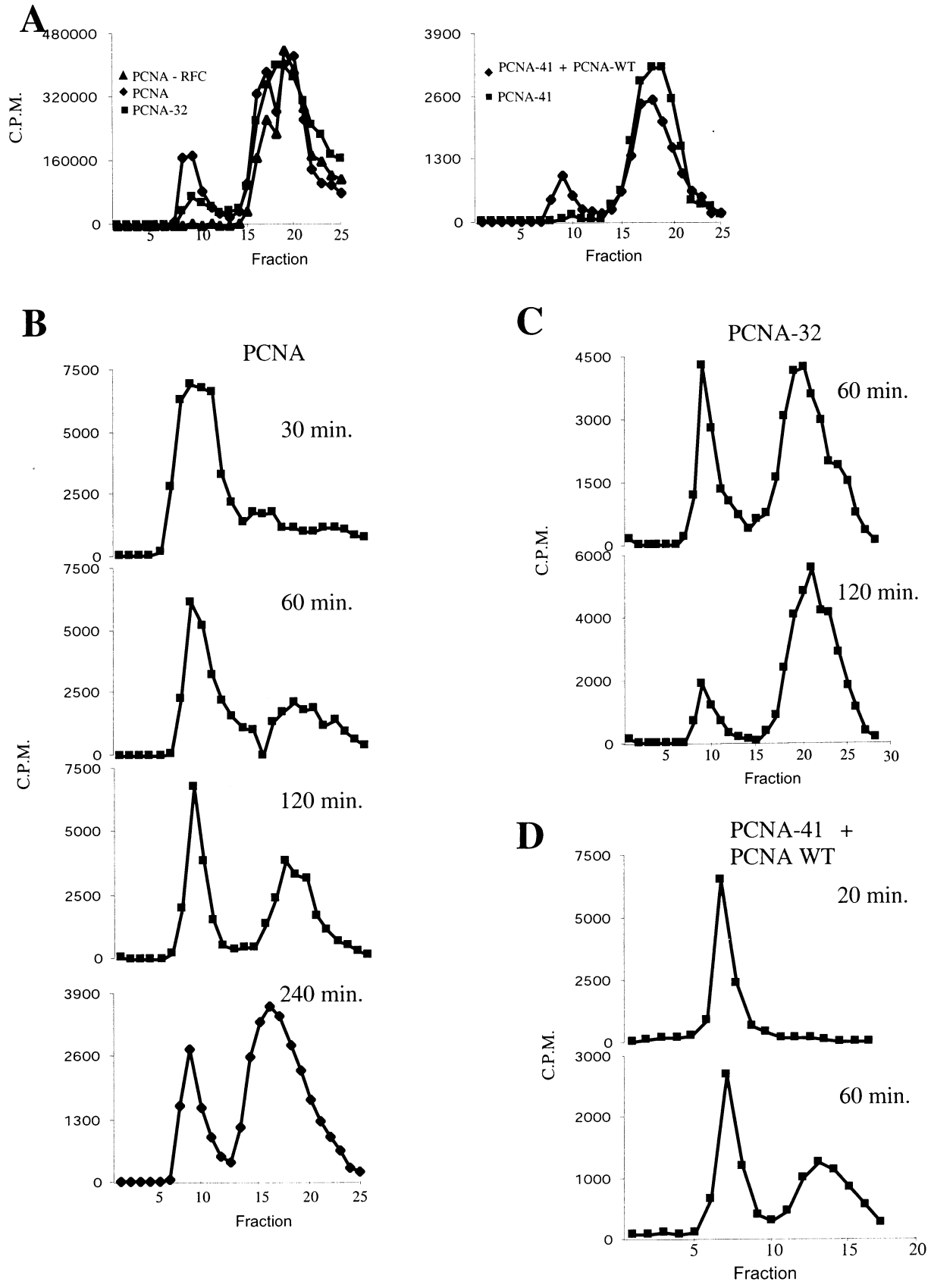

FIGURE 6: Loading of Ph-PCNAs onto nicked DNA molecules. (A) ^{32}P-Ph-PCNAs were incubated with or without RFC in reactions containing ATP and nicked DNA at 37 °C for 10 min. Loaded Ph-PCNA molecules were then separated from unloaded molecules by gel filtration. Fractions were collected, and aliquots were subjected to liquid scintillation counting. (B) Loaded Ph-PCNA molecules were recovered from the gel filtration fractions and then incubated at 25 °C for up to 3 h. Samples were then subjected to second round of gel filtration to assess status of the loaded molecules. (C) PCNA-32-DNA complexes were recovered from gel filtration fractions, incubated for up to 2 h at 4 °C and subjected to a second round of gel filtration. (D) PCNA-41 was premixed with PCNA, loaded onto DNA, and then assessed for stability as in panel C.

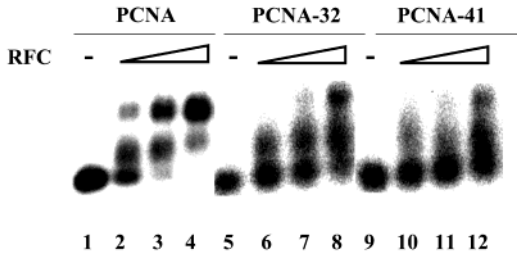

FIGURE 7: PCNA-41 is defective in its interaction with RFC. Mobility shift assays were performed with reactions containing 50 nM ^{32}P-Ph-PCNA, ATP, and varying concentrations of RFC: no RFC, lanes 1, 5, 9; 30 nM, lanes 2, 6, 10; 50 nM, lanes 3, 7, 11; 75 nM, lanes 4, 8, 12. Free PCNA and RFC-bound PCNA complexes were separated by gel electrophoresis and visualized by phosphorimage analysis.

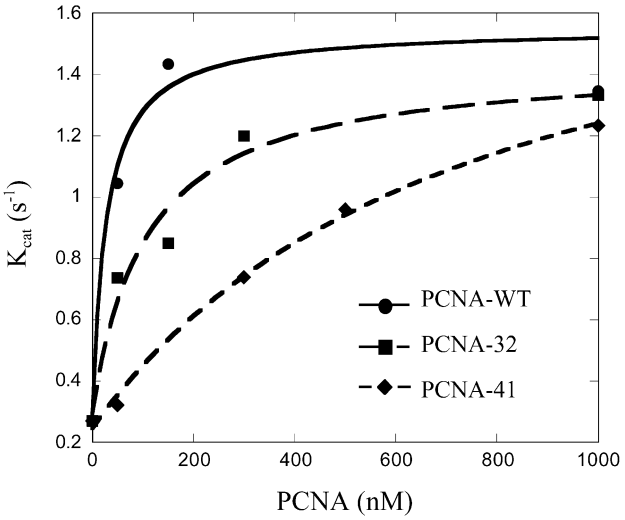

FIGURE 8: PCNA-41 is defective in stimulating the ATPase activity of RFC. RFC dependent ATPase assays were carried out as described under Experimental Procedures. Reaction rates for each PCNA concentration were derived from at least three independent experiments.

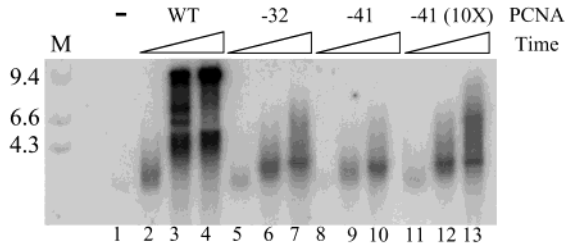

FIGURE 9: In vitro DNA polymerase delta dependent DNA replication assays. Replication assays were performed as described in Material and Methods and incubated at 30 °C for 5, 10, or 15 min. No PCNA, lane 1; 20 ng WT PCNA, lanes 2−4; 20 ng PCNA-32, lanes 5−7; 20 ng PCNA-41, lanes 8−10; 200 ng PCNA-41, lanes 11−13.

To further investigate the properties of the mutant PCNA proteins, we performed DNA polymerase *δ*-dependent in vitro DNA replication assays (Figure 9). Singly primed single stranded circular DNA substrates were included in reaction mixtures, including labeled nucleotides, RFC, PCNA, DNA polymerase delta, and single-stranded DNA binding protein. The reaction mixtures were incubated for up to 15 min, and the replication products were resolved on alkaline agarose gels. Whereas no replication activity was observed in the 20 min reaction sample that lacked PCNA (lane 1), full-length replication products were observed in the samples that contained WT PCNA (lanes 2−4). PCNA-32 was less effective at stimulating processive DNA replication (lanes 5−7), and the PCNA-41 reactions (lanes 8−10) had the least replicative activity (although still better than the reaction containing no PCNA at all). Even though the concentration of WT PCNA in these reactions was saturating (data not shown), greater replication activity could be achieved by increasing the concentration of the PCNA-41 10 fold. Therefore, PCNA-41 can function as a DNA polymerase *δ* processivity factor, albeit a weak one. On the basis of our gel filtration results, at the higher concentration of PCNA-41 (200 ng or 220 nM), roughly 30% of the molecules would be expected to be trimeric. This is comparable to the proportion of trimers in the 20 ng PCNA-32 reaction.

To investigate how the mutant PCNAs functioned in vivo, we generated a *pol30−41* allele that could be introduced into yeast by the plasmid shuffle technique. In brief, a *LEU2 pol30−41* plasmid was introduced into a yeast strain that harbored a chromosomal *pol30* deletion as well as a *URA3 POL30* plasmid. Transformants were then incubated on 5-FOA plates in order to select for those cells that could lose the *URA3 POL30* plasmid. Unexpectedly, we could readily recover 5-FOA resistant clones from the *pol30−41* strain, indicating that the PCNA-41 protein could function in vivo. A *pol30−41* strain was recovered and found to have a generation time identical to that of the *POL30* strain (100 minutes at 30 °C). It was not temperature sensitive for growth; nor did the cells exhibit any cell cycle related morphological defects that could be detected upon DAPI staining and microscopic analysis.

We then tested the *pol30−41* strain to determine whether it exhibited any defects related to aspects of DNA metabolism. Strains were assessed for mutator phenotypes by measuring the spontaneous frequency of occurrence of canavanine resistant clones. The *pol30−41* strain generated similar numbers of Can^r clones as did the *POL30* strain, indicating that the fidelity of the DNA replication process was not significantly affected in these mutants (data not shown). Next, we investigated the DNA repair proficiency of this strain. To do this, a set of four strains bearing the *POL30*, *pol30−32*, *pol30−41*, and the heterozygous *POL30 pol30−41* genotypes were tested for their sensitivities to UV irradiation and to the DNA damaging agent methylmethane sulfonate (MMS). Of the four, the *pol30−41* strain was the most sensitive to both UV- and MMS-induced DNA damage (Figure 10). This sensitivity phenotype was recessive, since the *POL30 pol30−41* strain exhibited a viability curve that was similar to that of the wild-type strain. This result indicates that the DNA repair pathways are sensitive to mutations that destabilize the trimeric PCNA clamp.

DISCUSSION

The creation and subsequent analysis of a monomeric form of yeast PCNA can provide insights concerning the structure and function of the ring-shaped sliding DNA clamp. The destabilizing effects of the A112T and S135F substitutions clearly indicate that these residues normally make contributions toward trimer assembly. While this can be readily appreciated for the A112 residue (which maps in the βI_1 beta-sheet, directly at a monomer-monomer interface), the position

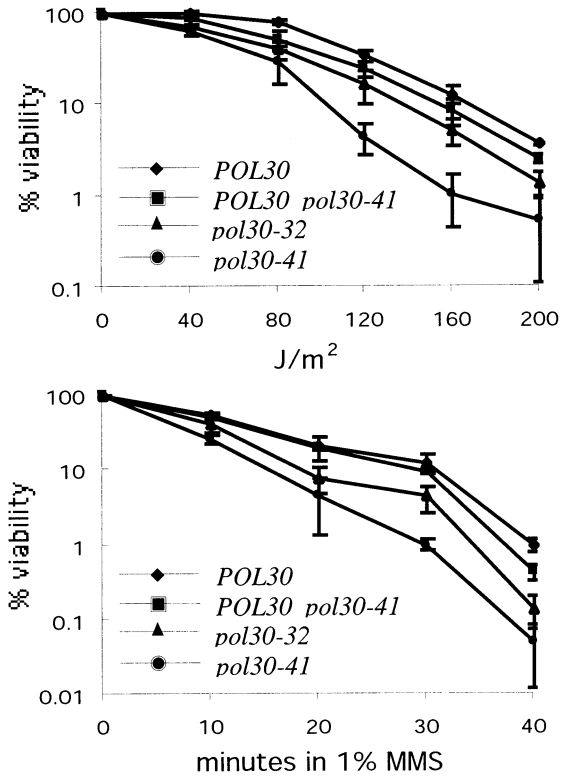

FIGURE 10: *pol30−41* mutants are sensitive to DNA damaging agents. Strains bearing the indicated genotypes were subjected to either UV irradiation or MMS treatment and scored for viability after 4 days of growth on YPD plates. Data points reflect mean values derived from at least three experiments.

of residue S135 places it at the beginning of the connecting loop between βA_1 and βI_1 (toward the interdomain connecting loop). Rather than functioning as a direct contact between adjacent subunits, this residue may help to stabilize the relative orientations of the two subdomains within each PCNA monomer. Given that double mutant PCNA-41 monomers can form mixed trimers with wild-type subunits, the overall structure of the PCNA-41 monomer is unlikely to be dramatically altered by the two substitutions. The destabilizing effects can be observed on the protein in isolation (by gel filtration and native gel analysis), as well as under conditions when it is loaded onto a DNA template. Whereas loaded wild-type PCNA was found to be stably associated with the template DNA for up to 2 h at 25 °C, the loaded mixed trimers were highly unstable at temperatures above 4 °C.

The analysis of the mutant, monomeric PCNAs can also provide insights into the nature of PCNA/RFC interactions. The observation that a monomeric PCNA interacts poorly with RFC argues against the model whereby RFC loads PCNA by recruiting three monomers and assembles them around the DNA template. Stable PCNA/RFC interactions likely require contacts with at least two PCNA subunits at a time, and these may well be mediated by multiple subunits of the RFC complex. In this regard, direct interactions have been reported between PCNA and multiple RFC subunits in both the yeast and human systems (*10*−*13*). The inability of a monomeric PCNA to interact effectively with RFC is in contrast to what has been observed for a monomeric form of the *E. coli* clamp (the β subunit of DNA polymerase III) (*5*−*7*). In that study, a monomeric β was found to interact with its clamp loader subunit (the δ subunit) 50 times greater than did the full circular clamp. Similar results were observed with the binding of the monomer to the whole γ complex (*9*). However, since the β clamp is a homo-dimer of subunits (each containing three similar subdomains)—as opposed to yeast PCNA, which is a homo-trimer of subunits containing two subdomains each—this difference may reflect a requirement for the interaction of at least three subdomains of a clamp with its clamp loader. It may also be that the different clamp geometries (homo-trimers in T4, yeast, and humans, vs homo-dimers in *E. coli*) are reflected in different mechanisms of clamp loading.

Similarly, the observation that monomeric PCNA does not effectively stimulate the ATPase activity of RFC suggests that cooperative interactions between multiple PCNA and RFC subunits may be required to trigger the conformational changes associated with elevated levels of ATP binding and hydrolysis. A model has been proposed whereby the loading reaction proceeds through an ordered set of reactions involving the stepwise binding of 4 ATP molecules to RFC (*14*). The sequential ATP binding reactions are coupled to conformational changes associated with the binding of RFC to both PCNA and DNA. ATP hydrolysis is thought to occur only at the end of the loading reaction, and it is associated with the release of RFC. Our findings are consistent with this model.

Since PCNA-41 could serve as a weak DNA polymerase delta processivity factor it presumably can be loaded onto DNA by RFC. The inability to detect this reaction in the loading assay is likely due to the PCNA-41 clamps falling off of the nicked DNA template during the subsequent gel filtration. Destabilized clamps could also be responsible for the low processivity observed in the in vitro DNA replication reactions.

One of the more surprising results from this study was that the double mutant *pol30−41* strain did not exhibit extensive DNA metabolism related phenotypes. Although the *pol30−41* strain did exhibit increased sensitivities to DNA damaging agents, we did not detect any obvious DNA replication defects. If we assume a 2 μm diameter of a yeast nucleus (*38*) and estimate that there may be some 1000 PCNA clamps/cell, then the internal concentration of PCNA would be around 400 nM—high enough for an appreciable fraction of the PCNA-41 molecules to form trimers. Local concentrations of PCNA could be even higher if it is concentrated at DNA replication or repair sites. Additionally, it is possible that in vivo, the trimeric structure of the mutant PCNAs is stabilized and maintained through interactions with other proteins. A previous analysis of another monomeric PCNA mutant (containing the S115P substitution), revealed that its in vitro DNA replication defects could be partially restored by the inclusion of the macromolecular crowding agent polyethylene glycol (*28*).

That DNA repair processes are preferentially more sensitive to destabilizing PCNA mutations may reflect differential requirements between replication and repair processes for stably loaded PCNA molecules. This may not be a function of stimulating DNA polymerase processivity per se, since DNA repair does not involve the synthesis of long stretches of DNA. Rather, DNA repair processes may depend on the presence of stably loaded (over time) DNA clamps to mark areas of the genome that will be targeted for subsequent

repair. Although crystallographic studies indicate that the 112 and 135 residues are buried, it is also possible that the A112T and S135F substitutions differentially affect the interactions between PCNA and the DNA replication and DNA repair proteins. In this regard, it will be interesting to investigate whether the mutant PCNA proteins can interact with other known PCNA-binding proteins.

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