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Requirement for PCNA in DNA Mismatch Repair at a Step Preceding DNA Resynthesis

Asad Umar,* Andrew B. Buermeyer,† Jeffrey A. Simon,† David C. Thomas,* Alan B. Clark,* R. Michael Liskay,† and Thomas A. Kunkel*
*Laboratory of Molecular Genetics
National Institute of Environmental Health Sciences
Research Triangle Park, North Carolina 27709
†Department of Molecular and Medical Genetics
Oregon Health Sciences University
3181 S.W. Sam Jackson Park Road
Portland, Oregon 97201-3098

Summary

A two-hybrid system was used to screen yeast and human expression libraries for proteins that interact with mismatch repair proteins. PCNA was recovered from both libraries and shown in the case of yeast to interact with both MLH1 and MSH2. A yeast strain containing a mutation in the *PCNA* gene had a strongly elevated mutation rate in a dinucleotide repeat, and the rate was not further elevated in a strain also containing a mutation in *MLH1*. Mismatch repair activity was examined in human cell extracts using an assay that does not require DNA repair synthesis. Activity was inhibited by p21*WAF1* or a p21 peptide, both of which bind to PCNA, and activity was restored to inhibited reactions by addition of PCNA. The data suggest a PCNA requirement in mismatch repair at a step preceding DNA resynthesis. The ability of PCNA to bind to MLH1 and MSH2 may reflect linkage between mismatch repair and replication and may be relevant to the roles of mismatch repair proteins in other DNA transactions.

Introduction

DNA mismatch repair plays a critical role in maintaining genome stability, and defects in mismatch repair genes are linked to cancer (Liu et al., 1996; and for review, see Kolodner, 1996). Several genes required for mismatch repair in eukaryotic cells have been identified and the functions of their products are partially understood (reviewed in Modrich and Lahue, 1996; Umar and Kunkel, 1996). For example, MSH2 and MSH6 (also called GTBP or p160 in humans) form a heterodimer designated hMutSα (Drummond et al., 1995; Iaccarino et al., 1996) that binds to and functions in repair of base•base and certain insertion–deletion mismatches. By analogy to the bacterial MutL homodimer, a heterodimer of MLH1 and PMS1 in yeast (Prolla et al., 1994b) or MLH1 and PMS2 in humans (designated hMutLα; Li et al., 1995), may interact with hMutSα and act as part of a multiprotein complex that excises the error in a newly replicated strand, which is then resynthesized correctly. Mismatch repair in human cells is strand specific, and, although the signal for strand-discrimination in vivo is unknown, repair in vitro can be directed to one strand by a nick in the DNA substrate (Holmes et al., 1990; Thomas et al., 1991). As in E. coli, the human mismatch repair system requires ATP, and excision and DNA resynthesis occur between the nick and the mismatch (Holmes et al., 1990; Thomas et al., 1991), which can be separated by as many as 1000 base pairs (hence the designation "long-patch" mismatch repair). As in E. coli, the human mismatch repair system has bidirectional excision capability (Fang and Modrich, 1993). The DNA synthesis step is sensitive to aphidicolin (Holmes et al., 1990; Thomas et al., 1991), suggesting the involvement of a polymerase that also participates in replication, i.e., pol α, δ, or ϵ.

The identities and functions of other eukaryotic mismatch repair proteins remain to be established. Possibilities include helicases, single strand DNA binding proteins, exonucleases, and the DNA polymerases mentioned above and their accessory proteins. In the present study, we initiated a search for proteins that interact with the MutL proteins of yeast and humans by independently screening expression libraries of the respective species. Both two-hybrid screens identified an interaction between MutL proteins and proliferating cell nuclear antigen (PCNA), a protein already known to function in both DNA replication (for review, see Stillman, 1994) and nucleotide excision repair (Nichols and Sancar, 1992; Shivji et al., 1992). A PCNA interaction was also detected with yeast MSH2. Moreover, a yeast strain containing a mutation in the PCNA gene was found to have a strongly elevated mutation rate in a dinucleotide repeat, and the rate was similar in a strain with an additional mutation in a known mismatch repair gene. These data indicate a role for PCNA in mismatch repair and further suggest that, perhaps via PCNA interactions with MSH2 and/or MLH1, this role might precede the DNA synthesis associated with mismatch repair. Biochemical studies of mismatch repair activity in human cell extracts provided evidence that this is indeed the case. The interactions of PCNA with MLH1 and MSH2 provide possible links between mismatch repair and other DNA transactions in vivo, such as replication and transcription-coupled nucleotide excision repair.

Results

PCNA Interacts with Mismatch Repair Proteins in the Yeast Two-Hybrid System

We have used a *lexA*-based two-hybrid system (Fields and Song, 1989; Vojtek et al., 1993; Hollenberg et al., 1995) to screen yeast and human cDNA expression libraries for proteins that associate with yeast PMS1 and MLH1 and human PMS2 (homolog of yeast PMS1). As "baits," we used the full coding sequence of individual MutL homologs fused to the DNA-binding domain of *lexA*. Additionally, since both the yeast and human MutL homologs are believed to function as heterodimers (Prolla et al., 1994a, 1994b; Li and Modrich, 1995), we used "bait/co-bait" plasmids (e.g. LexA–PMS1/MLH1) that coexpress PMS1 and MLH1, one (the bait) as a fusion with *lexA*, and the second (the co-bait) lacking the DNA-binding domain. The bait and co-bait are expected to

Table 1. Interactions of PCNA with Mismatch Repair Proteins

	Bait and Bait/Co-Bait Constructions[a]									
	LexA-PMS1		LexA-MLH1		LexA-PMS1/MLH1		LexA-MLH1/PMS1		LexA-MSH2	
GAD-Fusions[b]	HIS3[c]	β-Gal[d]	HIS3	β-Gal	HIS3	β-Gal	HIS3	β-Gal	HIS3	β-Gal
PCNA	−	NA	+	0.5	+	0.3	+	0.3	+	0.1
PMS1	−	NA	+	5.2	−	NA	+	1.9	−	NA
MLH1	+	4.9	−	NA	+	1.3	−	NA	−	NA
MSH2	−	NA	−	NA	−	NA	−	NA	−	NA

Expression of HIS3 and β-galactosidase induced by interactions between yeast PCNA and yeast DNA mismatch repair proteins was measured in a two-hybrid analysis as described in Experimental Procedures. NA, not applicable.

[a, b] See Materials for a description of these plasmids.

[c] Induction of HIS3 expression was determined by ability of yeast to grow on medium lacking histidine. + and − refer to growth detectable or not detectable, respectively, after incubation of plates for 3–4 days at 30°C.

[d] β-galactosidase activity was measured in yeast cells capable of growth on medium lacking histidine. The numbers listed are the average units (defined in Experimental Procedures) determined from three independent matings. The background β-galactosidase activity for control cells was 0.06 ± 0.02 unit.

interact in vivo and together form a target for protein-fusions expressed from the library plasmids (Toyoshima and Hunter, 1994). In a screen of $\sim 2 \times 10^7$ transformants using LexA–PMS2 as a bait, we isolated a full-length cDNA encoding human PCNA. Additionally, in a screen of approximately 3×10^6 transformants using the bait/co-bait construction LexA–PMS1/MLH1, we isolated a nearly complete cDNA encoding yeast PCNA (lacking the DNA encoding the first 5 amino acids).

To characterize further the interaction of yeast PCNA with individual mismatch repair proteins, the yeast PCNA–GAD fusion isolated in the library screen was tested against several yeast mismatch repair proteins, alone or in combination (summarized in Table 1). As reported previously (Prolla et al., 1994b) and shown here by growth without histidine and β-galactosidase activity, we detected an interaction between yeast PMS1 and MLH1, but could not detect interaction between these MutL proteins and yeast MSH2. However, the yeast PCNA–GAD fusion interacted with both of the bait/co-bait combinations containing yeast PMS1 and MLH1. The low β-galactosidase activity generated by the association of PCNA with the bait/co-baits suggests a weaker interaction between PCNA and the MutL homologs than between PMS1 and MLH1. Alternatively, the low β-galactosidase activity might result from toxicity due to overexpression of the PCNA–GAD fusion (Li et al., 1995). The interactions between yeast PCNA and the MutL bait/co-baits were specific as judged by the lack of growth without histidine when the PCNA–GAD plasmid was tested with the control bait LexA–Lamin and with the "empty" vector (pTRP1) which does not express a bait (data not shown).

In tests against individual proteins, yeast PCNA was found to interact with yeast MLH1 and with yeast MSH2, but not with PMS1 (Table 1). Although the signal detected between yeast PCNA and MSH2 was weak, only one other clone isolated in the interaction screens with MutL homologs was able to interact with both MLH1 and MSH2 (data not shown). Inability to detect interactions of yeast PCNA with PMS1 suggests that interaction of PCNA with the bait/co-baits is via contacts made only with MLH1. Therefore, the signal observed between PCNA–GAD and LexA–PMS1/MLH1 suggested that PCNA can interact with MLH1 complexed to PMS1. Although repeatable, the interaction between the human PCNA–GAD and LexA–PMS2 was weak (~0.2 units of β-galactosidase). However, we could not test whether human PCNA interacted with human MLH1 because full-length human MLH1 gave a very high nonspecific signal in the two-hybrid system (data not shown).

Effect of a Yeast PCNA Mutation on the Stability of Simple Repetitive DNA

Mutations in mismatch repair genes cause increased instability in simple repetitive DNA sequences, such as tracts of poly GT (Levinson and Gutman, 1987; Strand et al., 1993; Johnson et al., 1995). Additionally, several mutations have been described in yeast PCNA that increase slightly the spontaneous rate either of forward mutation to cycloheximide resistance or of mutation to inactivate the *SUP4-o* tRNA (Ayyagari et al., 1995). To gain support for a role for PCNA in mismatch repair, we determined whether mutations in yeast PCNA can destabilize simple repeats in a plasmid that contains a 51 bp poly GT sequence as an in-frame insertion in a fusion gene that encodes a protein with wild-type URA3 activity (Henderson and Petes, 1992; Strand et al., 1993). Plasmids with altered GT tract lengths represent frameshift mutations that inactivate the URA3 fusion and confer resistance to 5-fluoro-orotic acid (5-FOA) (Henderson and Petes, 1992). In the PCNA wild-type strain, the rate of mutation in the GT tract was 2.4×10^{-5} (Table 2), similar to rates reported previously (Henderson and Petes, 1992; Heale and Petes, 1996). Next we examined five different mutations in the *POL30* (PCNA) gene (Ayyagari et al., 1995) for effects on the stability of the poly GT tract. Four of the PCNA mutant strains tested did not destabilize the poly GT tract (data not shown). However, one mutant strain expressing the *pol30–52* mutant allele (a proline substituted for serine at amino acid 115) showed a rate of 5-FOA resistant colonies of 3.5×10^{-3}, representing a 150-fold increase relative to wild type (Table 2). The *pol30–52* mutation also causes a cold-sensitive phenotype and a 4.9-fold increase in the forward rate of mutation to cycloheximide resistance (Ayyagari et al., 1995). Similar to previous reports (Strand et al., 1993; Johnson et al., 1995), disruption of the yeast *MLH1* gene (PY50 *mlh1*Δ) resulted in a rate of tract alterations of 4.5×10^{-3}, a 190-fold increase relative to wild type (Table 2). In a strain containing both the

Table 2. Effect of the *pol30-52* Mutation on Rates of Alterations in poly GT Tracts in Wild-type and Mismatch Repair-Deficient Strains

Genotype	Median Number of Mutants/Culture	Total Number of Cells in 40 Cultures	Rate of Tract Instability[a]	Rate Relative to Wild Type
Wild type	32	1.6×10^7	2.4×10^{-5}	1
pol30-52	7560	1.1×10^7	3.5×10^{-3}	150
*mlh1*Δ	37260	3.5×10^7	4.5×10^{-3}	190
*pol30-52 mlh1*Δ	15997	1.6×10^7	4.4×10^{-3}	180

[a] Calculated as described in Experimental Procedures.

pol30–52 and *mlh1*Δ mutations, we observed a rate of 4.4×10^{-3}, a 180-fold increase over the wild-type strain (Table 2). Thus, the data are consistent with the *pol30–52* mutator effect reflecting a defect in the same process as that for *mlh1*Δ, i.e., a defect in mismatch repair.

Monitoring Mismatch Repair Processes Preceding DNA Resynthesis

Complementing the in vivo studies in yeast described above with biochemical analysis of mismatch repair in vitro must await development of assays to measure mismatch repair activity with yeast proteins. However, it is possible to examine the involvement of PCNA in mismatch repair using assays for activity in extracts of human (Holmes et al., 1990; Thomas et al., 1991) or mouse cells (Edelmann et al., 1996). In these assays, repair has been demonstrated to require a mismatch and is strand specific, with the signal for strand specificity provided by a nick (Holmes et al., 1990; Thomas et al., 1991). In human cells, MSH2, MLH1, and PMS2 are required for the initial steps in mismatch repair, ultimately leading to excision of nucleotides between the nick and the mismatch. The resulting gap is then filled by an aphidicolin-sensitive DNA polymerase (Holmes et al., 1990, Thomas et al., 1991), either pol α, δ, or ϵ. The processivity of the latter two polymerases is stimulated by PCNA, which binds to the polymerase and also topologically binds the DNA as a trimeric sliding clamp that encircles the duplex template-primer (Krishna et al., 1994). DNA synthesis associated with mismatch repair may require PCNA. However, because PCNA appears to interact with proteins involved in the initial steps in mismatch repair, it may also function prior to repair synthesis.

To test for a PCNA requirement prior to repair synthesis, we measured mismatch repair activity in human cell extracts using an assay (Thomas et al., 1991) that does not require DNA synthesis (Figure 1). An M13mp2 DNA substrate was used, containing a covalently closed (+) strand, a nicked (−) strand, and a G•G mispair at position 88 in the *lacZ*α gene coding sequence. The (+) strand codes for white plaques and the (−) strand codes for blue plaques. An unrepaired heteroduplex yields mixed plaques in an E. coli strain deficient in methyl-directed heteroduplex repair. Repair occurring in a repair-proficient human cell extract, but not in repair-deficient extracts (e.g., see Boyer et al., 1995), will reduce the percentage of mixed plaques and increase the white to blue plaques, since the nick directs repair to the (−) strand.

Importantly, detection of mismatch repair activity with our assay only requires excision of the mismatched base(s) in the nicked strand. Although DNA repair synthesis in the extract does occur under normal circumstances (Holmes et al., 1990; Thomas et al., 1991), synthesis is not required, because the gap generated by mismatch excision can be filled in by an E. coli polymerase after introduction of the DNA into the α-complementation host. In fact, when gapped substrates (constructed as described in Bebenek and Kunkel, 1995) encoding either blue or colorless plaque phenotypes are introduced into the *mutS* E. coli strain used for these studies, >99% of the plaques are pure bursts of the expected color (data not shown). That DNA resynthesis is not required can also be shown using well-known inhibitors of eukaryotic replicative polymerases, aphidicolin and N^2-(p-n-butylphenyl) deoxyguanosine triphosphate (BuPdGTP). Both compounds strongly inhibit the mismatch repair–associated DNA synthesis that occurs between the nick and the mismatch (Table 3) (see also Thomas et al., 1991). However, when the products of these inhibited reactions are scored for repair, the reduction in mixed bursts and increased ratio of colorless to blue plaques is similar to that observed in the uninhibited repair reaction (Table 3). This demonstrates that

Table 3. Uncoupling of Mismatch-Associated DNA Synthesis from Mismatch Excision

Addition to Repair Reaction	DNA Synthesis[a] (%)	Mixed Bursts (%)	Repair Efficiency[b] (%)	Ratio of White to Blue
Control[c]	—	38	—	0.19
None	100	11	71	1.3
1 μM BuPdGTP	84	10	74	1.1
10 μM BuPdGTP	26	13	66	0.9
100 μM BuPdGTP	11	11	71	1.0
50 μg/ml aphidicolin	0.7	13	66	0.8

Repair reactions (30 μl) and analysis of incorporation and repair were performed as described in Experimental Procedures and in Thomas et al., 1991, using a HeLa cell extract.

[a] The uninhibited reaction incorporated 127,000 cpm of [^{32}P]-α-dCTP. As illustrated earlier (see Figure 3 in Thomas et al., 1991), the vast majority of this incorporation is dependent on the presence of the mismatch (G•G) and occurs between the nick at nucleotide −264 and the mismatch at position 88.

[b] Calculated as described in Experimental Procedures.

[c] The substrate was introduced into E. coli *mutS* cells without prior incubation in the extract.

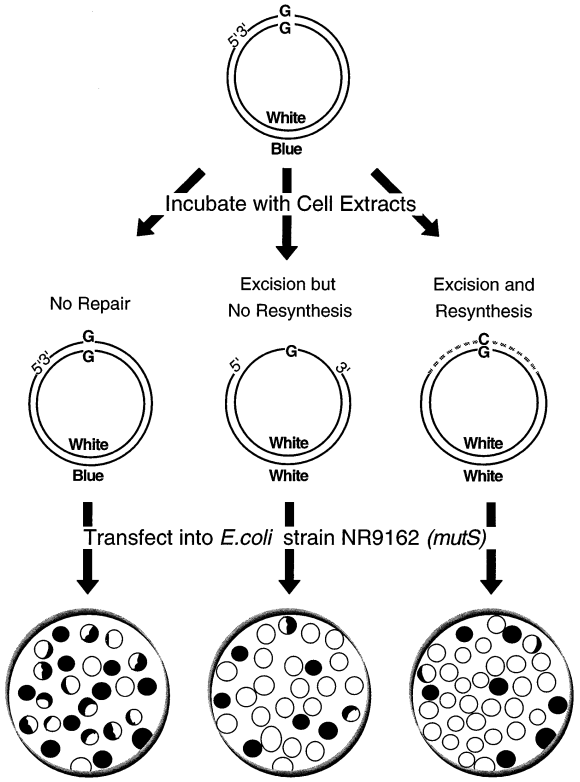

Figure 1. Assay for Mismatch Repair Activity
See Results for description.

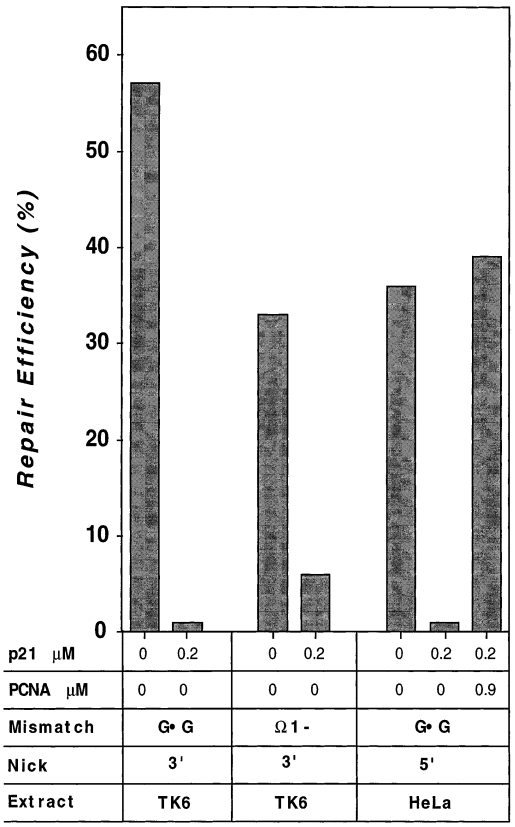

Figure 2. Inhibition of Mismatch Repair Activity by p21 and Reversal by Excess PCNA

Substrates contained either a G•G mismatch at position 88 or one unpaired T in the minus strand at positions 91–94 (a homopolymeric run) in the *lacZ* gene. The nick was either at position −264 (3′-nick) or at position 276 (5′-nick). Incubations were for 15 min, and reaction products were analyzed for repair activity as described in the text. Repair efficiency was calculated as described in Experimental Procedures, with each value reflecting at least 500 total plaques scored.

excision of the mismatch by the human cell extract can be observed in the absence of associated DNA repair synthesis, thus providing an assay to determine if PCNA has a synthesis-independent role in mismatch repair.

Inhibition of Mismatch Repair Activity by p21 or a p21 Peptide

To determine if PCNA is required for mismatch repair activity in vitro, we examined mismatch repair activity in an extract of HeLa cells in reactions to which was added p21^{WAF1}. p21 binds to PCNA and inhibits PCNA-dependent DNA replication and pol δ-catalyzed chain elongation on primed DNA templates (Flores-Rozas et al., 1994; Li et al., 1994; Waga and Stillman, 1994). Nucleotide excision repair of damaged DNA also requires PCNA (Nichols and Sancar, 1992; Shivji et al., 1992), and p21 also inhibits this reaction (Pan et al., 1995). In the present study, addition of p21 to human cell extracts at 0.2–2 μM, concentrations known to inhibit replication and nucleotide excision repair (e.g., see Pan et al., 1995), mismatch repair activity was strongly inhibited (Figure 2). Inhibition was observed with repair-proficient extracts of either HeLa or TK6 cells and with substrates containing either one unpaired nucleotide or a G•G mismatch. Repair of the G•G mismatch was inhibited whether the nick is located 5′ or 3′ to the mismatch.

To obtain additional evidence that p21 exerted effects via an interaction with PCNA, we exploited observations on the site of interaction between PCNA and p21. Using a two hybrid screen, Warbrick et al. (1995) demonstrated that the carboxy-terminal region of p21 interacts with the central loop of PCNA that connects the two domains of the PCNA monomer. Using synthetic peptides, they identified amino acids 144–151 of p21 as being critical for PCNA binding. A p21 peptide of only 20 residues (designated PBP for PCNA Binding Peptide) that contained this sequence bound to PCNA could capture PCNA from whole cell extracts and inhibited SV40 DNA replication in vitro. Studies of mutant peptides identified single amino acids that, when changed to alanine, reduced the inhibitory effect on replication. Furthermore, the ability to inhibit replication correlated with PCNA binding affinity.

Based on these results indicating that PBP is a PCNA-specific inhibitor, we examined the ability of PBP to inhibit mismatch repair activity in extracts. As shown in Figure 3, mismatch repair reactions catalyzed by extracts of HeLa cells, TK6 lymphoblastoid cells or ZR-75 breast cancer cells are all inhibited by addition of PBP. A substantially higher molar concentration of PBP as compared with intact p21 is required to inhibit mismatch repair to an equivalent extent (compare Figure 2 with Figure 3). This is similar to results demonstrating that

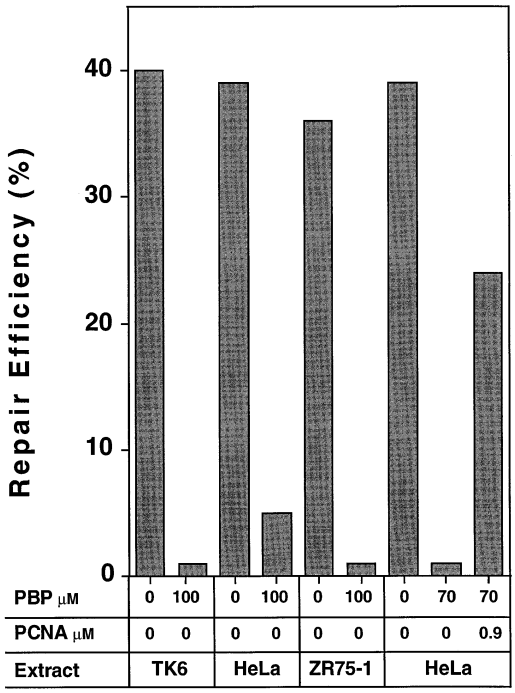

Figure 3. Inhibition of Mismatch Repair Activity by PBP and Reversal by PCNA

Reactions (25 μl, 15 min incubations) were performed with the indicated extracts and a G•G mismatch with a 3′-nick. PBP was used except for the right-most experiment, which used PBP_{bio}.

intact p21 is a more effective inhibitor of SV40 replication and nucleotide excision repair than is a PCNA-binding peptide derived from p21 (Pan et al., 1995). Note also that, compared with inhibition by PBP, much less inhibition of mismatch repair is obtained using equivalent amounts of a mutant peptide (PBP-A) containing an alanine substituted for a phenylalanine at amino acid 150 of p21 (Figure 4). This mutant peptide is known to bind to PCNA ~6-fold less avidly than PBP (Warbrick et al., 1995). Finally, no inhibition of mismatch repair activity is observed with a "jumbled" peptide (PBP-J) having the same amino acid composition as PBP but in a different order (Figure 4). Such a jumbled peptide has been shown to lack PCNA binding ability (Warbrick et al., 1995). The data obtained with the three peptides suggest that the inhibition of mismatch repair activity by PBP depends on its ability to bind PCNA.

Excess PCNA Reverses p21-Mediated Inhibition of Mismatch Repair

The addition of excess PCNA has been shown to reverse p21-mediated inhibition of SV40 replication (Waga and Stillman, 1994) and nucleotide excision repair in vitro (Pan et al., 1995). Here, when an inhibitory concentration of p21 was preincubated with the HeLa cell extract reaction mixture at 0°C and then excess PCNA was added and the reaction incubated at 37°C, repair was observed at levels found in the untreated extract (Figure 2, right). In a similar experiment, we observed that addition of excess PCNA also reversed the inhibitory effects of the PCNA binding peptide (Figure 3, right).

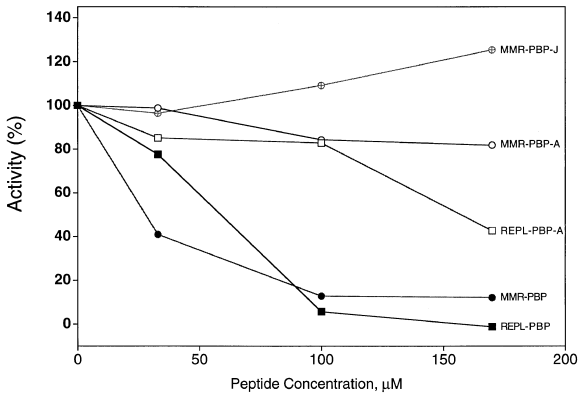

Figure 4. Effect of PBP, PBP-A, and PBP-J on Replication and Mismatch Repair Activity

Replication and repair reactions were performed as described in the legend to Figure 2 and in Results, using a HeLa cell extract and biotinylated peptides. PBP, PCNA Binding Peptide; PBP-A, PBP with alanine substituted for phenylalanine at residue 150; PBP-J, jumbled PBP; REPL, replication; MMR, mismatch repair.

PCNA Restores Mismatch Repair Activity to PCNA-Depleted Extracts

Warbrick et al. (1995) removed PCNA from a cell extract by incubation with PBP attached to streptavidine-agarose beads followed by centrifugation. Therefore, we incubated a mismatch repair–proficient extract of TK6 cells (three experiments) or HeLa cells (two experiments) with 100 μM biotinylated PBP, then added avidin attached to magnetic beads and removed the PBP and PBP-bound proteins using a magnet. The average G•G mismatch repair efficiency of the resulting extracts was 4% ± 5% (mean ± standard deviation, three independent determinations), as compared to the 67% ± 13% repair efficiency observed with untreated extracts. This represents a 94% inhibition of repair activity. When the treated extract was added to a reaction containing an untreated TK6 extract, repair activity was >100% of the untreated control (data not shown), indicating that the PBP was successfully removed using the magnetic beads. Finally and most importantly, addition of 0.9 μM PCNA to the treated, repair-deficient extract restored mismatch repair efficiency to 25% ± 2% (Figure 2), representing recovery of 36% of the original mismatch repair activity. Thus, mismatch repair activity was PCNA dependent. The same extent of activity was restored using 0.45 μM PCNA, and addition of PCNA to 1.8 μM did not further enhance activity (data not shown), suggesting that proteins other than PCNA may have been removed and were now rate limiting for mismatch repair activity.

Relative Sensitivity of MMR Activity and Replication to Inhibition by PBP

Consistent with a model wherein cell cycle arrest allows time to repair damage prior to replication (see Discussion), two previous studies have reported that SV40 replication is more sensitive to inhibition by p21 than is nucleotide excision repair (Li et al., 1994; Shivji et al., 1994). However, the fact that a major role for mismatch repair is correction of replication errors suggests that

replication and mismatch repair may be coordinated, and thus perhaps coordinately inhibited by PBP. To test this, we examined the ability of increasing concentrations of PBP to inhibit SV40 replication and G•G mismatch repair activity in parallel reactions in vitro (Figure 4). At the two highest PBP concentrations examined, both replication and repair are strongly inhibited. However, lesser inhibition is observed at 33 μM PBP, where replication activity is 78% of the control (i.e., in the absence of PBP), and mismatch repair activity is 41% of the control (Figure 4). The means and standard deviations for four independent determinations at 33 μM PBP yielded replication and mismatch repair activities that were 91% ± 15% and 58% ± 12%, respectively, of values obtained without PBP.

Discussion

The observations in the two-hybrid system presented here demonstrate that yeast PCNA can interact in vivo with MLH1 alone, with coexpressed yeast MLH1–PMS1, or with yeast MSH2 alone. These interactions are consistent with a role for PCNA in mismatch repair at steps in the pathway involving these proteins. Moreover, a missense mutation in yeast PCNA conferred a strong mutator phenotype, similar in magnitude to that resulting from disruption of the yeast *MLH1* gene. Furthermore, a strain containing both the MLH1 and PCNA mutations did not exhibit a synergistic increase in mutation, as would be expected if the PCNA mutation caused increased slippage during replication of the GT tracts. Rather, the mutation rate in the double mutant strain was similar to that observed in the single mutant strains (Table 2). Therefore, the data are consistent with the mutations in PCNA and MLH1 both affecting the same process, namely, DNA mismatch repair.

MSH2, MLH1, and PMS1 (hPMS2) all have essential roles in early steps of mismatch repair in eukaryotic cells. MSH2 initiates the pathway by binding to mismatches and insertion–deletion heteroduplexes (for review, see Modrich and Lahue, 1996), either alone or as part of a heterodimer. By analogy to the bacterial MutL homodimer, which has no known enzymatic function of its own, the eukaryotic MLH1•PMS1 (hPMS2) heterodimer participates in the reactions necessary to excise the DNA between the mismatch and the signal for strand discrimination. In fulfilling these functions, MLH1 and PMS1 may provide interactions with other proteins that are important for mismatch repair activity. In support of the idea that one of these required proteins is PCNA, p21, a known PCNA-binding protein that inhibits replication, also inhibits mismatch repair activity in vitro (Figure 2), using an assay (Figure 1) that does not require the DNA synthesis associated with mismatch repair (Table 3). That this inhibition is due to an interaction with PCNA and not some other protein is suggested by several observations: (i) a p21 peptide that interacts with PCNA also inhibits mismatch repair activity (Figure 3); (ii) a variant peptide than binds PCNA much less tightly inhibits mismatch repair activity much less effectively (Figure 4); (iii) a jumbled peptide of the same amino acid composition but different sequence that lacks PCNA binding does not inhibit mismatch repair activity (Figure 4); (iv) the inhibition of mismatch repair activity by p21 (Figure 2) or PBP (Figure 3) can be overcome by addition of excess PCNA; and (v) the repair activity of an extract rendered mismatch repair deficient by treatment with and then removal of PBP can be partially restored by addition of PCNA.

Although our results do not exclude that PCNA may participate as a processivity clamp for DNA polymerase δ or ε during mismatch repair-associated DNA synthesis, our data do suggest that PCNA has other functions earlier in the repair process. These could be prior to excision via the observed interactions with MSH2, MLH1, and/or PMS2, e.g., assisting in communication between the mismatch and the signal for strand discrimination or in loading a helicase. Another possibility is suggested by the observation that PCNA binds to and stimulates the activity of FEN1 (Li et al., 1995), a 5′-nuclease that removes RNA primers during DNA replication and whose yeast homolog, RTH1 (RAD27) has been suggested to participate in mismatch repair (Johnson et al., 1995). Thus, p21 may prevent excision of nucleotides between the mismatch and the signal for strand specificity by interfering with interactions between PCNA and a nuclease, possibly FEN1. In E. coli, different nucleases participate in the excision step depending on the location of the nick relative to the mismatch (for review, see Modrich, 1991). The observation that p21 inhibits mismatch repair in substrates containing nicks either 3′ or 5′ to the mismatch (Figure 2) implies that either PCNA interacts with more than one nuclease or a bidirectional nuclease or that direct PCNA–nuclease interactions are not involved.

In higher eukaryotic cells, the synthesis of p21 is transcriptionally activated by p53 in response to DNA damage. This leads to cell cycle arrest due to p21 binding to and inhibiting the functions of cyclin-dependent kinases and/or PCNA. This arrest presumably allows time to repair damage prior to replication. This model is consistent with two reports (Li et al., 1994; Shivji et al., 1994), indicating that replication is more sensitive to inhibition by p21 than is nucleotide excision repair. (However, see Pan et al., 1995). In contrast to the major role of the nucleotide excision repair pathway in removing lesions prior to replication, a major role for the mismatch repair pathway is to correct DNA replication errors. It is thus interesting to consider the relative sensitivity of mismatch repair versus replication to inhibition by the p21 peptide. Addition of 100 or 170 μM PBP to a human cell extract is sufficient to strongly inhibit both mismatch repair and SV40 replication in vitro (Figure 4). However, at 33 μM PBP, SV40 DNA replication was consistently inhibited to a lesser extent than was mismatch repair. This suggests the possibility that a (transient) concentration of p21 might arise in vivo that would allow replication under conditions of diminished mismatch repair. Given the critical role of mismatch repair in correcting replication errors, this could yield an elevated mutation rate.

PCNA is topologically bound to DNA and capable of interacting with and/or modulating the activities of DNA polymerases δ and ε, cyclins•cyclin-dependent kinase complexes (e.g., see Waga and Stillman, 1994, and references therein), the replication factor C protein complex

(Fukuda et al., 1995), FEN1 nuclease (Li et al., 1995), the damage induced protein Gadd45 (Smith et al., 1994; Hall et al., 1995), and now mismatch repair proteins. Thus, PCNA appears to be ideally suited to link the several DNA transactions in which it is now known to function. For example, although the signal for strand discrimination during eukaryotic mismatch repair is not known, it is possible that primer termini at the replication fork might serve this function. As a sliding clamp during replication, PCNA is associated with and enhances the processivity of replicative DNA polymerases. PCNA's role at an early step in mismatch repair might provide the physical link between mismatch repair and DNA replication that would allow primer termini at the fork to serve as strand discrimination signals for mismatch repair.

Finally, human cell lines containing mutations in the *hMSH2*, *hMLH1*, and *hPMS2* genes have recently been shown to be defective in transcription-coupled nucleotide excision repair (Mellon et al., 1996). PCNA is required for nucleotide excision repair (Nichols and Sancar, 1992; Shivji et al., 1992) and its ability to interact with MLH1 and MSH2 (Table 1) may facilitate their roles in this process. Mismatch repair proteins have also been suggested to function as sensors for genetic damage (Kat et al., 1993) and cells defective in MLH1 do not arrest in the G2 phase of the cell cycle following treatment with 6-thioguanine (Hawn et al., 1995). This connection between checkpoint control and mismatch repair may be mediated through PCNA's ability to interact with both cyclin•cyclin-dependent kinase complexes and mismatch repair proteins. PCNA's multiple functions and interactions with many different proteins lead to the expectation that amino acid substitutions at the site of interaction of PCNA with another protein could differentially interfere with one role but not another. For example, certain mutations in PCNA may allow replication without mismatch repair, a situation in human cells that is linked to cancer (for review, see Kolodner, 1996).

Experimental Procedures

Yeast Plasmids

The "bait" plasmids expressing mismatch repair proteins fused to the DNA-binding domain of LexA (see Table 1) were derived from pBTM116 (Bartel et al., 1993). "Bait/co-bait" constructions (e.g. LexA–PMS1/MLH1) contain, in addition to the bait, a co-bait expressed from the promoter of the yeast alcohol dehydrogenasegene (ADH) inserted at the PvuII site of pBTM116. The negative control plasmid pTRP1 is pBTM116 with the sequences corresponding to the ADH promoter and terminator and the LexA DNA-binding domain deleted. The "control" bait plasmid expressing LexA–lamin C has been described (Hollenberg et al., 1995). Plasmids expressing protein fusions containing the GAL4 activation domain (GAD fusions) were constructed in pGAD424 (Clontech, Palo Alto, CA).

For the mutator analysis, plasmid pRS313/TA4 was constructedby ligating an XmaI–SpeI fragment from pTA4 (Heale and Petes, 1996) into the XmaI–SpeI sites in pRS313 (*HIS3 CEN6 ARS4*) (Sikorski and Hieter, 1989). The plasmid pRS313/TA4 thus contains an *HIS3* selectable marker and an in-frame insertion of a 51 bp poly GT tract within the coding sequence of a gene encoding a fusion protein with wild-type URA3 activity. The disruption plasmid *mlh1Δ::LEU2* has been described (Prolla et al., 1994a). Plasmids for expression of yeast PCNA (*POL30* gene) pBL211(*POL30 URA3 ARS1 CEN4*), pBL230 (*POL30 TRP1 CEN6 ARS4*), and plasmids pBL230-6, -41, -45, -46, and -52 (each also *TRP1 CEN6 ARS4*) have been described (Ayyagari et al., 1995). Plasmid pBL245-52 (generous gift of P. Burgers, Washington University, St Louis) is identical to pBL230-52 except that the vector SalI site has been filled in.

Substrates for Mismatch Repair

The sources of materials for mismatch repair assays and the preparation of M13mp2 DNA heteroduplexes are described in Thomas et al. (1995). Substrates contained mismatches (as indicated in Table 2 and Figures 1 and 2) in the *lacZ*α gene and a nick in the minus strand at nucleotide −264 or +276 (where +1 is the first transcribed nucleotide of the *lacZ*α gene).

Cell Lines and Extracts

The origins of TK6, HeLa-S3, and ZR-75 cells are described in Boyer et al. (1995). Cells were grown in DMEM/F12 with 10% fetal bovine serum, and extracts were prepared as described (Roberts and Kunkel, 1993).

Proteins and Peptides

Purified human PCNA and p21 proteins were generous gifts from Bruce Stillman (Cold Spring Harbor Laboratory, New York). Peptide derivatives of p21 were synthesized by Research Genetics, Inc. (Huntsville, AL). The sequence of the peptide that includes the site of interaction between $p21^{WAF1}$ and PCNA (Warbrick et al., 1995) is KRRQTSMTDFYHSKRRLIFS (amino acids 141–160 of p21, designated PBP for PCNA Binding Protein). Two variant peptides were synthesized, one (PBP-A) with an alanine substituted for the phenylalanine at residue 150 (underlined above) and a second (PBP-J, for "jumbled") having the same amino acid composition as PBP but a different sequence, QDKTRYFHRTMSRSKSIRLF. Where indicated, experiments were performed with biotinylated peptides (PBP_{bio}). Peptides were dissolved in degassed H_2O at 5 or 10 mg/ml and stored at −80°C.

Yeast Two-Hybrid Analysis

The yeast cDNA expression library (generous gift of S. Elledge, Baylor College of Medicine, Houston) and a human lymphocyte cDNA library (Clontech) were screened as described (Hollenberg et al., 1995). Plasmid DNA was isolated (Ward, 1990) from His^+ colonies with detectable β-galactosidase activity (see below). Library plasmids were electroporated into Escherichia coli strain MC1066 and selected by growth on medium lacking leucine. Inserts in library plasmids were analyzed by restriction mapping and sequence inspection. Potential interacting clones isolated in the library screen were retested by a mating assay (Bendixen et al., 1994) or by cotransformation of bait and GAD fusion expressing plasmids (EZ yeast transformation kit, Zymo Research, Orange, CA). In either case, yeast-containing interacting fusions were selected on synthetic medium lacking histidine. Yeast containing noninteracting fusions do not grow in the absence of histidine. β-galactosidase activity was measured for colonies that grew on plates lacking histidine. The presence of β-galactosidase activity was determined in two ways, qualitatively by using a filter assay with X-gal as the substrate (Prolla et al., 1994b), and quantitatively using o-nitrophenyl-1-thio-β-D-galactopyranoside (ONPG) as a substrate. For the quantitative assay, yeast were resuspended in 500 μl of 0.1 M $NaPO_4$, 1 mM $MgSO_4$, .01% SDS, and 0.64 mg/mg o-nitrophenyl-1-thio-β-galactopyranoside (ONPG). Cells were permeablized with a drop of chloroform and incubated at 30°C until a yellow color developed (up to ~2 hr). Following addition of 200 μl of 1 M sodium bicarbonate to stop the reaction, cells were pelleted and β-galactosidase activity in the supernatant was measured as absorbance at 420 nm. To determine the approximate cell number, the yeast pellet was resuspended in one ml of water and the optical density at 600 nm was measured. A unit of activity (Table 1) is defined as $A_{420}/min/A_{600}$. The background β-galactosidase activity is 0.06 ± 0.02 units.

Measurement of Spontaneous Mutation Rates

Yeast strains were isogenic to PY50 (*MAT*α *can1–100ade2–1 lys2–1 ura3–52 leu2–3,112 his3-Δ 200 trp1-Δ901 pol30::hisG* [pBL211 (*POL30 URA3*)]; Ayyagari et al., 1995), except for alterations introduced by transformation. PY50 *POL30* and PY50 *pol30–52* were generated by transformation of PY50 with plasmid pBL230 (*POL30*)

or pBL245–52 (*pol30–52*) and subsequent segregation of plasmid pBL211 by growth on 5-flouro-orotic acid (5-FOA)–containing medium (Boeke et al., 1984). Disruption of *MLH1* in PY50 *POL30* and PY50 *pol30–52* was accomplished by transformation with linearized plasmid *mlh1Δ::LEU2* followed by selection on media lacking leucine. Disruption of the *MLH1* gene was confirmed by PCR with primers that bracket the deletion. The spontaneous frameshift rate was determined substantially as described (Heale and Petes, 1996). Yeast strains containing plasmid pRS313/TA4 are Ura^+ and sensitive to 5-FOA. Alterations in the length of the poly GT tract result in frameshift mutations that inactivate the *URA3* gene fusion and result in resistance to 5-FOA. To determine the rate of tract-length alterations, we plated yeast on omission medium (SD complete lacking tryptophan, leucine, and histidine) to force retention of the plasmids and to derepress the *LEU2* promoter of the *URA3*-fusion gene. Suitable dilutions of resultant colonies were plated on solid medium lacking tryptophan, leucine, and histidine (to determine cell number) and on identical medium containing 5-FOA (to determine the number of Ura^+ cells). The 5-FOA^R colonies were counted after 3 days growth at 30°C. For each rate measurement, the frequencies of 5-FOA^R colonies were measured for 10 colonies, and rate measurements were done for 4 independent transformants of each strain. Frequencies were converted to rates by using the method of the median (Lea and Coulson, 1949).

DNA Replication Fidelity and Mismatch Repair Assays

Procedures for measuring mismatch repair and SV40 replication activities have been described (Thomas et al., 1995; Roberts and Kunkel, 1993). Repair reactions (25 μl) contained 30 mM HEPES (pH 7.8), 7 mM $MgCl_2$, 4 mM ATP, 200 μM each CTP, GTP, and UTP, 100 μM each dATP, dGTP, dTTP, and dCTP, 40 mM creatine phosphate, 100 mg/ml creatine phosphokinase, 15 mM sodium phosphate (pH 7.5), 1 fmol of the indicated heteroduplex DNA, and 50 μg of cell extract protein. After incubating at 37°C for 15 min, reactions were processed and introduced into E. coli NR9162 (*mutS*), which were plated to score plaque colors. Repair efficiency is expressed in percent as 100 × (1 minus the ratio of the percentages of mixed bursts obtained from extract-treated and untreated samples). Replication reactions (25 μl) contained 30 mM HEPES (pH 7.8), 7 mM $MgCl_2$, 4 mM ATP, 200 μM each CTP, GTP, and UTP, 100 μM each dATP, dGTP, dTTP, and dCTP, 40 mM creatine phosphate, 100 mg/ml creatine phosphokinase, 15 mM sodium phosphate (pH 7.5), 40 fmol of SV40 origin–containing M13mp2 DNA, and 50 μg of cell extract protein. After incubating at 37°C for 2 hr, reactions were processed for incorporation as described (Roberts and Kunkel, 1993).

PCNA Restoration of Mismatch Repair Activity to PCNA-depleted Extracts

Biotinylated PBP (10 μg) was bound to 30 μg magnetic-avidin beads (5-micron size) (CPG Inc., NJ) for 1 hr at 4°C. The mixture was washed extensively in degassed dH_2O to remove unbiotinylated PBP and then incubated with an extract of TK6 cells (50 μg protein) for 30 min at 4°C. The extract was then cleared of biotinylated PBP by multiple magnetic separations. Parallel treatment without any peptide was performed as a control. Mismatch repair reactions were performed with a substrate containing a G•G mismatch.

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Note Added in Proof

Our recent analysis indicates that the pol30-52 PCNA mutant and the pol30-52/mlh1 double mutant strains show similar mutational spectra in the simple repeat assay as the mlh1 single mutant strain, i.e., alterations of one or two repeats. This further supports the involvement of PCNA in DNA mismatch repair.