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A novel ubiquitin binding mode in the *S. cerevisiae* translesion synthesis DNA polymerase η

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Abstract

The ubiquitin binding zinc finger (UBZ) domain in the C-terminal portion of Pol η has been found to interact with ubiquitin. However, the affinity between the Pol η UBZ and ubiquitin was shown to be low with a previously reported K_d of 73~81 μ M. This low-affinity binding between Pol η UBZ and ubiquitin has been difficult to reconcile with its presumed role in translesion synthesis as suggested by genetic and cell biology studies. In this work, we constructed a minimal *S. cerevisiae* Pol η UBZ domain and probed the Pol η UBZ-ubiquitin interaction using surface plasmon resonance (SPR) technique. Our quantitative binding data between the wild-type or mutant Pol η UBZ and ubiquitin revealed an interesting divergence between the Pol η UBZ from *S. cerevisiae* and humans. Moreover, we found that the C-terminal portion of yeast Pol η (amino acid 515~632) binds ubiquitin with a much higher affinity than the minimal UBZ domain. Further, distinct ubiquitin-binding kinetics were observed for the C-terminal portion of Pol η and the isolated UBZ domain. This observation raised the interesting possibility that the Pol η C-terminal portion binds ubiquitin in a novel mode that affords higher affinity. Our findings have broader implication in understanding the generally weak interaction between the known ubiquitin-binding domains and ubiquitin.

Keywords

Ubiquitin binding zinc finger; PCNA; translesion synthesis; polymerase η

Introduction

Ubiquitin, an 8.6 kDa protein, covalently modifies a target protein via an isopeptide bond between the C-terminal carboxylate of ubiquitin and the lysine ϵ -amino group in the target protein. Ubiquitination was initially discovered as a signaling mechanism for proteasome-mediated protein degradation¹. More recently, it was realized that ubiquitin is a highly versatile modifier that participates in a diverse array of cellular processes, including DNA damage response, transcription, nuclear transport, autophagy and immune response². Adding to the versatility of ubiquitin, a group of ubiquitin-like proteins (Ubls) also exists in eukaryotic cells and play essential roles in processes not related to protein degradation^{3, 4}.

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Ubiquitination, like phosphorylation, is a reversible process, catalyzed by ubiquitin ligase and deubiquitinating enzyme. However, ubiquitination possesses a higher degree of complexity intrinsic to ubiquitin as a modifier. It has been shown that ubiquitin can be conjugated to proteins in different forms, either as a monoubiquitin moiety or as polyubiquitin chains. Remarkably, polyubiquitin chains linked through one of the seven lysine residues on ubiquitin have been identified in cells⁵. The K48-linked polyubiquitin chains are known to target protein for proteasomal degradation. Monoubiquitination and polyubiquitination linked through several other ubiquitin lysine residues play nonproteolytic functions in endocytosis, vesicular trafficking and DNA damage response.

Central to the ubiquitin's diverse function is its ability to interact with proteins that contain ubiquitin-binding domain (UBD). To date at least sixteen ubiquitin-binding domains (or motifs) have been characterized. UBDs are usually small in size (20~150 a. a.) and many adopt an α -helical structure. Earlier studies found that many characterized UBDs interact with ubiquitin on a hydrophobic patch surrounding Ile44 with a low to moderate affinity (10–500 μ M)⁶. To rationalize the low-affinity interaction between most UBDs and ubiquitin, it has been proposed that multivalent interaction between UBD and multi-monoubiquitinated or polyubiquitinated proteins likely overcome the low affinity between UBD and a single ubiquitin moiety⁷. While it has been demonstrated that some UBDs bind polyubiquitin chain with higher affinity compared to monoubiquitin⁸, direct evidence for high-affinity multivalent interaction between UBD and monoubiquitinated protein has not been reported. It is therefore still not clear how UBDs achieve high-affinity interaction with monoubiquitinated proteins.

In eukaryotes, ubiquitination of proliferating cell nuclear antigen (PCNA) is essential for the normal cellular response to genotoxic stress. PCNA undergoes both monoubiquitination and polyubiquitination following UV-induced DNA damage^{9, 10}. Strikingly, K164 of PCNA is modified by either a single ubiquitin or by a K63-linked polyubiquitin chain. Available evidence suggests that monoubiquitination and polyubiquitination of PCNA likely channels the DNA damage response pathway to different branches by recruiting specific protein factors^{11, 12}. It is believed that the stalling of a replication fork by a DNA lesion leads to monoubiquitination of PCNA at Lys164, which in turn contributes to the recruitment of a specialized DNA polymerase to effect translesion synthesis (TLS)^{13–17}. Remarkably, many specialized polymerases contain either ubiquitin-binding zinc finger (UBZ) domain or ubiquitin-binding motif (UBM) that helps to recruit the specialized polymerase to the DNA damage site^{18–20}. Polyubiquitination of PCNA, on the other hand, is thought to trigger the error-free lesion bypass process and acts to restart the stalled replication fork^{9, 21–25}. The Rad6/Rad18 complex is responsible for monoubiquitination of PCNA on Lys164^{9, 10}. Polyubiquitination of PCNA requires Mms2-Ubc13 and the ubiquitin ligase Rad5⁹. More recently, monoubiquitination of PCNA at Lys107 was reported in the DNA ligase I deficient yeast cells²⁶. Instead of activating translesion synthesis, Lys107 ubiquitination might provide a signal for DNA damage on newly synthesized DNA strands. Besides ubiquitination, PCNA also undergoes SUMOylation at Lys164, and to a less extent at Lys127⁹. SUMOylation of PCNA recruits Srs2, a DNA helicase, and exerts an anti-recombinogenic effect^{27–29}.

Compared to many other ubiquitin binding domains, the function of UBZ domain is less well understood^{6, 7}. The UBZ domain was identified in TLS polymerase η from a number of eukaryotic organisms, ranging from humans to budding yeast *S. cerevisiae*. UBZ is on average 35 amino acids in size. A multiple sequence alignment of the Pol η UBZ domains from different eukaryotic organisms revealed that the sequences are highly diverged in the N-terminal 20 amino acids in UBZ (Fig. 1). In contrast, the C-terminal 15 residues are more conserved. Most Pol η UBZs contain a canonical C₂H₂ zinc-finger motif. A notable

exception is the *S. cerevisiae* Pol η (scPol η) UBZ. Instead of existing in a CXXC motif, the two cysteines in scPol η UBZ are juxtaposed next to each other. The second cysteine in the CXXC motif is replaced by Y555 in scPol η UBZ. Several residues, including D570 and A574 in scPol η , are conserved among the Pol η UBZ sequences analyzed.

The structure of human Pol η (hPol η) UBZ was solved by solution NMR technique³⁰. It contains two short antiparallel β -strands and a 12-amino acid α helix. Like the classical C₂H₂ zinc finger, a zinc ion was found sandwiched between the major α helix and fingertip formed by the antiparallel β -strands through coordination with the conserved residues, C635, C638, H650 and H654. One zinc ion per hPol η UBZ was determined by electrospray ionization (ESI) mass spectrometry³⁰. NMR titration experiment revealed that hPol η UBZ binds ubiquitin in the exposed surface of the α helix. A binding affinity of 73–81 μ M for ubiquitin was determined for hPol η UBZ by NMR titration and isothermal titration calorimetry (ITC)³⁰. The structure of scPol η UBZ has not been reported. However, a recent study revealed surprising divergence between the budding yeast and the human Pol η UBZ in the requirement of zinc ion for UBZ function³¹. It was found that the scPol η UBZ binds ubiquitin in a zinc-independent manner, which is in sharp contrast to the absolute requirement of zinc binding for ubiquitin interaction in its human counterpart. This observation raised the intriguing possibility that the Pol η UBZ from *S. cerevisiae* and humans are evolutionarily diverged and may possess different modes of ubiquitin binding.

In this study we carried out detailed interaction studies between the scPol η UBZ and ubiquitin. Further, we investigated the interaction of the C-terminal portion of scPol η with ubiquitin. Our quantitative approach revealed interesting difference in the ubiquitin binding mode between the isolated UBZ domain and the C-terminal portion of scPol η . Our results suggest that other element(s) in the scPol η C-terminal region may contribute to the ubiquitin binding in conjunction with the UBZ domain.

Results

Characterization of a minimal *S. cerevisiae* Pol η UBZ domain

The *S. cerevisiae* Pol η (632 a.a.) is constituted of a catalytic core domain (a.a. 1 to 514) and a C-terminal extension (a.a. 515 to 632), which is dispensable for Pol η 's polymerase activity. Compared to the well-characterized catalytic core domain of Pol η , detailed structural and functional information of the C-terminal portion of Pol η is still lacking. So far two motifs have been identified in the Pol η 's C-terminal sequence, *i.e.* the PCNA-interacting protein (PIP) motif and the ubiquitin-binding zinc finger (UBZ) domain (see Fig. 1). PIP resides at the very C-terminus of Pol η and is responsible for a high affinity interaction with PCNA³². The UBZ domain is located between the PAD (polymerase associated domain) in the catalytic core and the PIP motif. Although the high-resolution structure of the scPol η UBZ is not available, it was predicted that the scPol η UBZ likely adopts a zinc-finger motif similar to its human counterpart³³.

To better define the interaction between scPol η UBZ and ubiquitin, we introduced a series of point mutations into the potential ubiquitin-binding α helix of scPol η UBZ and determined UBZ's binding affinity to ubiquitin. We generated a minimal scPol η UBZ domain (a.a. 545–580) with a N-terminal GST tag. The scPol η UBZ was purified by sequential chromatography on glutathione-Sepharose and HiTrap Q columns. To ensure that the minimal scPol η UBZ domain is properly folded, we removed the GST tag by thrombin cleavage. Circular dichroism (CD) spectrum of the wild-type scPol η UBZ domain is indicative of a properly-folded protein domain (Fig. 2). The spectrum is consistent with that reported for a larger yeast Pol η UBZ construct (538–609)³¹. By NMR perturbation experiments, we demonstrated that the scPol η UBZ (a.a. 545–580) is functional in binding

ubiquitin. We determined the 2-D TROSY spectrum of N¹⁵-labeled ubiquitin alone or in complex with the GST-tagged scPol η UBZ (a.a. 545–580) (Supplementary Fig. 1). The chemical shift of the ubiquitin spectra was perturbed or attenuated at a number of residues, similar to that reported for the human Pol η UBZ and the larger scPol η UBZ construct^{30, 31}. Therefore, we conclude that the minimal scPol η UBZ domain retains normal ability in binding ubiquitin.

To probe the scPol η UBZ-ubiquitin binding, we selected several strictly conserved residues in the UBZ α helix for site-directed mutagenesis (see Fig. 1). We replaced the conserved A574 with a bulky residue phenylalanine. We also mutated the highly conserved D570 to alanine. Further, we introduced a proline residue at three different positions in the UBZ α helix (Q566P, A569P and A574P). We expect that the proline residue will disrupt the UBZ-Ub interaction by perturbing the structure of the UBZ α helix. All scPol η UBZ mutants were expressed as a GST fusion and purified to homogeneity as described in Experimental.

A recent report suggested that the scPol η UBZ interacts with ubiquitin independent of zinc binding³¹. We generated the scPol η UBZ mutants, HH568,572AA and CC552,553AA, with the potential zinc coordinating residues replaced with alanine. To confirm that both mutants no longer bind zinc, we used a colorimetric method to determine the content of zinc bound to the wild-type and mutant UBZ as described in Experimental. We observed approximately one zinc ion bound to the wild-type UBZ, in accord with the recent report for a larger scPol η UBZ construct³¹. In contrast, the CC552,553AA and HH568,572AA scPol η UBZ completely lost the zinc-binding ability (Fig. 3). Interestingly, the A574P scPol η UBZ showed substoichiometric zinc binding (0.4) (Fig. 3). This result indicates that disruption of the UBZ α -helix has an indirect effect on scPol η UBZ's zinc binding ability.

Binding affinity of scPol η UBZ domain to ubiquitin determined by SPR

We used surface plasmon resonance (SPR) to obtain quantitative binding information on wild-type and mutant scPol η UBZ interacting with ubiquitin by following a published protocol³⁴. scPol η UBZ with a N-terminal GST tag was flowed across the SPR surface immobilized with ubiquitin and the sensorgram was recorded. A fast binding and dissociation of GST-UBZ to immobilized ubiquitin was observed (Fig. 4a). Plotting the steady-state SPR response units against the different concentrations of scPol η UBZ revealed a hyperbolic binding isotherm. The K_d for scPol η UBZ binding to ubiquitin was obtained by fitting the binding isotherm to a one-site binding equation^{34, 35}. An equilibrium dissociation constant (K_d) of 14.6 ± 2.5 μ M was obtained for the wild-type scPol η UBZ (Fig. 4b).

Binding affinity of the scPol η UBZ mutants to ubiquitin was also determined by SPR following the same protocol. The K_d for UBZ mutants, CC552,553AA (22.3 ± 4.1 μ M) and HH568,572AA (22.2 ± 4.8 μ M), are only slightly larger than that of the wild-type scPol η UBZ. This observation suggests that zinc binding in scPol η UBZ is not required for ubiquitin interaction.

Next we determined the ubiquitin-binding affinity of the D570A and A574F scPol η UBZ mutants. The K_d of D570A UBZ (23.6 ± 2.4 μ M) is only slightly higher than that of the wild-type UBZ. More significant effect was observed for the A574F UBZ mutant with a K_d of 38.6 ± 8.6 μ M, which is 2.6-fold higher than that of the wild-type UBZ.

The most profound effect on ubiquitin binding was observed for the UBZ mutants with a proline introduced in the α helix. As summarized in Table 1, a large increase in K_d was observed for A569P (43.1 ± 10.2 μ M), Q566P (48.4 ± 7.2 μ M), and A574P (55.3 ± 5.5 μ M), corresponding to a 2.9- to 3.8-fold decrease in binding affinity. Notably A574P mutation had the largest impact on the ubiquitin-binding affinity among all mutants analyzed.

Assess the binding affinity of the C-terminal portion of Pol η to ubiquitin

Given the low-affinity interaction between the scPol η UBZ and ubiquitin ($K_d = 14.6 \mu\text{M}$), we are interested in determining whether other sequence motif(s) in the Pol η C-terminal portion contributes to the binding of ubiquitin. To address this question, we generated a truncated scPol η containing the C-terminal portion (a.a. 515 to 632) of Pol η with a N-terminal GST tag. Remarkably, we obtained a K_d of $0.44 \pm 0.05 \mu\text{M}$ for ubiquitin binding by the C-term scPol η following a similar SPR binding protocol (Fig. 4c & d). This represents a 33-fold increase in binding affinity for ubiquitin compared to the isolated scPol η UBZ domain.

Moreover, an inspection of the SPR sensorgram revealed marked differences in the binding and dissociation kinetics compared to the minimal scPol η UBZ domain (see Fig. 4c). While the scPol η UBZ domain binds to ubiquitin instantaneously, the C-term Pol η binds to ubiquitin with a much slower rate. Moreover, the dissociation of C-term Pol η from ubiquitin is extremely slow, which is in sharp contrast to the UBZ domain's fast dissociation from ubiquitin. A global fitting of the sensorgram to a simple Langmuir binding model afforded a satisfactory fit of both association and dissociation phases (Supplementary Fig. 2). The fitted kinetic constants of C-term Pol η -ubiquitin association and dissociation, $k_{\text{on}} = 9.9 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ and $k_{\text{off}} = 1.7 \times 10^{-3} \text{ s}^{-1}$, allowed us to calculate the equilibrium dissociation constant of $0.17 \mu\text{M}$, which is close to the K_d of $0.44 \mu\text{M}$ obtained from fitting the steady-state SPR binding data.

Next, we introduced the same set of UBZ mutations into the C-term scPol η and determined their affinity for ubiquitin by SPR. As summarized in Table 2, the HH568,572AA and CC552,553AA C-term scPol η mutants demonstrated a K_d of $0.59 \pm 0.14 \mu\text{M}$ and $0.30 \pm 0.10 \mu\text{M}$, respectively, which are close to that of the wild-type C-term scPol η . No significant change in K_d was observed for D570A C-term scPol η ($K_d = 0.30 \pm 0.08 \mu\text{M}$). In contrast, a significant (2.2-fold) increase in K_d was observed for the A574F C-term scPol η mutant ($K_d = 0.97 \pm 0.28 \mu\text{M}$), similar to the change observed for the A574F UBZ domain. We also observed a 2- to 4- fold increase in K_d for A574P, A569P and Q566P C-term scPol η mutants. The measured equilibrium dissociation constants for the three mutants are $1.65 \pm 0.88 \mu\text{M}$, $0.92 \pm 0.26 \mu\text{M}$ and $0.77 \pm 0.18 \mu\text{M}$, respectively.

Function of the Pol η UBZ mutants assessed by polymerase exchange assay

To probe the effect of the various UBZ mutations on the function of Pol η , we utilized the polymerase exchange assay to assess the ability of Pol η to switch with Pol δ under stalled condition^{15, 36}. We introduced the mutations, CC552, 553AA, HH568, 572AA, D570A, A574F, Q566P, A569P, and A574P into the full-length scPol η . The mutations in UBZ did not affect scPol η 's DNA synthesis activity when assayed on a singly primed ssM13 DNA template in the presence of PCNA and RFC (Supplementary Fig. 3).

In the polymerase exchange assay, we observed a $34 \pm 5\%$ decrease in DNA synthesis in the presence of 160 nM wild-type Pol η (Fig. 5). In accord with the interaction studies, mutations of CC552, 553AA, and HH568, 572AA in the zinc-binding residues did not adversely affect the function of Pol η in polymerase exchange. A $41 \pm 3\%$ and $39 \pm 2\%$ decrease in DNA synthesis by Pol δ was observed when the CC552, 553AA and HH568, 572AA scPol η were assayed, respectively (Fig. 5). In contrast, all three UBZ proline mutants, Q566P, A569P, A574P, differed significantly from the wild-type Pol η in effecting polymerase exchange. The extent of polymerase exchange observed for Q566P, A569P, and A574P Pol η ($20 \pm 3\%$, $18 \pm 4\%$, and $19 \pm 6\%$ respectively) was approximately 1.5- to 2-fold lower compared to the wild-type Pol η , which agrees well with the fold of difference observed for the wild-type and mutant C-term Pol η binding to ubiquitin. A574F mutation in Pol η also resulted in close to

two-fold decrease in polymerase exchange efficiency ($18 \pm 2\%$ versus $34 \pm 5\%$ as observed for the wild-type Pol η). Interestingly, D570A Pol η showed no significant decrease in polymerase exchange assay ($30 \pm 6\%$), agreeing with the ubiquitin binding data.

The entire C-terminal region of Pol η is required for high-affinity ubiquitin binding

Prompted by the observation of a large difference in ubiquitin binding affinity between the UBZ domain and the entire C-terminal portion of scPol η , we designed a series of scPol η mutants with different truncation in the C-terminal region (Fig. 6). We tested the sequences that flank the minimal scPol η UBZ domain in ubiquitin binding. The equilibrium dissociation constants (K_d) of the various constructs are summarized in Table 3. UBZ-C (545–608), which contains the 28 amino acid sequence C-terminal to the UBZ domain, binds ubiquitin with a K_d of $15.8 \pm 3.7 \mu\text{M}$, similar to the minimal UBZ domain (545–580). UBZ-N (515–580), which contains the 30 amino acids N-terminal to the UBZ binds ubiquitin with a K_d of $18.2 \pm 2.2 \mu\text{M}$, again close to that of the minimal UBZ domain. We then generated a construct, UBZ-CN (515–615), which contains sequences flanking both the N- and C-terminus of UBZ, but lacks the PIP motif. Surprisingly, UBZ-CN binds ubiquitin with much reduced affinity ($K_d = 11.1 \pm 1.8 \mu\text{M}$), 25-fold lower compared to the entire Pol η C-terminal portion (515–632). The large impact of removing the C-terminal 17 amino acids on the ubiquitin-binding affinity was unexpected. This observation suggests that the C-terminal 17 amino acid sequence of yeast Pol η may directly interact with ubiquitin, thus contribute to the higher affinity of Ub binding observed in the complete Pol η C-terminal region. Alternatively, the deletion of the very C-terminal 17 amino acid sequence in Pol η may have an indirect impact on ubiquitin binding.

Discussion

To date a number of ubiquitin-binding domains or motifs have been described. The large number of ubiquitin-binding domains and motifs existing in eukaryotic cells underlies the versatility of ubiquitin as a signaling molecule in many cellular processes. Despite extensive structural and functional characterization of UBD, it is not yet clear how the low-affinity interaction between most UBDs and ubiquitin can mediate the formation of functional protein complexes. One attractive proposal to account for this conundrum is that the known low-affinity UBDs may cooperate with other domain/motif to achieve higher affinity interaction with ubiquitin. Direct evidence for such a notion has been scarce, partly because many reported binding studies for UBDs utilized isolated ubiquitin-binding domain. Notably, a previous study showed that the CUE domain in Vps9 can form a domain-swap dimer that binds ubiquitin at two different sites, the canonical Ile-44 patch and the region defined by Ile-36/Leu-73³⁷. As a result, a higher affinity of Ub binding ($K_d = 1.2 \mu\text{M}$) was observed for Vps9 CUE than a monomeric CUE ($K_d = 155 \mu\text{M}$)³⁸. In the case of Vps9 CUE, the two UBDs are contributed by separate polypeptides. It is possible that multiple ubiquitin-binding domains or motifs in a single ubiquitin receptor protein contribute to Ub binding by interacting with different surface patches on ubiquitin. This binding mode results in larger buried surface between ubiquitin and the ubiquitin receptor protein, thus leading to higher binding affinity. We addressed this possibility by investigating the *S. cerevisiae* Pol η -ubiquitin interaction.

We first carried out quantitative ubiquitin-binding studies of a minimal scPol η UBZ. Our data showed that the minimal scPol η UBZ retains the ability to bind zinc ion despite that it contains an uncanonical zinc finger, agreeing with a recent report on a larger Pol η UBZ construct³¹. Our quantitative binding study of CC552, 553AA and HH568, 572AA scPol η UBZ provided further support that zinc binding by UBZ is dispensable for its interaction with ubiquitin. This observation underlines the difference between the hPol η and scPol η UBZ in the mode of zinc binding.

Given the marked divergence between the scPol η and hPol η UBZ in their sequences and the zinc-binding mode, the question remains whether scPol η UBZ binds ubiquitin similarly to its human counterpart. We found that the affinity of isolated scPol η UBZ for ubiquitin is comparable to that reported for hPol η ³⁰. Our mutational analyses of the scPol η UBZ in binding ubiquitin support a model proposed for hPol η UBZ with the α helix in UBZ being the major ubiquitin-binding site³⁰.

Remarkably, when we determined the binding affinity of the C-terminal portion of scPol η (a.a. 515–632) to ubiquitin a submicromolar K_d of 0.44 μ M was obtained, representing a 33-fold increase in ubiquitin-binding affinity compared to the UBZ domain alone. Abolishment in zinc binding had little effect on the C-term Pol η 's affinity for ubiquitin, similar to the minimal scPol η UBZ domain. Mutation in the UBZ α helix either by introducing proline at position A574, A569 and Q566, or by introducing a bulky group at A574 in the C-term Pol η had significant effect on ubiquitin binding. Our result agrees with the study on human Pol η UBZ suggesting that A574 and its equivalent residue is located at the interface between UBZ and ubiquitin. An unexpected observation was made for D570 in scPol η UBZ. Although the equivalent residue, D652, in human Pol η UBZ was shown to be important for ubiquitin binding¹⁸, mutation of D570 to alanine in the scPol η UBZ had little effect on ubiquitin binding. The normal function of D570A scPol η in polymerase exchange assay provided further support that the ubiquitin binding ability of D570A scPol η is not adversely affected.

We further investigated the possibility that the sequence flanking the UBZ domain may contribute to ubiquitin binding. After testing several truncations of the C-terminal portion of scPol η , we found that adding flanking sequences to either the N- or C-terminus of the minimal UBZ domain did not restore the ubiquitin binding affinity observed for the complete C-term Pol η . Strikingly, even a truncation of the C-terminal seventeen residues (a.a. 616–632) of scPol η resulted in a large decrease (25 folds) in its affinity for ubiquitin when compared to the complete C-terminal portion of Pol η . It is not clear whether the scPol η C-terminal seventeen amino acids directly binds ubiquitin, or it contributes to ubiquitin binding indirectly. Combined with the observation that the C-term Pol η binds to ubiquitin with a slower ubiquitin-binding kinetics than the minimal UBZ domain, it is possible that a conformational/structural rearrangement in the C-term Pol η precedes ubiquitin binding. This rearrangement may expose the UBZ α -helix for interaction with ubiquitin and lead to increase in the buried surface area between C-term Pol η and ubiquitin. High-resolution structural information on the C-terminal portion of scPol η will be required to address this possibility.

The Pol η UBZ domain has been implicated in polymerase switch between the high and low fidelity polymerases, such as Pol δ and Pol η ³⁹. However, biochemical studies addressing the functional and structural details of UBZ's role in polymerase switch have been lacking. We investigated the effect of mutation in the scPol η UBZ on polymerase switch. We found that mutation of the zinc-binding residues (CC552,553AA and HH568,572AA) had little effect on Pol η 's ability to exchange with Pol δ . This observation is in accord with the normal ability of both mutants in binding ubiquitin. More pronounced effect was observed for several mutants with mutation introduced in the UBZ α helix. The approximately two-fold decrease in polymerase exchange efficiency is in accord with the decrease in the ubiquitin-binding affinity observed for the C-term Pol η mutants. Notably, the impact of UBZ mutations on Pol η 's function is less significant compared to the Pol η PIP mutation. It has been shown that mutation of the conserved PIP Phe residues to Ala or truncation of the PIP motif largely abolished yeast Pol η 's ability to exchange with Pol δ ¹⁵. Therefore, the PIP motif provides the primary interaction between Pol η and PCNA in polymerase switch, consistent with the recent genetic studies on the human Pol η ⁴⁰.

Another interesting observation centers on Asp570 in scPol η . We found that D570A scPol η showed no significant decrease in the polymerase exchange efficiency, consistent with a minimal decrease in its affinity for ubiquitin. However, the D570A mutation resulted in elevated level of UV sensitivity and UV mutagenesis in yeast cells³³. This points to the possibility that D570 in scPol η UBZ may be required for interaction with proteins involved in DNA damage response other than ubiquitin. Indeed, a recent study found that PDIP38 interacts with Pol η through the UBZ domain and contributes to the localization of Pol η to the repair foci⁴¹. Our results support the notion that scPol η may interact with more than one proteins through its UBZ domain. Different sets of residues on UBZ may be responsible for the interaction with specific partners.

A high-resolution structure of the scPol η C-terminal portion is yet to be determined. Our attempt to identify the fold of the scPol η C-terminal region failed to retrieve any reliable structural homolog. Our limited proteolysis study revealed that multiple sites in the scPol η C-terminal sequence are vulnerable to proteolytic cleavage (J. Wang and Z. Zhuang, unpublished work). This result suggests that the C-terminal region of the scPol η likely adopts a flexible conformation. The flexibility of scPol η helps to explain the previous observation that monoubiquitination at several different positions on PCNA can lead to functional Pol η -(Ub)PCNA complex³⁶.

Our secondary structure analysis revealed that the region between UBZ and PIP motif is largely disordered, and likely acts as a hinge that allows movement of the UBZ relative to PIP motif once Pol η binds to Ub-PCNA. The available structural information of archaeal Dpo4 bound to PCNA1-PCNA2 dimer suggests a potentially flexible conformation because of the modular structure of Dpo4 connected by two peptide hinges⁴². It should be pointed out that in Dpo4, the little finger domain (or PAD) is juxtaposed to the PIP motif. In comparison, there are more than 100 amino acids embedded between the scPol η PAD and the PIP motif. The extra sequence likely contributes to a higher degree of flexibility in its conformation. Among the available eukaryotic Pol η sequences, the C-terminal region of Pol η is highly diverged compared to the more conserved catalytic core domain. Our observation that removing the C-terminal seventeen residues led to drastic decrease in scPol η 's ubiquitin binding affinity warrants further structural and functional elucidation of the C-terminal region of Pol η .

Experimental

Construction and purification of Pol η UBZ and truncated Pol η

The genes encoding the *S. cerevisiae* Pol η UBZ domain and the truncated Pol η C-terminal region were cloned into the pGEX-4T3 vector (Amersham Biosciences) with a N-terminal GST-tag. The Pol η UBZ (a.a. 545–580) was cloned using primer UBZ+ (5'-CTACCAGCTCGAAAGGATCCGAGAAAACCTCCGAAGTTGG-3') and UBZ- (5'-GGATGATTCTTCAGCCTCGAGTCAGCCTTCCGACAGTTTC-3'). The entire C-terminal portion of Pol η (C-term Pol η , a.a. 515–632) was cloned using primers, C-term Pol η + (5'-GTGCCGCGCGGCAGCGGATCCGTTGTAGATATGTTTGGC-3') and C-term Pol η - (5'-TAATTGTCTATTTGGCTCGAGTCATTTTTTCTTGTAATAAATG-3'). UBZ-C (a.a. 545–608) was cloned using primers UBZ-C+ (5'-ACCAGCTCGAAAGGATCCGAGAAAACCTCCGAAGTTGG-3') and UBZ-C- (5'-CTTCTGAGGTGTCTCGAGTCATTGTGAGTTTGGTCTTTTCCG-3'). UBZ-N (a.a. 515–580) was cloned using primers UBZ-N+ (5'-GTGCCGCGCGGCAGCGGATCCGTTGTAGATATGTTTGGC-3') and UBZ-N- (5'-GGATGATTCTTCAGCCTCGAGTCAGCCTTCCGACAGTTTC-3'). UBZ-CN (a.a. 515–615) was constructed by introducing a stop codon at amino acid position 616 of the C-term Pol η -pGEX4T3 construct using QuikChange PCR primers UBZ-CN+ (5'-

CTGCCACACCTCAGAAGTGACAAGTTACATCTTCC -3') and UBZ-CN- (5'-GGAAGATGTAACCTTGTCACCTTGAGGTGTGGCAG -3'). QuikChange PCR was used to introduce mutation (CC552,553AA, HH568,572AA, D570A, A574F, Q566P, A569P and A574P) into the UBZ domain and the C-term Pol η , respectively. The GST fusion protein was overexpressed in Rosetta(DE3) cells (Novagen) upon induction with 0.2 mM IPTG. Cell pellet was resuspended in lysis buffer (25 mM Tris•HCl, pH 6.9, 10% glycerol, 1 mM DTT, 1 mM EDTA) supplemented with protease inhibitor cocktail (Roche). Cells were lysed by sonication and cleared by centrifugation. Following incubation with glutathione resin (GE Biosciences), the resin was washed extensively with the lysis buffer. The target protein was eluted in elution buffer (25 mM Tris•HCl, pH 6.9, 10% glycerol, 33 mM Glutathione). The pooled protein fraction was further purified by 5 ml HiTrap SP-FF (GE Biosciences) for C-term Pol η (WT and mutants), UBZ-C, and UBZ-CN or by 5 ml HiTrap Q-FF (GE Biosciences) for UBZ (WT and mutants) and UBZ-N. The target protein was eluted with a salt gradient in 25 mM Tris•HCl, 10% glycerol, pH 6.9.

NMR Chemical shift perturbation

The ^{15}N -labeled *S. cerevisiae* ubiquitin was prepared using a bacterial expression system as previously described³⁶ except in M9 minimal media containing $^{15}\text{NH}_4\text{Cl}$ as the sole nitrogen source (Cambridge Isotope Laboratories, Andover, MA, USA). All samples for NMR experiments were prepared in a buffer containing 50 mM Tris•HCl, pH 7.0, 100 mM NaCl, 2 mM DTT and 10% D_2O . The two-dimensional ^1H - ^{15}N TROSY NMR spectra were acquired with a Bruker Avance AV600 spectrometer equipped with a 5 mm inverse CryoProbe at 25 °C. NMR data were processed by NMRpipe⁴³ and analyzed with Sparky 3. The residues in ubiquitin were assigned based on the previously published HSQC spectra^{30, 44}. Chemical shift perturbation experiments were performed by mixing a 2-fold molar excess of unlabeled GST-UBZ with ^{15}N -labeled ubiquitin. The changes in chemical shift ($\Delta\delta_{\text{H}}$ and $\Delta\delta_{\text{N}}$) were calculated for each identified residue, except for those residues severely attenuated in the scPol η UBZ-ubiquitin mixture.

Circular Dichroism of scPol η UBZ domain

For CD experiment, the UBZ domain was obtained by removing the GST tag from GST-UBZ with thrombin (GE Healthcare). The purified UBZ domain was dialyzed in KH_2PO_4 buffer (pH 7.5). Far-UV CD spectra (190~250 nm) were collected in a Jasco J-810 spectropolarimeter (Jasco Inc.) using in a rectangular quartz cuvette of 1.0 mm path length at 20 °C. Spectra were acquired at 50 nm/sec with a time constant of 4 sec and a band width equal to 1 sec. The KH_2PO_4 buffer was used as a blank. The mean residue molar ellipticity $[\theta]$ was determined using equation 1:

$$[\theta] = \theta / (C \times N \times l) \quad \text{eq. 1}$$

where θ is the measured ellipticity in millidegree, C is the molar concentration of protein, N is the number of amino acids, l is the cell path length in centimeter.

Determine the zinc content of wild-type and mutant scPol η UBZ

Wild-type and mutant UBZ were obtained after the removal of GST tag. The minimal UBZ domain was treated with DTT and zinc sulfate, and then dialyzed against HNG buffer (30 mM HEPES, pH 8.0, 10% glycerol, 350 mM NaCl, treated with Chelex 100 resin). 1 μM wild-type or mutant scPol η UBZ was added in HNG buffer containing 0.1 mM 4-(2-pyridylazo)-resorcinol (PAR). The mixture was titrated with *p*-hydroxymercuribenzoic acid sodium salt (PHMB) to release the zinc ion from the scPol η UBZ. The equivalent of

released zinc ion was determined by the absorbance at 500 nm using an ϵ_{500} value of 66,000 $\text{M}^{-1} \text{cm}^{-1}$ for the $(\text{PAR})_2\cdot\text{Zn}^{2+}$ complex. The HNG buffer was used as a control.

Determine the ubiquitin binding affinity by surface plasmon resonance

The real-time surface plasmon resonance (SPR) binding measurements were performed on the Biacore 3000 system (Uppsala, Sweden). The running buffer used for analysis contains 10 mM HEPES, pH 7.4, 150 mM NaCl, 0.005% P20 detergent (GE Healthcare). All buffer solutions were freshly prepared, degassed, and passed through a 0.22 μm pore size filter. Ubiquitin was immobilized on the CM5 chip surface following the standard EDC/NHS coupling chemistry as previously described^{34, 45}. The 1:1 mixture of 0.05 M N-hydroxysuccinimide (NHS) and 0.2 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was flowed through the flow cell for 20 min at a flow rate of 5 $\mu\text{l}/\text{min}$. Then ubiquitin (10 μM) in sodium acetate buffer (10 mM, pH 5.5) were passed over the surface at 5 $\mu\text{l}/\text{min}$ for 40 min, followed by a blocking step using ethanolamine (1 M, pH 8.5) at 5 $\mu\text{l}/\text{min}$ for 20 min. The SPR binding experiments were performed at 25 °C using a flow rate of 20 $\mu\text{l}/\text{min}$. For each experiment, a underivatized flow cell was used as the reference surface. Six different concentrations of each protein were injected over the experimental and reference flow cell. The NaCl solution (1 M) was used to regenerate the surface after each injection cycle. The sensorgrams were processed using the double-referencing technique as described by Myszkowski *et al.*⁴⁶ to correct against both the underivatized surface and the buffer component that could generate signal. To obtain the equilibrium dissociation constant (K_d), the steady-state SPR response of the wild-type or mutant Pol η UBZ binding to ubiquitin were fitted to equation 2:

$$R_{eq} = C \cdot R_{max} / (K_d + C) \quad \text{eq. 2}$$

where R_{eq} is the steady-state response, C is the analyte concentration, R_{max} is the maximum binding response and K_d is the equilibrium dissociation constant. For C-term Pol η binding to ubiquitin, the sensorgram did not reach saturation due to the intrinsically slow binding kinetics. Thus R_{eq} was obtained by fitting the sensorgrams to a 1:1 binding model using software BIAevaluation 3.2. K_d was obtained by fitting R_{eq} to equation 2 as described above. The binding and dissociation rate constants (k_{on} and k_{off}) were fitted using the software BIAevaluation 3.2 to a 1:1 binding with mass transfer model.

DNA polymerase exchange assay

A reaction mixture containing 12 nM Pol δ , 150 nM monoubiquitinated PCNA, 60 nM RFC, 2.3 nM singly primed ssM13 DNA, 1.4 μM single-stranded DNA binding protein (*E. coli* SSB), 1 mM ATP, 50 μM dATP and 50 μM dTTP was incubated in a reaction buffer (25 mM Tris, pH 7.5, 5 mM MgCl_2 , 10% glycerol, 50 mM NaCl, and 0.1 mg/ml BSA) at 37 °C for 1 minute to allow the formation of Pol η -PCNA holoenzyme on DNA. Then 160 nM wild-type or mutant full-length Pol η was added into the mixture and incubated for 1 min. DNA synthesis was initiated by adding 50 μM dNTPs containing [α -³²P]dGTP. The reaction was allowed to proceed for 30 seconds before being quenched with equal volume of 500 mM EDTA (pH 8.0). The DNA synthesis product was separated on a 1.2% alkaline agarose gel and quantified by PhosphorImager (Storm, GE Healthcare Bioscience) as previously described^{15, 36}.

Primer extension assay

A reaction mixture containing 5 nM singly primed ssM13 DNA, 2.5 nM Pol η , and 300 nM RPA were incubated in the presence or absence of 90 nM PCNA and 5 nM RFC in the assay buffer containing 40 mM Tris, pH 7.5, 8 mM MgCl_2 , 150 mM NaCl, 1mM DTT, 100 $\mu\text{g}/\text{ml}$

BSA, 0.5 mM ATP at 30 °C for 1 min. The reactions were then initiated by addition of 100 μ M dNTP and allowed to proceed for 10 min before quenched by equal volume of 500 mM EDTA (pH 8.0) solution. The reaction solution were treated with phenol-chloroform and precipitated with 100% ethanol. The DNA synthesis products were separated on a 10% urea-polyacrylamide gel and imaged by PhosphorImager (Storm, GE Healthcare Bioscience)

Conclusions

Ubiquitin-binding domains or motifs are essential mediators in a number of cellular processes. We demonstrated that the translesion synthesis DNA polymerase η in *S. cerevisiae* binds ubiquitin in a novel mode that has not been observed for the isolated UBZ domain. Quantitative binding study revealed high-affinity interaction between the C-terminal portion of Pol η and ubiquitin. Given the widespread presence of ubiquitin-binding zinc finger (UBZ) and ubiquitin-interacting motif (UIM) in many newly identified ubiquitin receptor proteins, our findings provided useful insights into how the high-affinity ubiquitin binding can be achieved through the otherwise low-affinity ubiquitin-binding domains/motifs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Sequence alignment of the Polη UBZ domains from different organisms. The UBZ domains of *Homo sapiens* (Hs), *Mus musculus* (Mm), *Rattus norvegicus* (Rn), *Drosophila melanogaster* (Dm), *Xenopus laevis* (Xl), *Schizosaccharomyces pombe* (Sp), and *Saccharomyces cerevisiae* (Sc) Polη were aligned. Highly conserved residues are highlighted. The potential zinc-binding residues are in red color.

Fig. 2. Far UV-CD spectrum of the wild-type scPolη UBZ domain (a.a. 545–580). The molar ellipticity was calculated as described in the Experimental section.

Fig. 3. Zinc binding of the wild-type and mutant scPol η UBZ domain. The molar concentration of zinc ion was determined by PAR colorimetric assay. Increasing concentration of PHMB was titrated into the solution containing scPol η UBZ. The absorbance at the plateau was used to calculate the stoichiometry of zinc binding. The titration curves of wild-type UBZ (diamond), CC552,553AA (triangle), HH568,572AA (square), A574P (circle) are depicted. The dialysis buffer was used as control.

Fig. 4. Surface plasmon resonance analysis of ubiquitin binding by Polη UBZ domain and C-term Polη. (a) Sensorgram of wild-type Polη UBZ binding to ubiquitin. Polη UBZ was injected at concentrations of 3.1, 6.2, 12.5, 25, 50 and 100 μM. (b) Fitting of the maximum response unit against the various Polη UBZ concentration to a one-site binding isotherm. (c) Sensorgram of wild-type C-term Polη binding to ubiquitin. C-term Polη was injected at concentrations of 0.06, 0.12, 0.25, 0.5, 1 and 2 μM. (d) Fitting of the maximum response unit against the various C-term Polη concentration to a one-site binding isotherm.

Fig. 5.

The effect of Pol η UBZ mutations on polymerase exchange between Pol δ and Pol η . The DNA synthesis product by the Pol δ holoenzyme in the presence of wild-type or mutant Pol η was separated by alkaline agarose gel electrophoresis. The absolute amount of DNA product was determined as the incorporation of [α - 32 P]dGMP. The decrease in DNA synthesis in the presence of wild-type or mutant Pol η was expressed as a percentage relative to the synthesis in the absence of Pol η . The data are average of at least three independent measurements.

Fig. 6.

A schematic illustration of the C-terminal portion of scPolη and the various constructs corresponding to the truncated Polη C-terminal region.

Table 1

K_d of Polη UBZ binding to ubiquitin

Polη UBZ	<i>K_d</i> (μM)	Fold of increase
WT	14.6 ± 2.5	1
HH568,572AA	22.2 ± 4.8	1.5
CC552,553AA	22.3 ± 4.1	1.5
D570A	23.6 ± 2.4	1.6
A574F	38.6 ± 8.6	2.6
A574P	55.3 ± 5.5	3.8
A569P	43.1 ± 10.2	2.9
Q566P	48.4 ± 7.2	3.3

Table 2 K_d of C-term Polh binding to ubiquitin

C-term Polh	K_d (μ M)	Fold of increase
WT	0.44 ± 0.05	1.0
HH568,572AA	0.59 ± 0.14	1.3
CC552,553AA	0.30 ± 0.10	0.7
D570A	0.30 ± 0.08	0.7
A574F	0.97 ± 0.28	2.2
A574P	1.65 ± 0.88	3.7
A569P	0.92 ± 0.26	2.1
Q566P	0.77 ± 0.18	1.8

Table 3

K_d of truncated C-term Polh binding to ubiquitin

Truncated C-term Polh	K_d (μ M)	Fold of increase
C-term Polh (515~632)	0.44 ± 0.05	1
UBZ-N (515~580)	18.2 ± 2.2	41
UBZ-C (545~608)	15.8 ± 3.7	36
UBZ-CN (515~615)	11.1 ± 1.8	25