Ubiquitin mediates the physical and functional interaction between human DNA polymerases η and ı

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ABSTRACT

Human DNA polymerases η and ı are best characterized for their ability to facilitate translesion DNA synthesis (TLS). Both polymerases (pols) co-localize in ‘replication factories’ in vivo after cells are exposed to ultraviolet light and this co-localization is mediated through a physical interaction between the two TLS pols. We have mapped the polı-ı interacting region to their respective ubiquitin-binding domains (UBZ in polı and UBM1 and UBM2 in polı), and demonstrate that ubiquitination of either TLS polymerase is a prerequisite for their physical and functional interaction. Importantly, while monoubiquitination of polı precludes its ability to interact with proliferating cell nuclear antigen (PCNA), it enhances its interaction with polı. Furthermore, a pol-ubiquitin chimera interacts avidly with both polı and PCNA. Thus, the ubiquitination status of polı, or polı plays a key regulatory function in controlling the protein partners with which each polymerase interacts, and in doing so, determines the efficiency of targeting the respective polymerase to stalled replication forks where they facilitate TLS.

INTRODUCTION

Most types of DNA damage block the progression of a replication fork. To circumvent these blocks, cells recruit specialized DNA polymerases to facilitate translesion DNA synthesis (TLS) past the damaged DNA, thus allowing completion of genome duplication (1–3). While many human DNA polymerases (pols) have some capacity to promote TLS (4), the most proficient TLS enzymes belong to the Y-family of DNA polymerases (5). Polη, the best-characterized Y-family DNA polymerase, is defective in humans with the sun-sensitive cancer-prone xeroderma pigmentosum variant (XP-V) syndrome (6,7). Polη can replicate efficiently and with high accuracy through ultraviolet (UV)-induced cyclobutane pyrimidine dimers (CPDs) (8–10). Polη-deficient XP-V cells manifest high levels of cellular mutagenesis after exposure to UV radiation (11), indicating that polη normally prevents UV-induced mutations and cancer. It has been postulated that in the absence of a functional polη, other low-fidelity pols facilitate TLS of CPDs with mutagenic consequences (2). The most likely candidates are Y-family pols ı and ı and the B-family polı (12,13).

Structural studies (10,14–19) have shown that compared with replicative polymerases, TLS polymerases share a more open catalytic site. As a consequence, most Y-family polymerases display low-fidelity DNA synthesis when copying undamaged DNA (20,21). The regulation of their activities in a living cell is, therefore, critical to maintain genomic stability.

The current working hypothesis postulates that when the cell’s replication machinery is stalled at damaged DNA site, the replicative polymerase is replaced by a TLS polymerase in a process called ‘polymerase switching’ (5,22). In eukaryotic cells, such replacement is mediated by the proliferating cell nuclear antigen (PCNA)
processivity factor, which is recruited to the stalled fork. All four human Y-family polymerases (polη, polζ, polκ and Rev1) have been shown to interact directly with PCNA (23–27). PCNA is also subject to a DNA damage-dependent monoubiquitination event that helps targeting of polη to the stalled replication forks (28,29). PCNA monoubiquitination occurs at K164 via Rad6, a E2-ubiquitin-conjugating enzyme and Rad18, a E3-ubiquitin ligase (30). Polη has a higher affinity for monoubiquitinated PCNA than unmodified PCNA suggesting that ubiquitination of PCNA helps target polη to stalled replication forks (28,29). The non-covalent association of polη with ubiquitin (and monoubiquitinated PCNA) is mediated via its Ubiquitin-binding-zinc-finger (UBZ) motif (31,32). Mutations within the UBZ block the interaction with ubiquitin and reduce the ability of polη to accumulate into damage-induced foci, or so-called ‘replication factories’ (31). Like polη, polζ, polκ and Rev1 also interact with ubiquitin (26,31,33). Polη and Rev1, however, contain structurally different ubiquitin-binding motifs termed ‘UBMs’ (26,31,33,34). Similar to polη UBZ mutants, mutations in the polη or Rev1 UBMs not only block the interaction with ubiquitin but also inhibit the accumulation of the TLS polymerases into replication factories (26,31,33).

In addition to a non-covalent interaction with ubiquitin through their respective UBZ and UBMs, both polη and polζ can be covalently monoubiquitinated at specific residues in the respective enzyme (31). The sites of ubiquitination in polη are currently unknown. However, recent studies have indicated that polζ can be mono-ubiquitinated at four separate lysine residues near its C-terminus (K682, K686, K694 and K709) (35). Monoubiquitination of polζ plays an important regulatory function, as it precludes an interaction with PCNA (35). Interestingly, monoubiquitinated polζ is de-ubiquitinated upon DNA damage, thereby allowing an interaction with PCNA at stalled replication forks, when the TLS activity of polζ is most needed (35).

Polη and polζ have also been shown previously to physically interact and co-localize into replication factories at sites of DNA damage (36), although the kinetics with which the two polymerases reside in these replication factories differs (37). The region within polη and polζ responsible for the physical interaction has been loosely mapped to their respective ~200 C-terminal residues (25,36). We were interested in mapping the sites of the polη–t interaction more precisely, so as to potentially begin to elucidate the structural basis for the interaction, as has recently been reported for the polη–Rev1 interface (38,39). We report here that these interactions occur via the respective UBZ and UBMs of polη and polζ. Rather than a direct UBZ–UBM interaction, we present evidence that the polη–t interaction is actually mediated through ubiquitin. Thus, the monoubiquitination status of polη and t is likely to determine which protein partner(s) the respective polymerase interacts with and how efficiently it is recruited to replication factories at sites of DNA damage where they facilitate TLS.

MATERIALS AND METHODS

Saccharomyces cerevisiae two-hybrid vectors and interaction analysis

Two-hybrid vectors carrying full-length human polη, polζ, PCNA or ubiquitin, were described earlier (25,33,36). Vectors expressing variants of human polη or polζ were either generated by site-directed mutagenesis, or gene synthesis of the mutant allele as a service provided by Genscript Inc. (Piscataway, NJ, USA) and subsequently sub-cloned into the original expression vector (Supplementary Table S1). Interactions between proteins were demonstrated in vivo using the Saccharomyces cerevisiae two-hybrid Matchmaker III system (Clontech, Palo Alto, CA, USA). pACT2, pGADT7, pGBK7 and various derivatives were co-transformed into the S. cerevisiae strain AH109. Transformants were selected on DOBA-Trp-Leu plates. Colonies were subsequently replica plated on DOBA-Trp-Leu-His-Ade plates, to confirm the activation of the reporter genes.

Escherichia coli expression vectors and protein purification

Full-length His-tagged human polη was expressed in the Escherichia coli strain RW644 (40) from plasmid pJM868 (41). Plasmids expressing pol variants F507S (pJRM97), P511R (pJRM102), P680A (pJRM86) and P692R (pJRM108) were generated by sub-cloning the desired synthesized allele (Genscript) into pJM868 (Supplementary Table S2). Wild-type His-polη and mutant variants were purified on Ni²⁺-charged nickel-nitrioltriacetic acid His-Bind Resin (Qiagen, Valencia, CA, USA) as recommended by the manufacturer. The eluate containing pol was dialyzed in buffer A (20 mM sodium phosphate pH 7.3, 10 mM sodium chloride, 10% glycerol, 10 mM 2-mercaptoethanol) and applied to an HP Q-Sepharose column (GE Healthcare, Piscataway, NJ, USA). Pol was eluted in a step gradient of NaCl and the pol-containing fractions were aliquoted and stored at −80°C.

Fluorescent vectors, transfection and foci formation assay

The fluorescent construct carrying full-length wild-type polη (pECFP-C1-polη) was described earlier (36). Derivatives carrying F507S (pJRM23), or P511R (pMGB9) in polη, or pol-Ub (pJRM128) were generated by sub-cloning the desired synthesized allele (Genscript) into pECFP-C1-polη wt (Supplementary Table S3). The fluorescent constructs were transfected into transformed MR6.6 C5 fibroblasts (TurboFectin 8.0) according to the manufacturer’s protocol (Origene, Rockville, MD, USA). Twenty hours after transfection, cells were irradiated at 7 J/m² and incubated for a further 6h. Fixation of cells was carried out as described earlier (36). Fluorescence images of cell nuclei were acquired on a Zeiss Axiosphot2 microscope (Carl Zeiss) equipped with an Orca ER CCD camera (Hamamatsu) using Simple PCI software. Images were captured by excitation at 436 nm and detection of CFP emission at 480 nm. At least 200 nuclei were analyzed for each cell line and treatment in 2–5 independent experiments.
In vitro transcription/translation of proteins

In vitro transcription/translation of pol\(\eta\), pol (wild type and variants), PCNA or ubiquitin, was performed using a TNT-Quick Coupled Transcription/Translation System (Promega, Madison, WI, USA) according to the manufacturer’s instructions. The expression vectors encoding pol\(\eta\) (pAVR65), PCNA (pAVR18), ubiquitin (pBP129), pol wt (pAR110), pol\_F507S (pNEO155), pol\_P511R (pJRM65), pol\_P680A (pJRM64) were added separately to the reaction mixtures and incubated for 90 min at 30°C in the presence of \(\text{[}^{35}\text{S}\text{]}\) methionine (Perkin Elmer, Waltham, MA, USA). Reaction products were analyzed directly by SDS–PAGE and used in the far-Western assay.

Far-Western analysis

Purified His-tagged pol\(\eta\) proteins or K63-linked Ub-chains were separated by 4–20% SDS–PAGE (Invitrogen, Carlsbad, CA, USA) and transferred to nitrocellulose membranes (Invitrogen). Membranes with His-pol\(\eta\) proteins were incubated at 4°C overnight with \(\text{[}^{35}\text{S}\text{]}\)-labeled pol\(\eta\), PCNA or ubiquitin and membranes with K63-linked ubiquitin chains with \(\text{[}^{35}\text{S}\text{]}\)-labeled pol. Following incubation, membranes were washed three times at 4°C, dried briefly and scanned with a FujiFilm FLA-5100 phosphoimager. The amount of loaded protein was verified by staining membranes with Ponceau S (Sigma, St Louis, MO, USA).

Model building

The images of the murine UBM1 and human UBM2 structures in complex with ubiquitin were generated using Pymol (The PyMOL Molecular Graphics System, Schrödinger, LLC) with PDB files 2KWV and 2KHW, respectively.

FLAG pull-down assay

Mammalian expressing constructs carrying full-length wild-type pol\(\eta\) (pJRM46) or pol\(\eta\)-Ub chimera (pJRM140) and pol\(\eta\) (pJRM56) were generated by sub-cloning the desired synthesized allele (Genscript) into pCMV6AN-DDK and pCMV6AN-HA vectors, respectively (Origene) (Supplementary Table S3). Constructs were transfected into HEK293T cells using Turbofectin 8.0 according to manufacturer’s instructions (Origene). Twenty-four hours after transfection, cells were harvested and lysed. The presence of FLAG- and HA-tagged proteins in cell extracts was verified using Western blot. For the pull-down assay, respective cell extracts were incubated overnight at 4°C with EZView Red ANTI-FLAG M2 Affinity Gel (Sigma-Aldrich, St. Louis, MO, USA), washed three times and analyzed directly by SDS–PAGE and Western blot.

RESULTS

Identification of a region in pol\(\eta\) involved in binding pol\(\eta\)

We previously reported that human pol\(\eta\) and pol physically interact through their C-termini (36). In particular, the last 230 amino acids of pol\(\eta\) are sufficient to interact with pol (25). To more precisely determine the amino acid residues involved in the pol\(\eta\)--pol\(\eta\) interaction, we first used a yeast two-hybrid approach. As shown in Figure 1, only cells expressing the pol\(\eta\) construct with a deletion between residues S587-L641 failed to grow on selective medium. Interestingly, this deletion contains the N-terminal part of the pol\(\eta\) UBZ domain (Figure 2A), consistent with the idea that an intact UBZ domain is required for the pol\(\eta\) and pol interaction.

To investigate this hypothesis, we then generated base substitutions in the UBZ domain of full-length pol\(\eta\) and assayed their ability to interact with pol in the two-hybrid assay. Pol\(\eta\) variants with a double C635A/C638A substitution, or individual C635A, C638A or D652A substitutions eliminated the interaction with pol. The inability of these mutants to interact with pol is specific, as similar to wild-type pol\(\eta\), they retained their ability to interact with PCNA (36) (Figure 2B). In contrast, and as reported earlier (33), the pol\(\eta\) H654A UBZ mutant lost its ability to interact with ubiquitin, but still retained its ability to interact with pol.

Identification of regions in pol\(\eta\) that interact with pol\(\eta\)

Having identified a region in pol\(\eta\) that appears necessary for the interaction with pol\(\eta\), we were interested in identifying the reciprocal region in pol\(\eta\) that interacts with pol\(\eta\). As wild-type pol\(\eta\) cannot interact with the C635A/C638A UBZ pol\(\eta\) mutant, we hypothesized that if we were able to identify a suppressor mutation in pol\(\eta\) that gained an ability to interact with the UBZ mutant, then the pol\(\eta\) ‘suppressor’ would most likely be a compensatory mutation at, or close to, the pol–pol\(\eta\) interface. To identify such a suppressor, we randomly mutagenized the activating domain plasmid expressing full-length pol\(\eta\) and screened for colonies in the two-hybrid assay that were able to interact with the C635A/C638A pol\(\eta\) mutant. Several interacting clones were identified and one carrying a single nucleotide mutation that leads to a P692L substitution in pol\(\eta\) was chosen for further study (Supplementary material for experimental details). The pol\(\eta\) P692L mutant is fully functional and interacts with the C635A/C638A double mutant and the C635A, C638A and D652A single pol\(\eta\) UBZ mutants, as well as wild-type pol\(\eta\) (Figure 2B).

Proline 692 is located in the center of pol\(\eta\)’s UBM2 motif (31), raising the intriguing possibility that pol\(\eta\) and pol might interact through their respective UBZ and UBM2 motifs. To test this hypothesis and potentially identify additional residues in pol\(\eta\)’s UBM2 involved in the pol\(\eta\)--pol interaction, we made additional substitutions at several highly conserved residues in pol\(\eta\)’s UBM2 motif (Figure 3B) and assayed their ability to interact with pol\(\eta\) in addition to PCNA, or ubiquitin, as controls (Figure 3C). Growth of the yeast strains was determined after 4 and 6 days of incubation at 30°C and compared with the growth of the wild-type pol construct to give a qualitative idea of the protein–protein interactions. Most mutants appear to be correctly folded, since like wild-type pol\(\eta\), they gave a positive interaction with PCNA after 4–6 days of growth (Figure 3C). The
The main exception was the V687A/F688A construct, which interacted poorly with PCNA, even after 6 days of incubation. P680A also appeared to have a somewhat reduced ability to interact with PCNA, as it took 6 days to observe good growth with this mutant, compared with 4 days for the wild type and other mutants. As expected, given their location in the UBM2 motif, many of the pol\_i substitutions disrupted the ability of the mutant to physically interact with ubiquitin (Figure 3C). Interestingly, and in support of the notion that the UBM2 motif is the region in pol\_i that interacts with pol\_Z, many of the UBM2 mutants that had reduced or no interaction with ubiquitin were also unable to interact with pol\_Z (Figure 3C), including P680A, I683A/D684A, L691A/P692A, Q696A and E698A. Our data, therefore, identify pol\_i UBM2 as a region within pol\_i that interacts with both ubiquitin and pol\_Z. However, these interactions are not necessarily dependent upon each other since in a previous study (33), we identified P692R in UBM2 as a substitution that selectively disrupts pol\_i’s interaction with ubiquitin, whilst retaining its ability to interact with pol\_Z (Figure 3C; 33).

Pol\_i has two UBM\_s (31) and given that UBM2 appears to be important for pol\_i to interact with both ubiquitin and pol\_Z, we wanted to determine what effect, if any, substitutions in pol\_i’s UBM\_1 (Figure 3A) might have on the ability of the protein to interact with ubiquitin and pol\_Z. We focused on two substitutions: P511R, which would be analogous to the ubiquitin-binding-deficient, but pol\_Z-binding-proficient P692R mutant in UBM2, and F507S, as this is a naturally occurring single nucleotide polymorphism (SNP) found in ~3% of humans (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=3218786). Interestingly, both UBM\_1 substitutions interacted with ubiquitin, yet both showed a reduced ability to interact with pol\_Z (Figure 3C).

**Far-Western analysis of pol\_i mutants**

To confirm the altered protein–protein interactions observed in the yeast two-hybrid assay, we performed ‘far-Western’ analysis of the interactions (Figure 4). We first determined the ability of the pol\_i mutants to interact...
with polh (Figure 4A). In general, the results were consistent with the yeast two-hybrid analysis, with pol F507S, P511R and P680A all exhibiting a reduced ability to interact with polh (~25–45% of the wild-type protein).

We then compared the mutant pol’s ability to bind to PCNA (Figure 4B). Again, the data confirmed the two-hybrid analysis. P511R, which exhibited good growth after 4 days of incubation in the two-hybrid assay, also showed a strong interaction with PCNA (similar to wild-type polh). F507S and P680A, which exhibited delayed growth with PCNA in the two-hybrid assay, also interacted less efficiently with PCNA in the far-Western assays (~50–75% of that observed with wild-type polh: Figure 4B).

Finally, we assayed for an interaction with free ubiquitin and K63-linked ubiquitin chains (Figure 4C and D). As expected from the two-hybrid assay, P511R showed a strong interaction with free ubiquitin, whereas P680A, which took longer to reveal an interaction with ubiquitin in the two-hybrid assay, exhibited the weakest interaction with ubiquitin (~40% of wild type). F507S also exhibited a reduced ability to interact with free ubiquitin (~50% of wild-type levels), but nevertheless retained its normal capacity to interact with K63-linked poly-ubiquitin chains (Figure 4D).

Location of polh residues within UBM1 and UBM2 implicated in interacting with polh

The solution structures of human UBM2 (34) and murine UBM1 (42) have previously been determined. The locations of the human polh UBM1 and UBM2 mutants...
The two UBM1 mutants (F507S and P511R) are located at the interface between Poli and ubiquitin (Figure 5A). From a structural point of view, it is hard to reconcile that these mutants retain their ability to interact with ubiquitin, unless the interaction is mediated through the intact UBM2 motif (see below for further discussion).

The Poli UBM2 mutants fall into three classes (Figure 5B). The main class consists of mutants that simultaneously affect binding to Poli and ubiquitin. These mutants are colored yellow in Figure 5B and are clustered at the interface between Poli and ubiquitin. The second class of UBM2 mutant (K697D, A701D and R705D) retains the ability to interact with both Poli and ubiquitin. These residues are colored blue in Figure 5B and are located on the outside surface of the long A-helix 1 of UBM2. The third and final class of UBM2 mutant exhibits split phenotypes/properties. For example, P692R (colored red in Figure 5B) is completely defective in binding ubiquitin, yet has a near normal ability to bind Poli [Figures 3C and 4C; (33)]. This observation is also hard to reconcile from a structural point of view, unless the interaction with Poli is mediated through the intact UBM1 motif.

Our finding that many mutants in Poli UBM2 are simultaneously defective in binding ubiquitin and Poli despite possessing an intact UBM1 indicates that the primary binding site for both proteins in vitro is the Poli UBM2 motif. However, our observation that a single mutation in UBM2 (P692R) blocks the interaction with ubiquitin, but not Poli, suggests that Poli can also interact with Poli via UBM1. This suggestion is supported by the finding that the Poli F507S UBM1 mutant is unable to interact with Poli.

Figure 4. In vitro far-Western assay verifying the interactions between Poli UBM1 (F507S and P511R) and UBM2 (P680A) substitutions with Poli, PCNA, ubiquitin and K63-linked ubiquitin chains. Purified His-tagged wild-type Poli and the indicated mutants were separated by SDS-PAGE, transferred to nitrocellulose and incubated with in vitro translated 35S-labeled Poli (A), PCNA (B) and ubiquitin (C). Densitometric analysis of far-Westerns (top panels) compares the strength of interaction with wild-type Poli and mutants with the 35S-labeled proteins; the 35S band intensities (middle panels) were normalized to their respective Ponceau-stained bands (bottom panels). (D) Wild-type Poli and UBM mutants interact with K63-linked Ub chains; 15 μg of K63-linked ubiquitin chains (Boston Biochem) were separated by SDS-PAGE, transferred onto nitrocellulose and incubated with 35S-labeled wild-type Poli or the indicated UBM mutant.
and leads to the prediction that a double mutant in both UBM1 and UBM2 would be unable to bind ubiquitin or pol\(\eta\). Indeed, as shown in Figure 5C, the pol\(i\) F507S/P692R (UBM1/UBM2 double mutant) is unable to interact with either protein in the two-hybrid assay, yet exhibits a strong interaction with PCNA.

Reduced accumulation of pol\(i\) into replication factories in UBM1 mutants unable to interact with pol\(\eta\)

It has previously been reported that upon DNA damage, pol\(i\) accumulates into damage-induced foci (36,37) that are believed to represent subcellular ‘replication factories’ (5). The number of damaged-induced pol\(i\) foci drops significantly in pol\(\eta\)-deficient XP-V cells, leading to the hypothesis that pol\(\eta\) is required to physically target pol\(i\) into replication factories (36). However, because of their defect in pol\(\eta\), XP-V cells are blocked in S-phase after UV-irradiation (43), and the lack of accumulation of pol\(i\) into foci might simply result from the indirect consequence of delayed post-replication repair and altered cell cycle signaling, rather than a direct, physical role for pol\(\eta\) in targeting pol\(i\) into replication factories. We tested this hypothesis directly in cells expressing wild-type pol\(\eta\) by assaying the ability of pol\(i\) mutants that are unable to interact with pol\(\eta\) to accumulate into replication factories. To do so, we generated eCFP-tagged pol-fusions (36) with single missense mutations in UBM1 (F507S or P511R) as these mutants exhibited a significantly reduced ability to interact with pol\(\eta\), whilst retaining the ability to interact with ubiquitin and compared foci formation to the wild-type eCFP-tagged pol (Figure 6). In these experiments, ~12% of undamaged cells and 30% of UV-irradiated cells exhibited foci formation when transfected with wild-type pol\(i\). In contrast, when cells were transfected with the pol\(i\) UBM1 mutants they exhibited very limited foci formation (<5% of cells), even after being exposed to UV irradiation. We attribute this phenotype to the reduced ability of the pol\(i\) UBM1 mutant to physically interact with pol\(\eta\). However, in the case of pol\(i\) F507S, we cannot exclude the possibility that its slightly reduced ability to interact with PCNA (Figures 3 and 4), may also contribute to its inability to accumulate into replication factories in vivo (25).

The interaction between pol\(i\)--pol\(\eta\) is mediated via ubiquitin

Our current studies have shown that in addition to facilitating the interaction with ubiquitin and ubiquitinlated PCNA, the respective UBZ and UBMs in pol\(\eta\) and pol\(i\) are required for a physical and functional interaction between the two TLS polymerases. However, it is unclear if these protein–protein interactions are direct or indirect. For

![Figure 5. Pol\(i\) interacts with pol\(\eta\) through its UBM domains. Ribbon diagrams show the structure of the UBM1s (green) interacting with Ubiquitin (bronze). (A) Location of UBM1 mutants. The model of human UBM1 was generated based upon the closely related murine UBM1 structure (PDB 2KWV). Residues that impair the interaction with pol\(\eta\) are highlighted in purple. (B) Location of UBM2 mutants. The human UBM2-ubiquitin structure was generated using PDB 2KHW. Residues that simultaneously disrupt the interaction with ubiquitin and pol\(\eta\) are highlighted in yellow. Residues that do not impair the interaction with ubiquitin or pol\(\eta\) are highlighted in blue. The P692 residue, which when changed to Arg selectively disrupts the interaction with ubiquitin, is highlighted in red. (C) A two-hybrid assay demonstrating that the F507S/P692R UBM1-UBM2 double mutant does not interact with pol\(\eta\) or ubiquitin, whilst retaining its ability to interact with PCNA. Yeast strain AH109 was transformed separately with the GAL4-AD expression vectors pACT2, pACT2-pol\(i\) wild type (pAR116) and pACT2-poli F507S/P692R (pJRM142) in combination with one of the following GAL4-BD expression vectors: pGBK7, pGBK7-pol\(\eta\) wild type (pAVR65), pGBK7-PCNA (pAVR18) and pGBK7-ub (pBP129) as indicated. Several colonies from each transformation were grown overnight at 30°C in selective medium, and a sample was spotted on to a DOBA-Trp-Leu-His-Ade plate and incubated at 30°C for 6 days.
example, both polymerases are monoubiquitinated in vivo (31,35) and it is plausible that the interaction between the two polymerases is mediated by a monoubiquitinated form of each enzyme binding to the UBZ or UBM of its partner.

It has previously been shown that polγ can be monoubiquitinated at four different lysine residues (K682, K686, K694 and K709) located near its C-terminus and that mutant forms of polγ, in which the four-lysine residues have been changed to alanine (4K→A), cannot be monoubiquitinated in vivo (35). To test the hypothesis that pol might interact with monoubiquitinated polγ we introduced the 4K→A substitutions into our polγ two-hybrid vector and assayed for an ability to interact with polγ, PCNA and ubiquitin. As shown in Figure 7, the 4K→A polγ mutant retains its ability to interact with PCNA, yet has completely lost its ability to interact with either ubiquitin or pol. Our observations, therefore, support the hypothesis that pol interacts with a monoubiquitinated form of polγ via its UBM.

The monoubiquitination sites in pol are currently unknown, so it is not possible to perform the reciprocal experiments in which monoubiquitination of pol is blocked. To circumvent this obstacle, we instead constructed a chimeric protein in which ubiquitin is fused to the C-terminus of pol (pol-Ub) (Figure 8A). The ubiquitin moiety lacks the terminal glycine residues (G75/G76) and cannot be covalently linked to another substrate. A similar chimeric construct was previously reported for polη (35). Interestingly, like wild-type pol, the pol-Ub chimera exhibited a strong interaction with polγ, but was unable to interact with ubiquitin, presumably because the ubiquitin moiety of the chimera occupies pol’s UBM2, thereby precluding any further interactions with free ubiquitin (Figure 8A). To prove that the interaction between polγ and pol-Ub is dependent upon the fused ubiquitin moiety, we made an I44A substitution in ubiquitin. The I44 residue is normally located at the center of the interface between ubiquitin and polγ’s UBZ (32), and the I44A substitution abolishes the interaction between polγ and pol-Ub (Figure 8A).

Interestingly, a strong interaction between polγ and the pol-Ub chimera was apparent after 2 days growth, compared with 4 days required for wild-type pol, suggesting that polγ has a tighter affinity for pol-Ub than with wild-type pol. Indeed, a physical interaction between polγ and pol has proven historically difficult to demonstrate in traditional pulldown experiments with extracts from human cells [Figure 8C; (36)]. However, in experiments where FLAG-tagged pol was expressed in human HEK293T cells and a portion (~10%) of the protein is clearly ubiquitinated, we were able to pull down small amounts of polγ (Figure 8B, track 2). Furthermore, the amount of polγ pulled-down increased significantly in the presence of pol-Ub (Figure 8B, track 4). Unlike polγ, where ubiquitination inhibits an interaction with PCNA (35), the pol-Ub chimera showed no diminished capacity to interact with PCNA, indicating that ubiquitination of pol does not preclude an interaction with PCNA (cf. Figure 8C, tracks 2 and 4).
We have previously shown that an interaction between poli and polη is required for poli to accumulate into replication factories (Figure 6), and our observations above indicate that the interaction between poli and polη is strengthened when poli is ubiquitinated (Figure 8A and B). We therefore hypothesized that the poli-Ub chimera might accumulate into replication factories more efficiently than the wild-type protein. As seen in Figure 8D, this proved to be the case, as we observed a 2-fold increase in the number of undamaged cells exhibiting eCFP-poli foci and similar levels of damage-induced foci. We note that this is in contrast to a ~3-fold decrease in the number of cells exhibiting GFP-polη-Ub foci (35). Thus, the effect of ubiquitination of poli at its C-terminus is opposite to that of polη. Rather than hindering re-localization, ubiquitination at the C-terminus of poli actually increases its sub-cellular re-localization.

DISCUSSION

It has been known for over a decade that poli and polη physically interact (36), and the regions responsible for the
interaction were previously loosely mapped to the C-terminal ~200 amino acids of each protein (25,36). Although the two polymerases clearly co-localize at sites of DNA damage, the kinetics of their re-localization differs, suggesting that the two polymerases are not tightly associated in a living cell (37). Our studies begin to shed light on how such an interaction is facilitated and regulated. We identified the regions responsible for the pol\(\eta\)–\(i\) interaction as their respective UBZ and UBMs (Figures 1–3). Pol\(\eta\) is known to be monoubiquitinated \textit{in vivo} (31,35) and we considered the possibility that the physical interaction between the two polymerases might be mediated though the monoubiquitinated form of the polymerases and their respective UBZ or UBMs. To test this hypothesis, we generated a mutant pol\(\eta\) (4K→A) that cannot be monoubiquitinated. Interestingly, the mutant pol\(\eta\) protein was completely defective in its ability to interact with pol\(i\). Monoubiquitination of pol\(\eta\), therefore, appears critical for the interaction with pol\(i\).

The fact that we observe an interaction between wild-type pol\(\eta\) and pol\(i\) in the two-hybrid assays suggests that at least a fraction of human pol\(\eta\) is likely to be subject to monoubiquitination in the yeast cells used for the \textit{in vivo} two-hybrid assay. Furthermore, if monoubiquitination is a prerequisite for the interaction, how do we explain that we observe an interaction with the \textit{in vitro} translated proteins in the far-Western assays? The answer lies in the fact that a significant fraction of the radiolabeled pol\(\eta\) and pol\(i\) synthesized in the coupled transcription-translation assay is also concomitantly ubiquitinated \textit{in vitro} (Supplementary Figure S1). Thus, the data presented are entirely consistent with the hypothesis that the preferred partner for pol\(i\) is a monoubiquitinated form of pol\(\eta\).

We identified the region in pol\(\eta\) responsible for the interaction with pol\(i\) as its UBZ (Figures 1 and 2). As pol\(i\) is also known to be monoubiquitinated \textit{in vivo} (31), we hypothesized that the preferred partner of pol\(i\) might actually be a ubiquitinated form of pol\(\eta\). To test this hypothesis, we generated a chimera in which the N-terminus of ubiquitin was fused to the C-terminus of pol\(i\). The mutant chimera lacked the two C-terminal glycine residues, and therefore only allows for non-covalent interactions. The chimera interacts avidly with pol\(\eta\) in the two-hybrid assays and this interaction was dependent upon I44 of ubiquitin (in the pol\(i\)-Ub chimera) (Figure 8A). When expressed in human HEK293T cells, the pol\(i\)-Ub chimera was able to ‘pull-down’ considerably more pol\(\eta\) than wild-type pol\(i\) (Figure 8B). We therefore conclude that the preferred partner for pol\(\eta\) is indeed, a ubiquitinated form of pol\(i\). The mobility of the ‘pulled-down’ pol\(\eta\) suggests that it is the non-ubiquitinated pol\(\eta\). That being the case, it appears that the interaction between pol\(\eta\) and pol\(i\) is enhanced when either pol\(\eta\) (Figure 7), or pol\(i\) (Figure 8B), is ubiquitinated. Based upon our observations presented here, it appears that pol\(\eta\) and pol\(i\) can interact in a variety of ways through ubiquitinated forms of either protein via their respective UBZ or UBMs (Figure 9).

**Figure 9.** Cartoon explaining how the various interactions between pol\(i\), pol\(\eta\) and PCNA can be modulated by ubiquitin. The polymerases are indicated as a rod with functional domains/motifs colored as follows: catalytic domain of pol\(i\), light blue; catalytic domain of pol\(\eta\), dark blue; PIP-box, purple rectangle; PCNA, purple disk; wild-type UBM1/UBM2/UBZ, green rectangle; mutant UBM1/2, red rectangle; wild-type ubiquitin, orange ellipsoid; I44A Ubiquitin mutant, red ellipsoid. (A) pol\(i\) interacts with ubiquitinated pol\(\eta\) predominantly via UBM2. Pol\(i\) can still bind PCNA via is PIP-box, but ubiquitinated pol\(\eta\) is unable to bind PCNA (35); (B) when UBM2 is unavailable, pol\(i\) can potentially interact with ubiquitinated pol\(\eta\) via UBM1; (C) pol\(i\) cannot interact with ubiquitinated pol\(\eta\) when both UBMs are mutated; (D) mutation of pol\(\eta\)’s natural ubiquitination sites blocks the interaction between pol\(\eta\) and pol\(i\); (E) the pol\(i\)-Ub chimera binds to the UBZ of pol\(\eta\). Both polymerases are able to interact with PCNA; (F) the I44A mutation in the pol\(i\)-Ub chimera inhibits the interaction between pol\(i\) and pol\(\eta\), but allows for an interaction between ubiquitin and UBM2.
The functional importance of the polη-1 interaction is clearly demonstrated by the fact that mutants of polη that are unable to interact with polη exhibit reduced accumulation into replication factories (Figure 6). Conversely, the polη-Ub chimera, which exhibits a tighter interaction with polη shows an enhanced accumulation into replication foci (Figure 8D).

Given the complex set of protein–protein interactions that polη and poli are known to participate in (35), it is reasonable to predict that the ubiquitination status of the pols allows a cell a variety of ways to regulate the formation of TLS complexes. For example, mono-ubiquitination of polη is known to inhibit an interaction with ubiquitinated PCNA (35), but as shown here, it enhances its interaction with poli. Upon DNA damage, polη is de-ubiquitinated and this will lead to a reduced ability to interact with poli, but a concomitant increased ability to interact with ubiquitinated PCNA. This might explain why the polymerases exhibit different sub-cellular mobility in a living cell (37).

In summary, we have shown here that the physical and functional interaction between pols η and i occurs between ubiquitinated forms of either polymerase via their respective UBZ or UBMs. We see no reason to exclude the possibility that similar protein–protein interactions might occur between the various TLS pols (not polη and poli exclusively) and monoubiquitinated repair proteins, or the monoubiquitinated TLS pols and repair enzymes containing UBZ or UBMs, thereby enabling the TLS pols to be efficiently targeted to sites of DNA damage where they can facilitate TLS, or possibly channeled into an ever-growing myriad of different repair pathways, such as nucleotide excision repair, homologous recombination and intra-strand crosslink repair, in which they are known to participate (5).

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online: Supplementary Tables 1–3 and Supplementary Figure 1.

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REFERENCES