A Ubiquitin-binding Motif in the Translesion DNA Polymerase Rev1 Mediates Its Essential Functional Interaction with Ubiquitinated Proliferating Cell Nuclear Antigen in Response to DNA Damage

Received for publication, March 20, 2007, and in revised form, May 4, 2007 Published, JBC Papers in Press, May 21, 2007, DOI 10.1074/jbc.M702366200

Adam Wood, Parie Garg 1, and Peter M. J. Burgers 2
From the Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110

During normal DNA replication, the proliferating cell nuclear antigen (PCNA) enhances the processivity of DNA polymerases at the replication fork. When DNA damage is encountered, PCNA is monoubiquinated on Lys-164 by the Rad6–Rad18 complex as the initiating step of translesion synthesis. DNA damage bypass by the translesion polymerase Rev1 is enhanced by the presence of ubiquitinated PCNA. Here we have carried out a mutational analysis of Rev1, and we have identified the functional domain in the C terminus of Rev1 that mediates interactions with PCNA. We show that a unique motif within this domain binds the ubiquitin moiety of ubiquitinated PCNA. Point mutations within this ubiquitin-binding motif of Rev1 (L821A,P822A,I825A) abolish its functional interaction with this domain binds the ubiquitin moiety of ubiquitinated PCNA. Point mutations within this ubiquitin-binding motif of Rev1 (L821A,P822A,I825A) abolish its functional interaction with PCNA. We show that a unique motif within this domain binds the ubiquitin moiety of ubiquitinated PCNA. Point mutations within this ubiquitin-binding motif of Rev1 (L821A,P822A,I825A) abolish its functional interaction with ubiquitinated PCNA in vitro and strongly attenuate damage-induced mutagenesis in vivo. Taken together, these studies suggest a specific mechanism by which the interaction between Rev1 and ubiquitinylated PCNA is stabilized during the DNA damage response.

During normal progression of the cell cycle, replicative DNA polymerases are charged with the task of faithfully replicating host DNA. In eukaryotes, the B-family DNA polymerase δ (pol δ) 3 and DNA polymerase ε synthesize the bulk of newly formed DNA (reviewed in Ref. 1). Both DNA polymerases possess a highly restrictive active site to promote proper Watson-Crick base pairing between the template strand and incoming bases (2). However, the restrictive nature of these enzymes also makes it inherently difficult for them to deal with DNA damage in the template DNA, and in general, the presence of DNA damage blocks the progression of the replication fork. This stalling activates one of several post-replication repair mechanisms that are designed to bypass damage in either an error-free or error-prone manner (reviewed in Refs. 3–5). These pathways are initiated by monoubiquitination of the proliferating cell nuclear antigen (PCNA) on Lys-164 by Rad6–Rad18.

During normal replication, PCNA serves as a processivity factor for the replicative DNA polymerases and coordinates the functions of enzymes on the lagging strand that are involved in the maturation of Okazaki fragments (reviewed in Refs. 6, 7). In order to carry out its DNA-associated functions, PCNA is loaded by the clamp loader replication factor C (RFC) in an ATP-dependent reaction (reviewed in Ref. 8). Elegant genetic studies have shown that mono-ubiquitination of PCNA activates translesion synthesis (TLS) by translesion DNA polymerases (9–11). The more open active site of TLS polymerases, particularly those in the Y-family of DNA polymerases, permits bypass of a variety of DNA lesions present on the template DNA (reviewed in Ref. 12).

TLS in yeast consists of two branches, both of which require mono-ubiquitinated PCNA (PCNA Ub 1 ) for function. Bypass of UV damage, particularly cis-syn pyrimidine dimers, is mediated by pol η, the xeroderma pigmentosum variant DNA polymerase (13, 14). In mammalian cells, pol η specifically interacts with PCNA Ub 1 at sites of damage (15, 16). Although unmodified PCNA has been shown to serve as a cofactor for damage bypass by pol η, ubiquitination of PCNA increases the rate of bypass in vitro (17, 18).

TLS of most forms of DNA damage involves the participation of three DNA polymerases, pol δ, pol ζ, and Rev1, and may require additional activation by the Cdc7/Dbf4 protein kinase that normally functions in cell cycle progression (12, 19). This pathway is largely responsible for DNA damage-induced mutagenesis in eukaryotic cells. However, spontaneous mutagenesis and mutagenesis resulting from defects in the replication machinery is also largely dependent on this pathway (20, 21). Several models for TLS of damage have been proposed in which one DNA polymerase carries out the insertion step across the lesion, and a second polymerase extends from the lesion (22, 23).

Although pol δ is actually a high fidelity DNA polymerase, its requirement for mutagenesis follows from genetic studies of its third subunit pol 32 (24, 25). pol ζ is an error-prone DNA polymerase that can bypass damage (26). In vitro, the enzyme...
shows a high frequency of misincorporation and a high propensity to extend mismatched template-primer termini, making it the most error-prone B-family DNA polymerase (27–29). The third required polymerase is the Rev1 deoxycytidyltransferase. Rev1 shows the highest catalytic activity opposite template guanines and abasic sites (30, 31). This enzyme is primarily responsible for inserting dC residues opposite abasic sites during mutagenesis (25, 32, 33).

Although the requirement for PCNA mono-ubiquitination in damage-induced mutagenesis follows conclusively from genetic studies, the exact role of PCNA\textsuperscript{Ubi} remains to be established. All three DNA polymerases required for mutagenesis (pol\textsubscript{δ}, pol\textsubscript{ζ}, and Rev1) show interactions with unmodified PCNA, but only in the case of Rev1 have we been able to detect altered interactions upon ubiquitination of PCNA (18). PCNA and PCNA\textsuperscript{Ubi} show identical properties with regard to loading by RFC, in stimulating processive DNA synthesis by pol\textsubscript{δ} and by pol\textsubscript{ε}, and in coordinating Okazaki fragment maturation by pol\textsubscript{δ}, the flap endonuclease FEN1, and DNA ligase I (18). Translesion synthesis by pol\textsubscript{ζ} is stimulated by PCNA via an unknown motif (34). This interaction is likely important for mutagenesis as PCNA mutants deficient for functional interactions with pol\textsubscript{ζ} are also defective for mutagenesis (21). Surprisingly, however, ubiquitination of PCNA does not alter its functional interactions with pol\textsubscript{ζ} (18).

Recently, we identified Rev1 as a possible target for PCNA\textsuperscript{Ubi} in mutagenesis (18). PCNA\textsuperscript{Ubi} stimulates TLS by Rev1 more efficiently than PCNA does. Our model suggests that ubiquitination of PCNA may serve to localize Rev1 to stalled replication forks, which in turn recruits other components of the TLS machinery. This recruitment is likely mediated through the multiple interactions that Rev1 shows with TLS DNA polymerases, including pol\textsubscript{ζ} (35, 36). However, following a similar biochemical approach to ours, others have failed to detect specialized interactions between PCNA\textsuperscript{Ubi} and Rev1 (37). Therefore, it is important to establish both a genetic and biochemical correlation between PCNA ubiquitination and Rev1 function.

Recent studies have shown that several Y-family DNA polymerases contain a conserved ubiquitin-binding motif (UBM), and these UBM motifs contribute to increased binding of the polymerases to artificial linear ubiquitin-PCNA fusion proteins (38, 39). However, whether these UBM motifs are important for functional interaction with the physiologically relevant form of PCNA, i.e. with ubiquitin attached to Lys-164 of PCNA, remains to be determined. Yeast Rev1 contains at least two sequences that mirror these conserved UBM\textsubscript{S}. In order to determine whether these motifs play a role in the interaction of Rev1 with PCNA\textsuperscript{Ubi}, and whether this interaction is physiologically relevant for mutagenesis, we have generated a series of Rev1 deletion and point mutants that either delete or mutate these motifs. By measuring on one hand the ability of these Rev1 mutants to physically and functionally interact with PCNA\textsuperscript{versus} PCNA\textsuperscript{Ubi}, and on the other hand to promote mutagenesis in vivo, we conclude that a single ubiquitin-binding motif is responsible for regulating the interactions between Rev1 and PCNA\textsuperscript{Ubi}. This motif is close to or embedded in a domain of Rev1 that mediates basic interactions with PCNA regardless of its ubiquitination state.

**Experimental Procedures**

**DNA Substrates**—All oligonucleotide substrates were prepared as described (34). The linear oligonucleotide templates contained a biotin at both ends. They are as follows: V9, 5’-Bio-CCTTGGCAATTCCT\textsubscript{25}GGGCTCCCTTCTCTCCTCCCTCCTCCCTCCT\textsubscript{30}-Bio; V9AP1, 5’-Bio-CCTTGGCAATTCCT\textsubscript{25}GGC0CTCCCTTCTTCTCTTCTCCCTCTCCTCCCTCCT\textsubscript{30}-Bio; and V9AP2 (where 0 indicates a tetrahydrofuran moiety), 5’-Bio-CCTTGGCAATTCCT\textsubscript{25}GGC0CTCCCTTCTTCTCTTCTCCCTCTCCTCCT\textsubscript{30}-Bio. Primer C12 (5’-AGGGAAGGGAGGAGGGAGGAAAAGGAG) was 5’-32P-labeled and hybridized to the templates followed by addition of a 2-fold molar excess of streptavidin to block the biotin ends.

**Enzymes**—Replication protein A (RP-A), RFC lacking the N-terminal domain of Rfc1 (RFC-1A273), and PCNA were purified as described previously (34, 40, 41). Overexpression and purification of Uba1, Rad6–Rad18, and Rev1 were as described (42). PCNA\textsuperscript{Ubi} was prepared in an *in vitro* ubiquitination reaction, as described, and purified by nickel-agarose chromatography, followed by purification over a MiniQ column (18). The MiniQ column separates PCNA\textsuperscript{Ubi} from unmodified PCNA and from partially co-migrating Rad6– Rad18, and two or three successive MiniQ purification steps were often required to remove all Rad6–Rad18. The resulting preparations contained in general 5–20% unmodified PCNA.

**Rev1 Mutants**—Point mutations in *REV1* were made in plasmid pJN60 (2 μm ori GAL1-10 URA3 GST-REV1) by PCR mutagenesis (30). C-terminal truncation mutants were made in the same fashion by inserting stop codons by PCR mutagenesis at the sites of truncation. The desired mutant was verified by sequencing and subcloning of the relevant (mutant) section of the gene. This series of mutant plasmids was named pBL822-x. Mutants were transferred from pBL822-x to series pBL820-x plasmids (pRS315-based: Bluescript SKII\textsuperscript{+} LEU2 CEN6 ARSH4 rev1-x) for genetic analysis of rev1-x mutants. Plasmids and sequences are available from authors upon request.

**Purification of Rev1 and Rev1 Mutants**—Yeast strain BJ2168 (MATa ura3-52 trp1-289 leu2-3,112 prb1-1122 prc1-407 pep4-3) was transformed with pBL820-x, and transformants were grown on selective glycerol-lactate media and induced with galactose as described (43). The cells (~40 g) were lysed by blending in ice with 20 ml of 3X lysis buffer (1X lysis buffer = 25 mM potassium phosphate, pH 7.8, 1 mM EDTA, 5% glycerol, 5% ethylene glycol, 1 mM dithiothreitol, 10 mM NaHSO\textsubscript{4}, 10 μM pepstatin A, 10 μM leupeptin, 0.5 mM phenylmethylsulfonyl fluoride). After thawing of the lysate, 5 mM NaCl was added to a final concentration of 1 M and 10% Triton X-100 to a final concentration of 1%. All subsequent steps were at 0–4 °C. The lysate was spun for 30 min at 18,000 rpm in a Sorvall SS34 rotor. The supernatant was poured in a clean tube and spun again for 30 min at 18,000 rpm. The supernatant was incubated by gentle rocking motion for 2 h to 1 ml of glutathione-Sepharose, equilibrated in buffer B\textsubscript{1000} (B\textsubscript{0} + 1000 mM NaCl; B\textsubscript{0} = 50 mM potassium phosphate, pH 7.2, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 10 mM NaHSO\textsubscript{4}, 10 mM pepstatin A, 10 μM leupeptin, 0.5 mM phenylmethylsulfonyl fluoro-
Interaction of Rev1 with Ubiquitinated PCNA

ride, 1% Triton X-100). The beads were loaded in a 10-ml column and washed at 1 ml/min with 100 ml of buffer B500, followed by 10 ml of B500 + 5 mM magnesium acetate + 1 mM ATP, followed by 10 ml of B100. The Mg-ATP wash removes contaminating Ssa1 heat shock protein. The column was eluted at 0.1 ml/min with 5 ml of B300 containing 20 mM glutathione at pH of 8.0. Fractions containing GST-Rev1 were treated overnight at 4 °C with 80 units of thrombin (Sigma). The digest was diluted with 2 volumes of buffer B100 and fractionated over a 1 ml MonoS column (GE Healthcare) using a 15-ml gradient of B100 to B500 for elution. Pure Rev1 eluted at ~200–250 mM NaCl. Purification of the Rev1 mutants proceeded similarly.

Ubiquitination of °32P-PCNA—PCNA containing an N-terminal phosphorylatable tag (MRRASVGSS-PCNA) was °32P-labeled by phosphorylation with cyclic AMP-dependent protein kinase (catalytic subunit; New England Biolabs) and [γ-°32P]ATP and purified as described (40). Ubiquitination was carried out in a 300-μl reaction containing 25 mM HEPES, pH 7.6, 50 mM NaCl, 0.1 mg/ml bovine serum albumin, 1.5 mM ATP, 8 mM MgAc2, 600 fmol of deca-primed SKII single-stranded DNA (6 pmol of template-primer termini), 60 pmol of RPA, 4 pmol of RFC, 10 pmol of Rad6–Rad18, 20 pmol of Ubα1, 20 pmol of His6-ubiquitin, and 3 pmol of °32P-PCNA, and the reactions were incubated for 1 h at 30 °C. An identical control reaction was set up, but lacking ubiquitin. After incubation, EDTA was added to 5 mM and dithiothreitol to 10 mM final concentration, and incubation was continued for 15 min to discharge ubiquitin-activating enzyme and ubiquitin carrier protein intermediates. The reaction was directly loaded onto a 0.8-ml MiniQ column and °32P-PCNA and °32P-PCNA°ubi eluted with a linear gradient from 100 to 500 mM NaCl. These partially purified preparations were used in the Rev1 binding assays.

In Vitro Binding Assays—Binding studies of Rev1 (mutants) with PCNA and PCNA°ubi were performed in 200-μl reactions containing 20 mM HEPES, pH 7.6, 75 mM NaCl, 1% glycerol, 1 mM EDTA, 8 mM magnesium acetate, 0.01% Triton X-100, and PCNA or PCNA°ubi was loaded by RFC prior to pre-loading the clamp. Reactions were then started by the addition of 100 fmol of Rev1 or mutant Rev1 as indicated and allowed to proceed for the indicated time. Products were resolved on a 10% polyacrylamide, 7 M urea gel and quantitated using a Storm PhosphorImager.

UV-induced Mutagenesis—The pBL820-based Rev1 plasmids were transformed into strain into strain SFY-1 (MATα arg4-17 his3Δ1 leu2-3 trp1Δ ura3-52 rev1Δ::hisG) obtained from Christopher Lawrence (Rochester University). Transformants were grown for 2 days to saturation in selective minimal media. The cells were washed with sterile water and either 106, 107, or 108 cells plated in patches on selective minimal media lacking arginine. The plates were either not irradiated or irradiated with 20 J/m2 of UV254 and immediately incubated at 30 °C in the dark. Plates were photographed after 3 days. The same series of plasmids was also transformed into strain pY201 (MATα his3Δ1 leu2-3 trp1Δ ura3-52 rev1Δ::hisG) and grown similarly. Serial dilutions were plated on selective plates (for the plasmid) with or without 80 μg/ml canavanine and irradiated with 0, 10, 20, or 30 J/m2 of UV light. Survival was measured on the plates without canavanine, spontaneous frequencies to canavanine resistance on unirradiated plates, and UV-induced frequencies to canavanine resistance on irradiated canavanine plates. Colonies appearing after 3 days of growth at 30 °C were counted.

RESULTS

Salt Sensitivity of the Rev1 Assay—We have previously observed that Rev1 activity is stimulated by PCNA and hyper-stimulated by PCNA°ubi. However, another group did not observe stimulation of Rev1 activity by PCNA nor by PCNA°ubi (37). In order to understand the variabilities in the assay that might produce such disparate results, we measured Rev1 activity with or without clamp as a function of the salt concentration. The 101-nucleotide-long oligonucleotide template contained biotin residues at both ends. The template was primed in the middle with a 30-mer primer, and the template ends were blocked with streptavidin moieties. After coating of the single-stranded template regions with the single-stranded binding protein RPA, PCNA or PCNA°ubi was loaded by RFC prior to initiating DNA synthesis at t = 0 by addition of Rev1. The terminal biotin-streptavidin anchors are important in preventing the loaded PCNA from sliding off the DNA (34). In the assay in Fig. 1, the primer was positioned such on the template that Rev1 has to insert one dCMP opposite a template guanine prior to encountering the abasic site (Fig. 1A).

Remarkably, at 75 mM NaCl Rev1 is proficient in abasic site bypass even in the absence of PCNA, and no further stimulation of Rev1 activity by the addition of PCNA was observed (Fig. 1, B and C). We attribute this lack of stimulation to the prolonged presence of the clamp loader RFC on the template, preventing association of Rev1 with PCNA or possibly even with the primer terminus. Such competition between the clamp loader and a DNA polymerase for interaction with the clamp has been well documented in Escherichia coli and also exists in eukaryotes (44, 45). In order to reduce interference by RFC for Rev1-PCNA-DNA complex formation, we have taken a combination of two approaches. First, in our studies we use a form of
RFC, designated RFC-1Δ273, that lacks an N-terminal DNA binding domain of Rfc1 that is dispensable for loading in vitro and in vivo (41). RFC-1Δ273, although perfectly proficient for clamp loading, shows reduced interactions with the DNA thereby decreasing interference. Second, at higher salt concentrations, RFC is very rapidly dissociated from the DNA-PCNA complex after completion of loading (46). Consequently, at higher salt levels Rev1 could be properly recruited to the DNA-PCNA complex, and a strong stimulation by PCNA and hyperstimulation by PCNAUbi were observed (Fig. 1, panel wt, lanes 1–6). PCNA stimulated Rev1 activity 6-fold, whereas PCNAUbi caused a 15-fold stimulation. At 100 mM NaCl, we could easily and reproducibly detect basal activity by Rev1 alone, as well as stimulation by PCNA and hyper-stimulation by PCNAUbi. Therefore, we have used 100 mM NaCl throughout the remainder of our lesion bypass experiments.

**Design of Mutants for Rev1 Interaction Analysis**—In order to determine which domains of Rev1 may play a role in mediating its interaction with ubiquitinated PCNA, we systematically mutated several functional domains within Rev1, some of which have previously been shown to affect DNA damage-induced mutagenesis. One of these domains is a BRCT domain that is required for mutagenesis. The rev1-1 mutant contains a G193R mutation in a conserved hairpin turn in the BRCT domain; the mutant is defective for mutagenesis, and the mutant Rev1-1 protein has reduced polymerase activity (47). Recent studies have shown that Y-family DNA polymerases harbor highly conserved UBM containing an invariant Leu-Pro within the sequence (38). We identified two potential motifs in Rev1 that may serve this same function (UBM-1 and UBM-2, shown in Fig. 2A). We used triple alanine substitutions to generate point mutations within these two domains that we refer to as rev1-11 (UBM-1 mutant; L809A,P810A,M813A) and rev1-12 (UBM-2 mutant; L821A,P822A,I825A) (Fig. 2A). In addition, we made two C-terminal truncation mutants based upon multiple sequence alignment analysis of 14 divergent sequences (48). The truncations were made between blocks of conserved sequences. Truncation mutant rev1-5 (Δ866–985) retains the two putative UBM motifs and a proposed PCNA interaction motif (see below) but removes the proposed pol ζ interaction motif, based upon studies with mouse Rev1 (36). A more extensive truncation mutant, rev1-4 (Δ780–985), removes both UBMs and the proposed C-terminal PCNA interaction motif.

Conflicting data exist with regard to the domain of Rev1 that binds PCNA. In one study, the PCNA-binding domain of human Rev1 was mapped, by two-hybrid analysis, to amino acids 923–1047, corresponding approximately to yeast amino acids 775–845. Therefore, the PCNA-binding domain proposed by Sale and co-workers (49) should be retained in Rev1-5 but not in the Rev1-4 truncation mutant. However, based upon two-hybrid analysis and pull-down assays, Guo et al. (50) concluded that PCNA binding is mediated by an N-terminal 240-amino acid fragment of Rev1 that also contains a BRCT domain, and binding was abrogated when the BRCT domain contained a glycine → arginine mutation analogous to the mutation in yeast rev1-1 that is defective for mutagenesis.

Finally, we generated a catalytic null mutant of Rev1, rev1-3 (Y319A,F320A) with mutations in a β-strand that enters the active site of the enzyme (51). Rev1-3 shows no detectable polymerase activity with or without the clamp present (data not shown). All mutants were stably expressed in yeast and were purified to homogeneity from a yeast overexpression system (data not shown).

**A Conserved UBM within Rev1 Mediates Stimulation by PCNAUbi in Vitro**—Using our collection of Rev1 mutants, we investigated which mutants were unable to be stimulated by PCNA or ubiquitinated PCNA. We used two types of template-primers. The first template-primer (V9AP1/C12) has a model abasic site positioned directly behind the primer terminus. This “standing start” assay likely reflects the substrate encountered in vivo when replicative polymerases stall at an abasic site. The rate of dCMP insertion at the abasic site by wild type Rev1 is stimulated by PCNA and hyper-stimulated by PCNAUbi (Fig. 2C, panel wt, lanes 1–6). The second substrate has a GCGG template sequence downstream of the primer terminus. Guanines are the preferred template residues for replication by Rev1 (31), and indeed, Rev1 alone replicated only the first two template G positions (Fig. 2C, panel wt, lanes 7, 10, and 13). However, addition of PCNAUbi, but not PCNA, stimulated Rev1 to replicate the template C residue and proceed to the next template G residue (Fig. 2C, lanes 9, 12, and 15). Therefore, the detection of these extended replication products is diagnostic for PCNAUbi function.

Removal of the C-terminal 120 amino acids of Rev1 (Rev1-5) did not affect its basic catalytic properties nor its stimulation by
PCNA and hyper-stimulation by PCNA\textsuperscript{Ubi} on either template-primer (Fig. 2C, compare panel wt with panel 5). However, deletion of an additional 86 amino acids while not affecting the basal activity of Rev1, completely eliminated the ability of the mutant Rev1-4 protein to be stimulated by PCNA or PCNA\textsuperscript{Ubi} (Fig. 2C, panel 4 versus panel 5). These data suggest that both the functional PCNA-binding motif and the functional ubiquitin interaction motifs are wholly or partially located in the 780–865 domain of Rev1. This region contains the two putative UBMs. We next investigated the properties of Rev1-11 and Rev1-12 with point mutations in each of these UBMs.

Although Rev1-11, the UBM-1 mutant, displayed abasic site bypass activities similar to those of wild type, the UBM-2 mutant Rev1-12 still maintained the ability to be stimulated by PCNA, but hyper-stimulation by PCNA\textsuperscript{Ubi} was abolished (Fig. 2C, panel 11 versus panel 12; also compare F with D and E). Thus, mutations within the UBM-2 motif render Rev1 insensitive to the presence of the ubiquitination moiety on PCNA, and this UBM may serve to bind and recognize this specific modification of PCNA.

Mutation rev1-1 within the BRCT domain of Rev1 has been shown previously to affect its polymerase activity (47). Consistent with this, the activity of Rev1-1 at an abasic site was only about 20% that of wild type (Fig. 2G). However, Rev1-1 activity was stimulated by PCNA and hyper-stimulated by PCNA\textsuperscript{Ubi} indicating that functional interactions with these two forms of the clamp were preserved in the mutant.

**UBM-2 Enhances Binding of Rev1 to PCNA\textsuperscript{Ubi}**—In order to probe for direct interactions between Rev1 and the clamps, GST-Rev1 was incubated with \(^{32}\)P-labeled PCNA and PCNA\textsuperscript{Ubi}, and binding was observed after affinity capture of GST-Rev1 on glutathione beads and separation of bound proteins by SDS-PAGE, followed by PhosphorImager analysis (Fig. 3).

Wild type Rev1 has a strong preference for PCNA\textsuperscript{Ubi} compared with unmodified PCNA (Fig. 3, lane 5), and the small truncation mutant Rev1-5 also showed wild type-like binding properties (lane 8). Importantly, the larger truncation mutant Rev1-4 failed to bind PCNA\textsuperscript{Ubi} preferentially over PCNA (Fig. 3, compare lanes 7 and 8). However, in several experiments, PCNA binding by this mutant was significantly higher than background, indicating residual PCNA binding in the remaining part of Rev1 (Fig. 3, compare lanes 4 and 7). The UBM-1 mutant Rev1-11, which showed no defect in stimulated bypass synthesis, also displayed a strong binding preference of evaluated for wild type (wt) Rev1, and the mutants are shown. Reactions were carried out for the indicated times. U indicates PCNA\textsuperscript{Ubi}. C–E, using template-primer V9AP1/C12, time course assays were performed to determine the initial activities of wild type Rev1 (D), UBM-1 mutant Rev1-11 (E), and UBM-2 mutants Rev1-12 (F). G, activity of rev1-1 in DNA damage bypass. Reaction times were extended to 8 min; the BRCT domain mutant is much less efficient at insertion across the abasic site in template V9AP1.
PCNA\textsuperscript{Ubi} over PCNA. In contrast, the UBM-2 mutant Rev1-12 that was defective for hyper-stimulation by PCNA\textsuperscript{Ubi} also was defective for preferential binding of PCNA\textsuperscript{Ubi}. However, binding of PCNA was comparable to wild type and significantly higher than background (Fig. 3, compare lanes 4 and 10). Interestingly, the BRCT domain mutant Rev1-1 still maintains preferential binding to PCNA\textsuperscript{Ubi}, although it does bind both PCNA and PCNA\textsuperscript{Ubi} less efficiently than wild type Rev1 does (Fig. 3, lane 6).

**Damage-induced Mutagenesis Is Linked to PCNA\textsuperscript{Ubi} Binding by Rev1**—We used two genetic targets to measure the role of the various Rev1 domains in mutagenesis. The ochre allele arg4-17 has been used extensively to study Rev1 function in mutagenesis, and mutations in REV1 reduced UV-induced reversion of arg4-17 by as much as 1000-fold (52). In contrast, the forward mutation assay to canavanine resistance samples a wide variety of alterations that cause inactivation of the CAN1 gene. A patch plating assay was used to measure arg4-17 reversion in response to 20 J/m\textsuperscript{2} of UV\textsubscript{254} treatment (Fig. 4A). As expected from previous studies, the catalytic null mutant (rev1-3) was proficient for UV mutagenesis (49, 53). However, both truncation mutants were defective for mutagenesis, as was the original rev1-1 allele with a mutation in the BRCT domain (52). Interestingly, mutations in the UBM-1 domain that did not disrupt

**FIGURE 3.** Binding of PCNA and PCNA\textsuperscript{Ubi} to Rev1 mutants. A, 12 fmol each of \textsuperscript{32}P-PCNA and \textsuperscript{32}P-PCNA\textsuperscript{Ubi} were preincubated with 1 pmol of GST-Rev1 as described under “Experimental Procedures.” Bound proteins were affinity-purified using glutathione-Sepharose, and the bound fractions were resolved by SDS-PAGE and visualized using a Storm PhosphorImager. Lanes 1 and 2 show the input PCNA and PCNA\textsuperscript{Ubi}, and lane 3 shows 10% of a typical binding reaction before addition of beads. Lane 4 contained no GST-Rev1. B, quantitation of the data for the negative control (left two bars, no Rev1, lane 3 in A) and wild type (wt) or the indicated mutants. The error bars show the standard deviations for three independent experiments. The PCNA\textsuperscript{Ubi} preparation contained 12% unmodified PCNA. Since in vitro ubiquitination of PCNA monomers is random, to a first approximation the preparation contains 88% of trimers containing two ubiquitinated monomers and one unmodified PCNA. These mixed trimers are expected to bind Rev1 as avidly as fully ubiquitinated trimers, and therefore, a correction was applied to the measured PCNA signal in order to subtract the signal originating from binding of mixed trimers of PCNA\textsuperscript{Ubi}.

**FIGURE 4.** Ability of REV1 mutants to support DNA damage-induced mutagenesis. A, SFY1-derived strains (rev1-3 arg4-17) containing control vector or plasmid containing wild type REV1 or the indicated mutant were plated at the indicated densities onto plates lacking arginine. Cells were then grown without damage or after irradiation with 20 J/m\textsuperscript{2} of UV\textsubscript{254}, and growth after 3 days was recorded. Survival was >30% for all strains (not shown). B and C, survival rates (B) and survival-corrected mutation frequencies (C) in PY201-derived strains (rev1-3 CAN1\textsuperscript{Ubi}) containing control vector or plasmid containing wild type REV1 or the indicated mutant. See “Experimental Procedures” for details.
Interaction of Rev1 with Ubiquitinated PCNA

physical and functional interactions also did not affect mutagenesis. However, allele rev1-12 with triple point mutations in UBM-2 that do disrupt these interactions was defective for UV mutagenesis. These phenotypes were mirrored in the canavanine resistance assay, although in this assay residual UV mutagenesis was observed in the UBM-2 mutant rev1-12 (Fig. 4C). Consistent with these results, mutants rev1-3 and rev1-11 that were proficient for mutagenesis were also the only mutants that showed UV resistance comparable with wild type (Fig. 4B).

DISCUSSION

Two studies have recently been published (38, 39) that investigate the importance of the UBM of Y-family DNA polymerases for their function in vivo, and correlate these genetic results with in vitro interaction studies with ubiquitinated PCNA. However, the in vitro studies were performed with a chimeric protein that contains ubiquitin fused to the N terminus of PCNA. Surprisingly, such an artificial ubiquitin-PCNA fusion protein displayed increased physical interactions, compared with PCNA alone, with several Y-family enzymes, including Rev1. However, it remained to be established whether the physiologically relevant form of PCNA 

addition, the Rev1-PCNA 

interaction studies are in accord as well; the mammalian Rev1 UBMs from extracts from UV-irradiated cells (39). Our results with purified proteins show that this observed defect in extracts can be attributed to a large decrease in binding between PCNA 

and the UBM-2 mutant Rev1-12, although the residual interaction with PCNA remains.

The studies with the other mutants support our conclusions. The Rev1-5 mutant with a small C-terminal truncation retains both UBM-2 as well as the PCNA-binding domain based upon mapping studies with human Rev1 (49) and was therefore expected to show no defects in vitro. This was indeed observed. However, this mutant lacks the interaction domain with pol ζ (36). Therefore, this interaction appears to be essential for DNA damage-induced mutagenesis to function in yeast. The larger truncation, Rev1-4, eliminates both UBMs and the enhanced binding to PCNA 

Curiously, a significant binding to PCNA remains in this mutant even though this binding shows no functional relevance (compare Fig. 3A, lane 4, with 7).

In a recent study, Haracska et al. (37) reported that they were unable to reproduce our earlier study of the functional interactions of Rev1 with PCNA and PCNA 

they neither observed stimulation of Rev1 by PCNA nor by PCNA 

and in addition did not detect binding of Rev1 to PCNA or PCNA 

It is unlikely that this difference can be attributed to the assay conditions, which were quite similar between the two studies. Moreover, minor differences in salt concentrations between 75 and 150 mM NaCl (Fig. 1). One possible reason for the discrepancy is that Haracska et al. (37) used human rather than yeast ubiquitin to monoubiquitinate yeast PCNA. There are only three amino acid changes between yeast and human ubiquitin, and separate mutations at each of these positions that change the yeast to the human sequence do not affect yeast viability (54). However, whether these changes still sustain wild type DNA repair and mutagenesis capacity to our knowledge has not been determined.

A recent study suggested that ubiquitin binding to Rev1 may be mediated in part through its N-terminal BRCT domain (50). Indeed, we also observed decreased binding of the BRCT mutant Rev1-1 to PCNA and to PCNA 

However, Rev1-1 was also partially defective for DNA polymerase activity (Fig. 2G) (47). Interestingly, the mutant polymerase was still stimulated by PCNA and hyper-stimulated by PCNA 

suggesting that functional stimulation by the ubiquitinated clamp remained. One possibility suggested by these diverse results is that the BRCT domain participates in stabilizing interactions with both the polymerase and the C-terminal domain. In the Rev1-1 mutant in which the BRCT domain is likely unfolded, destabilization of the other domains may result in a general dysfunction of all activities of the protein. Whether this general dysfunction is so severe to cause the known complete defect in mutagenesis in vivo, or whether the BRCT domain in addition shows specific essential interactions with other factors in mutagenesis remains to be determined.
Acknowledgments—We thank John Majors for critical discussions during the progress of this work and Carrie Stith for invaluable technical assistance.

REFERENCES

1. Garg, P., and Burgers, P. (2005) Crit. Rev. Biochem. Mol. Biol. 40, 115–128
2. Franklin, M. C., Wang, J., and Steitz, T. A. (2001) Cell 105, 657–667
3. Broomefield, S., Hryciw, T., and Xiao, W. (2001) Mutat. Res. 486, 167–184
4. Lehmann, A. R. (2003) Cell Cycle 2, 300–302
5. Hocegger, H., Sonoda, E., and Takeda, S. (2004) BioEssays 26, 151–158
6. Kao, H. I., and Bambara, R. A. (2003) Crit. Rev. Biochem. Mol. Biol. 38, 433–452
7. Garg, P., and Burgers, P. M. (2005) Cell Cycle 4, 221–224
8. Majka, J., and Burgers, P. M. (2004) Prog. Nucleic Acids Res. Mol. Biol. 78, 227–260
9. Hoege, C., Pfander, B., Moldovan, G. L., Pyrowolakis, G., and Jentsch, S. (2002) Nature 419, 135–141
10. Stelter, P., and Ulrich, H. D. (2003) Nature 425, 188–191
11. Haracska, L., Torres-Ramos, C. A., Johnson, R. E., Prakash, S., and Prakash, L. (2004) Mol. Cell. Biol. 24, 4267–4274
12. Prakash, S., Johnson, R. E., and Prakash, L. (2005) Annu. Rev. Biochem. 74, 317–353
13. Johnson, R. E., Prakash, S., and Prakash, L. (1999) Science 283, 1001–1004
14. Masutani, C., Araki, M., Yamada, A., Kusumoto, R., Nogimori, T., Maekawa, T., Iwai, S., and Hanaoka, F. (1999) EMBO J. 18, 3491–3501
15. Kannouche, P. L., Wing, J., and Lehmann, A. R. (2004) Mol. Cell 14, 491–500
16. Watanabe, K., Tateishi, S., Kawasuji, M., Tsurimoto, T., Inoue, H., and Yamaitzumi, M. (2004) EMBO J. 23, 3886–3896
17. Haracska, L., Kondratchick, C. M., Unk, I., Prakash, S., and Prakash, L. (2001) Mol. Cell 8, 407–415
18. Garg, P., and Burgers, P. M. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 18361–18366
19. Pessoa-Brandao, L., and Sclafani, R. A. (2004) Genetics 167, 1597–1610
20. Quab, S. K., von Borstel, R. C., and Hastings, P. J. (1980) Genetics 96, 819–839
21. Northam, M. R., Garg, P., Baitin, D. M., Burgers, P. M., and Scherbakova, P. V. (2006) EMBO J. 25, 4316–4325
22. Prakash, S., and Prakash, L. (2002) Genes Dev. 16, 1872–1883
23. Friedberg, E. C., Lehmann, A. R., and Fuchs, R. P. (2005) Mol. Cell 18, 499–505
24. Gerik, K. J., Li, X., Pautz, A., and Burgers, P. M. (1998) J. Biol. Chem. 273, 19747–19755
25. Gibbs, P. E., McDonald, J., Woodgate, R., and Lawrence, C. W. (2005) Genetics 169, 575–582
26. Nelson, J. R., Lawrence, C. W., and Hinkle, D. C. (1996) Science 272, 1646–1649
27. Johnson, R. E., Washington, M. T., Haracska, L., Prakash, S., and Prakash, L. (2000) Nature 406, 1015–1019
28. Haracska, L., Prakash, S., and Prakash, L. (2003) Mol. Cell. Biol. 23, 1453–1459
29. Zhong, X., Garg, P., Stith, C. M., McElhinny, S. A., Kissing, G. E., Burgers, P. M., and Kunkel, T. A. (2006) Nucleic Acids Res. 34, 4731–4742
30. Nelson, J. R., Lawrence, C. W., and Hinkle, D. C. (1996) Nature 382, 729–731
31. Haracska, L., Prakash, S., and Prakash, L. (2002) J. Biol. Chem. 277, 15546–15551
32. Zhao, B., Xie, Z., Shen, H., and Wang, Z. (2004) Nucleic Acids Res. 32, 3984–3994
33. Otsuka, C., Kunitomi, N., Iwai, S., Loakes, D., and Negishi, K. (2005) Mutat. Res. 578, 79–87
34. Garg, P., Stith, C. M., Majka, J., and Burgers, P. M. (2005) J. Biol. Chem. 280, 23446–23450
35. Murakumo, Y., Ogura, Y., Ishii, H., Numata, S., Ichihara, M., Croce, C. M., Fishel, R., and Takahashi, M. (2001) J. Biol. Chem. 276, 35644–35651
36. Guo, C., Fischhaber, P. L., Luk-Paszyc, M. J., Masuda, Y., Zhou, J., Kamiya, K., Kiser, C., and Friedman, E. C. (2003) EMBO J. 22, 6621–6630
37. Haracska, L., Unk, I., Prakash, L., and Prakash, S. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 6477–6482
38. Bienko, M., Green, C. M., Crosetto, N., Rudolf, F., Zapat, G., Coull, B., Kannouche, P., Wider, G., Peter, M., Lehmann, A. R., Hofmann, K., and Dikic, I. (2005) Science 310, 1821–1824
39. Guo, C., Tang, T. S., Bienko, M., Parker, J. L., Bielen, A. B., Sonoda, E., Takeda, S., Ulrich, H. D., Dikic, I., and Friedman, E. C. (2006) Mol. Cell. Biol. 26, 8892–8900
40. Eisenberg, J. C., Ayyagari, R., Gomes, X. V., and Burgers, P. (1997) Mol. Cell. Biol. 17, 6367–6378
41. Gomes, X. V., Gary, S. L., and Burgers, P. M. (2000) J. Biol. Chem. 275, 14451–14459
42. Bailly, V., Lauder, S., Prakash, S., and Prakash, L. (1997) J. Biol. Chem. 272, 23360–23365
43. Bylund, G. O., Majka, J., and Burgers, P. M. (2006) Methods Enzymol. 409, 1–11
44. Naktinis, V., Turner, I., and O’Donnell, M. (1996) Cell 84, 137–145
45. Yuzhakov, A., Kelman, Z., Hurwitz, J., and O’Donnell, M. (1999) EMBO J. 18, 5189–5199
46. Gomes, X. V., and Burgers, P. M. (2001) J. Biol. Chem. 276, 34768–34775
47. Nelson, J. R., Gibbs, P. E., Nowicka, A. M., Hinkle, D. C., and Lawrence, C. W. (2000) Mol. Microbiol. 37, 549–554
48. Edgar, R. C. (2004) Nucleic Acids Res. 32, 1792–1797
49. Ross, A. L., Simpson, L. J., and Sale, J. E. (2005) Nucleic Acids Res. 33, 1280–1289
50. Guo, C., Sonoda, E., Tang, T. S., Parker, J. L., Bielen, A. B., Takeda, S., Ulrich, H. D., and Friedman, E. C. (2006) Mol. Cell 23, 265–271
51. Nair, D. T., Johnson, R. E., Prakash, S., Prakash, S., and Aggarwal, A. K. (2005) Science 309, 2219–2222
52. Lemontt, J. F. (1971) Genetics 68, 21–33
53. Haracska, L., Unk, I., Johnson, R. E., Johansson, E., Burgers, P. M., Prakash, S., and Prakash, L. (2001) Genes Dev. 15, 945–954
54. Sloper-Mould, K. E., Jemc, J. C., Pickart, C. M., and Hicke, L. (2001) J. Biol. Chem. 276, 30483–30489