

Intrinsic Flexibility of Ubiquitin on Proliferating Cell Nuclear Antigen (PCNA) in Translesion Synthesis*^[5]

Received for publication, June 8, 2012, and in revised form, August 29, 2012. Published, JBC Papers in Press, September 18, 2012, DOI 10.1074/jbc.M112.389890

Richard G. Hibbert¹ and Titia K. Sixma²

From the Division of Biochemistry and Center for Biomedical Genetics, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

Background: The conjugation of ubiquitin to a target is a common signaling mechanism, but structures of ubiquitinated substrates are lacking.

Results: Native ubiquitinated PCNA can be produced enzymatically to study its structure in solution.

Conclusion: The ubiquitin moiety is flexible when conjugated to PCNA, allowing efficient signaling.

Significance: Flexibility is likely to be a general feature of conjugated ubiquitin.

Ubiquitin conjugation provides a crucial signaling role in hundreds of cellular pathways; however, a structural understanding of ubiquitinated substrates is lacking. One important substrate is monoubiquitinated PCNA (PCNA-Ub), which signals for recruitment of damage-tolerant polymerases in the translesion synthesis (TLS) pathway of DNA damage avoidance. We use a novel and efficient enzymatic method to produce PCNA-Ub at high yield with a native isopeptide bond and study its Usp1/UAF1-dependent deconjugation. In solution we find that the ubiquitin moiety is flexible relative to the PCNA, with its hydrophobic patch mostly accessible for recruitment of TLS polymerases, which promotes the interaction with polymerase η . The studies are a prototype for the nature of the ubiquitin modification.

Conjugation of ubiquitin to a target protein is a critical post-translational modification that is involved in most cellular processes. The reaction is catalyzed by a cascade of E1/E2/E3 ubiquitin-conjugating enzymes. The enzymes can monoubiquitinate a substrate or can form ubiquitin chains on the target. The vital role of ubiquitin conjugation is comparable with other major post-translational modifications, such as phosphorylation or glycosylation, but the physical nature of the modification is less well understood. Phosphorylation is primarily a defined and structured modification found at a binding interface (1). In contrast, oligosaccharides at glycosylation sites are usually flexible (2). It remains to be resolved where ubiquitin modification lies between these extremes of flexibility. Ubiquitinated PCNA (PCNA-Ub)³ within the translesion synthesis (TLS) pathway is a candidate to address this.

TLS is a DNA damage response pathway in which specialized TLS polymerases can accurately replicate past sites of damage at blocking lesions and hence allow replication to be completed (3, 4). One of the earliest steps in the pathway is the monoubiquitination of PCNA, which specifically occurs on lysine 164. This modification is exclusively performed by E2/E3 ubiquitin-conjugating enzymes Rad6 and Rad18 in yeast (5), but low levels of Rad18-independent PCNA ubiquitination can be detected in higher eukaryotes (6), with E3 ligases Rnf8 and CLR4 (Cdt2) proposed as candidates for this activity (7, 8). The modification provides a signal for recruitment and regulation of the TLS polymerases at DNA damage sites (9). Most TLS polymerases contain conserved ubiquitin binding motif or ubiquitin binding zinc finger ubiquitin binding domains that are required for recognition of the PCNA-Ub, and *in vivo* activity (10, 11). Structural studies have shown that most of the ubiquitin binding domains interact with the hydrophobic patch of ubiquitin surrounding isoleucine 44 (10, 12). The ubiquitin binding motifs work in tandem with PCNA-interacting peptide motifs to recruit TLS polymerases to ubiquitinated PCNA (13). An extended area surrounding the PCNA-interacting peptide motifs is likely to provide the majority of the binding energy of the interaction (14, 15). PCNA ubiquitination is reversible, with Usp1 providing the deconjugation activity (16) and UAF1 serving as an activator (17). The Usp1/UAF1 complex is recruited to PCNA-Ub via an interaction of UAF1 with Elg1, an alternative component of the clamp loader (18).

Interest in both the molecular mechanisms of TLS and in the structure of ubiquitinated substrates has inspired several methods to produce PCNA-Ub. Native ubiquitinated PCNA can be produced enzymatically, but current protocols have low yields and do not reach completion (19–22). These methods have enabled biochemical reconstitution of parts of the TLS pathway, including polymerase binding, exchange, and activation (19, 20, 23), but are not suitable to produce PCNA-Ub for structural studies. Alternatively, chemical mimics of PCNA-Ub are available. These include an intein-based method to cross-link

* This work was supported by the European Union RUBICON Network of Excellence and an European Research Council advanced grant (to T. K. S.).
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^[5] This article contains supplemental Figs. S1–S4 and additional references.

¹ To whom correspondence may be addressed. Tel.: 31205121957; E-mail: r.hibbert@nki.nl.

² To whom correspondence may be addressed. Tel.: 31205121959; E-mail: t.sixma@nki.nl.

³ The abbreviations used are: PCNA, proliferating cell nuclear antigen; PCNA-Ub, ubiquitinated PCNA; Bis-Tris propane, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; HSQC, heteronuclear single quantum

coherence; MALS, multiangle light scattering; MMT, malic acid-MES-Tris; PDB, Protein Data Bank; Pol, polymerase; SAXS, small angle x-ray scattering; SEC, size exclusion chromatography; TAMRA, tetramethylrhodamine; TCEP, Tris (2-carboxyethyl)phosphine; TLS, translesion synthesis.

the C terminus of modified ubiquitin and a single-cysteine variant of PCNA with a disulfide bond (23); “split” PCNA-Ub, where the N terminus of PCNA is co-expressed with a linear fusion of ubiquitin with the C terminus of PCNA (24), and a “click chemistry”-derived mimic (25). However, the nature of these mimics limits their applicability because they are altered at the site of covalent attachment of ubiquitin to PCNA.

These chemical mimics have facilitated early structural studies on PCNA-Ub (24, 26). A crystal structure of split PCNA-Ub has been elucidated (24). In this structure, the hydrophobic patch of ubiquitin packs against the side of PCNA. However, because the ubiquitin moiety is not available for recruitment of ubiquitin-binding TLS polymerases in this arrangement, the state that has been captured in the crystal is probably not relevant to TLS. The study highlights a fundamental problem of studying the structure of the ubiquitin modification using x-ray crystallography: crystallization of flexible complexes may select specific conformations that are favored by crystal contacts but not relevant in solution or in the cell.

A recent study, conducted independently of this paper, addresses the structure of split and cross-linked PCNA-Ub in solution using small angle x-ray scattering (SAXS) and molecular modeling (26). The study estimates that PCNA-Ub is ~70–80% ordered. The isolated crystal structure is not consistent with the SAXS data, but by considering an ensemble of several different orientations of the ubiquitin moieties, including contributions from the crystal structure and defined orientations derived from molecular modeling, the fits to the SAXS profiles improve (26). SAXS is evolving as an important technique for studying the structure of proteins in solution, but such ensemble-driven SAXS methods require further validation.

Here, we achieve efficient and specific PCNA monoubiquitination, using ubiquitin-conjugating enzyme UbcH5c. Our method allows production of large amounts of native PCNA-Ub for the first time. The physiological linkage of the complex enables study of the Usp1/UAF1-dependent deubiquitination of PCNA. Finally, we analyze the conformation of natively modified PCNA in solution using biophysical techniques including SAXS and NMR and find the ubiquitin to be flexible with respect to the PCNA and thus able to promote the interaction with polymerase η .

EXPERIMENTAL PROCEDURES

Plasmids—The expression plasmids for full-length human ubiquitin, PCNA, E1 (Uba1), UbcH5c (S22R), ^{21–785}Usp1 (G671A,G672A), ^{6–677}UAF1, and ^{602–713}Pol η have been described (15, 21, 27, 28). The PCNA K164R point mutant was generated by a QuikChange reaction (Agilent Technologies), according to the manufacturer’s instructions.

Protein Expression and Purification—Human ubiquitin, PCNA, E1 (Uba1), UbcH5c (S22R), and Pol η were expressed in BL21 (DE3) *Escherichia coli* cells (Merck) and were purified as described (15, 21, 27). Minimal medium containing ¹⁵N-NH₄Cl (Cambridge Isotope Laboratories, Cambridge, MA) was used to express the ¹⁵N-ubiquitin for the NMR experiments. Usp1 and UAF1 were expressed in Sf9 insect cells (Invitrogen) and purified as described (17). N-terminally carboxytetramethylrhod-

amine (TAMRA)-labeled ubiquitin was synthesized and purified as described (29).

Ubiquitination of PCNA—Initial conjugation reactions contained 80 nM E1, 2 μ M UbcH5c (S22R), 16 μ M ubiquitin, and 8 μ M PCNA or PCNA (K164R) in a buffer containing 50 mM Tris or malic acid-MES-Tris (MMT) buffer, pH 8.0, 100 mM NaCl, 3 mM MgCl₂, 0.5 mM Tris-(2-carboxyethyl)phosphine (TCEP), and 3 mM ATP. Experiments were performed for 2 h at 37 °C in a 10- μ l volume. The reaction was systematically optimized, and the final conditions contained 80 nM E1, 32 μ M UbcH5c (S22R), 32 μ M ubiquitin, and 16 μ M PCNA in a buffer containing 50 mM MMT pH 9.0, 25 mM NaCl, 3 mM MgCl₂, 0.5 mM TCEP and 3 mM ATP.

Ubiquitinated PCNA was initially purified using a 6-ml Resource Q column (GE Healthcare) via a 20-column volume linear gradient from 100 to 500 mM NaCl in 10 mM HEPES, pH 7.5, 0.5 mM TCEP. The PCNA-Ub eluted at ~300 mM NaCl. The product was finally purified using an S200 16/60 size exclusion chromatography column (GE Healthcare) using 10 mM HEPES, pH 7.5, 150 mM NaCl, 0.5 mM TCEP as the running buffer. The gel filtration column was calibrated using bovine thyroid thyroglobulin, rabbit muscle aldolase, hen egg ovalbumin, and bovine pancreas ribonuclease A (GE Healthcare), to calibrate the elution volume to the molecular mass of the analyte. Nonglobularity of PCNA and PCNA-Ub causes the proteins to elute earlier from a SEC column than spherical proteins of the same molecular mass. This results in an overestimation of the SEC-derived masses.

Deubiquitination of PCNA—The deubiquitination reaction was performed at 37 °C in a 10- or 20- μ l volume. The reaction conditions contained 3 μ M PCNA-Ub and 150 or 50 nM Usp1 or Usp1/UAF1 in a buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 0.5 mM TCEP, and 1 mM EDTA, unless otherwise stated.

Quantification of Enzyme Reaction Rates—Reactions mixtures were analyzed on homemade 12.5% or 15% SDS-PAGE gels that were made using 40% 19:1 acrylamide/bisacrylamide (Bio-Rad) according to standard protocols and resolved using Coomassie Brilliant Blue solution, unless otherwise stated. All experiments were performed at least twice and were quantified using a ChemiDoc (Bio-Rad) under conditions that were within the linear range of detection. The fluorescent signal from the experiment using TAMRA-labeled ubiquitin was detected prior to Coomassie staining. Error bars show the difference in extent of reaction between the two experiments, normalized to the extent of reaction within a lane, unless otherwise stated. Michaelis-Menten kinetics were calculated by fitting the data to the Michaelis-Menten equation using Graphpad Prism. The amount of product produced at a single early time point was used as the initial rate of reaction. This assumes a constant reaction rate to this time point.

Size Exclusion Chromatography (SEC) in Line with Multi-angle Light Scattering (MALS)—MALS experiments were performed at 4 °C on a Mini-Dawn light scattering detector (Wyatt Technology) online with a Superdex S200 10/30 column (GE Healthcare) in 10 mM HEPES, pH 8.0, 150 mM NaCl, 0.5 mM TCEP buffer. Refractive index and light scattering detectors were calibrated against toluene and BSA. Scattered light was

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detected at angles of 41.5°, 90.0°, and 138.5°. The molecular mass and root mean square radius measurements and errors thereof were calculated using the Zimm method (30) according to manufacturer's protocols. The column was calibrated using molecular mass standards, to calibrate the elution volume with molecular mass, and to confirm the calibration of the detectors.

SAXS—PCNA and PCNA-Ub were purified using an S200 16/60 size exclusion column using a buffer containing 10 mM HEPES, pH 8.0, 150 mM NaCl, 0.5 mM TCEP, to buffer-exchange the samples and to remove any aggregates. Samples of the running buffer were collected for use as a blank measurement in the SAXS analysis. SAXS data were collected on Bio-SAXS beamline ID14-3 at the European Synchrotron Radiation Facility or the X33 beamline at the Deutsches Elektronen Synchrotron (Hamburg, Germany) on several occasions during the optimization of conjugation reaction. Data were recorded on both PCNA and PCNA-Ub samples at a range of concentrations from ~0.5 to ~12.5 mg/ml. To ensure that radiation damage was not altering the scattering curves, sequential frames of 10-s exposure were collected, compared, and averaged. The data were processed using the ATSAS Package (31). Molecular masses were calibrated using BSA as a standard. Estimates of the radii of gyration for the PCNA and PCNA-Ub used a Guinier analysis that was linear over the selected points. The shapes of the scattering curves were similar for each protein regardless of the concentration. However, low concentration samples contained little high resolution data, whereas the highest concentration samples deviated at low angles. The scattering curves presented here were prepared by merging three sets of data for both PCNA and PCNA-Ub: low resolution data from ~1 mg/ml samples, medium resolution data from 3–6 mg/ml samples, and high resolution data from ~12.5 mg/ml samples. Theoretical scattering curves of PCNA or ubiquitinated PCNA were calculated using Crysol in the ATSAS package (31).

Nuclear Magnetic Resonance (NMR)—NMR experiments were performed in NMR buffer (20 mM Bis-Tris propane, pH 7.0, 200 mM NaCl, 0.5 mM TCEP, 5% D₂O) on a Bruker Avance2 750 MHz NMR spectrometer, at 298 K. The ¹H-¹⁵N HSQC spectra were recoded with a protein concentration of 300 μM. Ubiquitin chemical shift assignments were based on previous resonance assignments (Biological Magnetic Resonance Data Bank entry 6457). Normalized chemical shift changes were calculated as $\Delta\delta_{\text{ppm}} = ((\Delta\delta_{\text{HN}})^2 + (\Delta\delta_{\text{N}}/5)^2)^{1/2}$. Data were analyzed using SPARKY (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco).

Analytical Gel Filtration—Experiments were performed on an ÄKTA micro using a Superdex 200 PC 23.2/30 column (GE Healthcare) in a buffer containing 10 mM HEPES, pH 7.5, 100 mM NaCl, 2 μM ZnCl₂, and 0.5 mM TCEP. PCNA (50 μM), PCNA-Ub (50 μM), and ⁶⁰²⁻⁷¹³Polη (100 μM), or preincubated complexes thereof, were loaded in a volume of 50 μl. The elution profile was analyzed at 280 nm. Fractions of each run were collected and visualized on a 15% SDS-PAGE by Coomassie staining.

Molecular Modeling and Graphics—Models of PCNA or PCNA-Ub were prepared using the available structures of native human PCNA (PDB code 1VYM (32)) and ubiquitin (taken from the structure with PDB code 1KJH (33)). The ubiq-

uitin was manually placed at different orientations relative to the PCNA using Swiss PDB Viewer. Alternatively, the trimer of split PCNA was prepared by adding the symmetry mates to the crystal structure (PDB code 3LOW (24)). The radii of gyration of the different models were estimated using Yasara. Figures of the molecular models were generated using PyMOL (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC).

RESULTS

We investigated whether PCNA-Ub can be produced using recombinant UbcH5c, which is active toward PCNA with E3 enzymes Rnf8 and CRL4 (Cdt2) (7, 8). Surprisingly, UbcH5c will ubiquitinate PCNA, even in the absence of an E3 enzyme (Fig. 1 and supplemental Fig. S1). We studied whether UbcH5c retains specificity for monoubiquitination of the Lys-164 site. We did not detect modification of a K164R point mutant of PCNA under any conditions (Fig. 1 and supplemental Fig. S1). The reaction favors monoubiquitination of PCNA when wild-type UbcH5c is used as the E2 enzyme, and we exclusively detected monoubiquitination of PCNA with an S22R point mutant of UbcH5c that does not form ubiquitin chains (27). This shows that the reaction is specific for monoubiquitination of lysine 164 (Fig. 1 and supplemental Fig. S1).

We show that the rate of the reaction increases with UbcH5c and PCNA protein concentrations and reaction temperature. The reaction is optimal under alkaline conditions and in low ionic strength buffers (Fig. 1 and supplemental Fig. S1). Using optimized conditions based on the results of this screen, we can rapidly and efficiently produce and purify PCNA-Ub in milligram amounts (Fig. 1D).

Usp1 is the physiologically relevant deubiquitinating enzyme for PCNA-Ub and is activated by UAF1 (16, 17). We reconstituted the Usp1/UAF1-dependent PCNA-Ub deubiquitination *in vitro*, using a published construct of Usp1 that lacks 20 N-terminal residues and an autocleavage site, together with almost full-length UAF1 (17). Whereas UAF1 displays no detectable activity, and isolated Usp1 shows only low levels of activity toward PCNA-Ub, stoichiometric mixtures of Usp1 and UAF1 are highly active, confirming that the PCNA-Ub can be targeted by Usp1/UAF1 (Fig. 2). We performed a preliminary estimation of the Michaelis-Menten kinetics of the reaction, using a single time point assay (supplemental Fig. S2) and found that UAF1 predominantly activates the catalytic turnover (k_{cat}) of Usp1, without a significant effect on the affinity (K_m) for PCNA-Ub. The kinetic constants are consistent with published constants, estimated using Ub-AMC, a fluorescent mimic of a ubiquitin conjugate (supplemental Fig. S2 and Ref. 17), although our calculated k_{cat} values are somewhat lower. These data support models in which the Usp1/UAF1 complex is indirectly recruited to PCNA-Ub (18).

The availability of natively modified PCNA-Ub allows study of the structure of the molecule to understand whether the ubiquitin is flexible or ordered with respect to the PCNA. Several biophysical techniques were used to study the properties of PCNA-Ub in solution (Fig. 3). SEC was used to separate PCNA and PCNA-Ub based upon their radius of gyration (Fig. 3, A and E) and MALS in-line with SEC allowed quantification of these

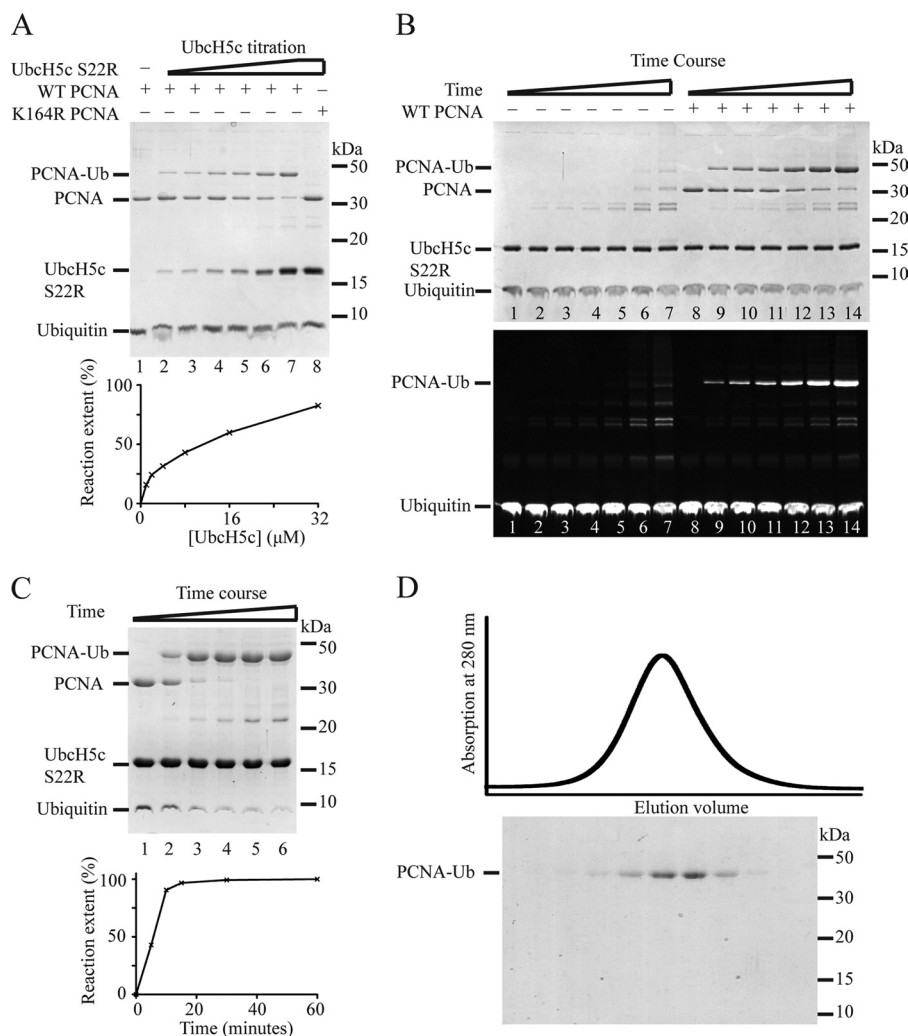


FIGURE 1. Lys-164-linked PCNA-Ub can be produced enzymatically. *A*, the rate of reaction increases with UbH5c (S22R) concentrations of 0, 1, 2, 4, 8, 16, and 32 μM in lanes 1–7 in reactions containing wild-type PCNA. Lane 8 contains 32 μM UbH5c (S22R) with K164R-PCNA. The extent of the reaction is shown as a Coomassie-stained SDS-polyacrylamide gel and a quantified representation. The K164R mutant of PCNA is not modified by 32 μM UbH5c (S22R) under these conditions, showing the high degree of specificity of the reaction for this lysine residue. *B*, time course of enzyme reaction in the absence (lanes 1–7) or presence (lanes 8–14) of PCNA, using TAMRA-ubiquitin. The reaction was performed using optimized conditions, except with 150 mM NaCl, at time points of 0, 5, 10, 15, 30, 60, and 120 min. The reaction was followed by Coomassie staining (top) or via the fluorescent TAMRA signal (bottom) and demonstrates that the reaction is highly specific for monoubiquitination of PCNA. *C*, kinetics of the UbH5c-dependent PCNA ubiquitination reaction under optimized conditions (80 nM E1, 32 μM UbH5c (S22R), 32 μM ubiquitin, and 16 μM PCNA in a buffer containing 50 mM MMT, pH 9.0, 25 mM NaCl, 3 mM MgCl_2 , 0.5 mM TCEP, and 3 mM ATP) at time points 0, 5, 10, 15, 30, and 60 min are shown in lanes 1–6. PCNA-Ub can be produced efficiently under these optimized conditions. *D*, elution profile and SDS-PAGE gel from anion exchange chromatography performed on the reaction mixture. Although other minor ubiquitinated species are produced during the reaction, these are readily separated from PCNA-Ub. Error measurements are differences between two independent measurements.

radii of gyration (Fig. 3, *B* and *E*). Interestingly, these data suggest that PCNA-Ub is larger than would be expected if the conjugated ubiquitins were compact and ordered on the PCNA-Ub molecule (Fig. 3*G*).

We analyzed the SAXS profiles of PCNA and PCNA-Ub. The PCNA-Ub had less pronounced inflection points in its scattering curve, which can indicate flexibility (Fig. 3*C*). We used the Guinier method to study the radius of gyration of the two molecules (31), and the data are in good agreement with the MALS analysis (Fig. 3*E*). Finally, we estimated the interatomic probability distribution function for PCNA and PCNA-Ub to understand the dimensions of the proteins (Fig. 3*D*). For PCNA, both the estimates of the longest dimension and a simulation of the scattering profile are in good agreement with the crystal structure (PDB code 1VYM;

Fig. 3*D* and supplemental Fig. S3). However, the SAXS data for PCNA-Ub are not consistent with a compact conformation (Fig. 3 and supplemental Fig. S3).

To understand the structural states of PCNA-Ub that are consistent with our biophysics data, we prepared four molecular models of PCNA-Ub with the ubiquitin moieties in different orientations: the crystal structure of split PCNA (24), a closed conformation with the ubiquitins in the groove at the PCNA subunit interface, an extended structure and an intermediate structure. The final two models represent flexible states because they contain no significant contacts between the ubiquitin and PCNA to stabilize the conformations. Both the split and closed PCNA-Ub models are in poor agreement with the experimental data, whereas structures that represent flexibility fit the data considerably better (Fig. 3*G* and supplemental Fig.

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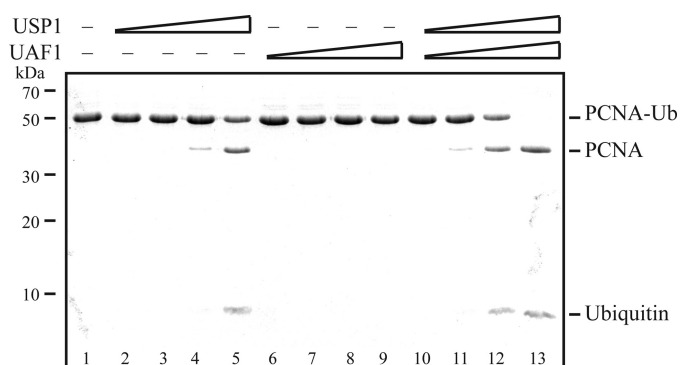


FIGURE 2. UAF1 activates Usp1 toward PCNA deubiquitination. The availability of native PCNA-Ub allows characterization of the deubiquitination reaction. The effect of Usp1 and UAF1 concentrations on PCNA-Ub deubiquitination is shown on a Coomassie-stained SDS-PAGE gel. Usp1 (lanes 2–5), UAF1 (lanes 6–9), and USP1/UAF1 (lanes 10–13) concentrations were 10, 40, 160, and 640 nM. Lane 1 also shows the quality of the final PCNA-Ub product.

S3). We interpret that, to a first approximation, ubiquitin is flexible when conjugated to PCNA.

We used NMR to analyze the PCNA-Ub molecule. We produced PCNA-Ub in which ubiquitin is ^{15}N -labeled and collected ^1H - ^{15}N HSQC experiments. This method allows direct study of the properties of the conjugated ubiquitin on PCNA-Ub. We compared the chemical shifts of PCNA- ^{15}N -ubiquitin with free ^{15}N -ubiquitin because chemical shift changes can provide information about preferred contacts between the PCNA and ubiquitin within the PCNA-Ub molecule. As expected, all of the largest chemical shift changes map to a site that includes the C-terminal tail of ubiquitin, which is directly conjugated to PCNA (Fig. 4). This large continuous patch also includes residues somewhat further from the C terminus including leucine 8. Several additional residues on opposite faces of ubiquitin were also perturbed. These chemical shift changes are likely to indicate transient interactions between the ubiquitin and the PCNA, but the small magnitude of the shifts is not consistent with a stable interaction between the hydrophobic patch of ubiquitin and PCNA (Fig. 4B and supplemental Fig. S4).

Traditional NMR is sensitive to the rate of motion and hence the rotational correlation time of residues in a protein: large proteins have broad NMR peaks. Surprisingly, and despite PCNA-Ub being a large complex (112 kDa), the peaks in the HSQC spectra are sharp. This directly demonstrates that ubiquitin is much more mobile than would be expected if there was a stable interaction between the PCNA and ubiquitin: the ubiquitin is able to move independently of PCNA and hence has sharp peaks in our spectra (Fig. 4A and supplemental Fig. S4A). Importantly, this flexible ubiquitin moiety will have its hydrophobic patch available for recruitment of Pol η (supplemental Fig. S4B).

Finally, we studied the interactions of PCNA and PCNA-Ub with Pol η , using analytical gel filtration (Fig. 5). We used a published construct of Pol η that contains the ubiquitin binding zinc finger and PCNA interacting region but lacks the polymerase domain (15). As expected, a transient interaction between PCNA and Pol η is indicated by a small shift in the PCNA peak to higher molecular mass. However, PCNA-Ub forms a tighter interaction with Pol η , as demonstrated by a

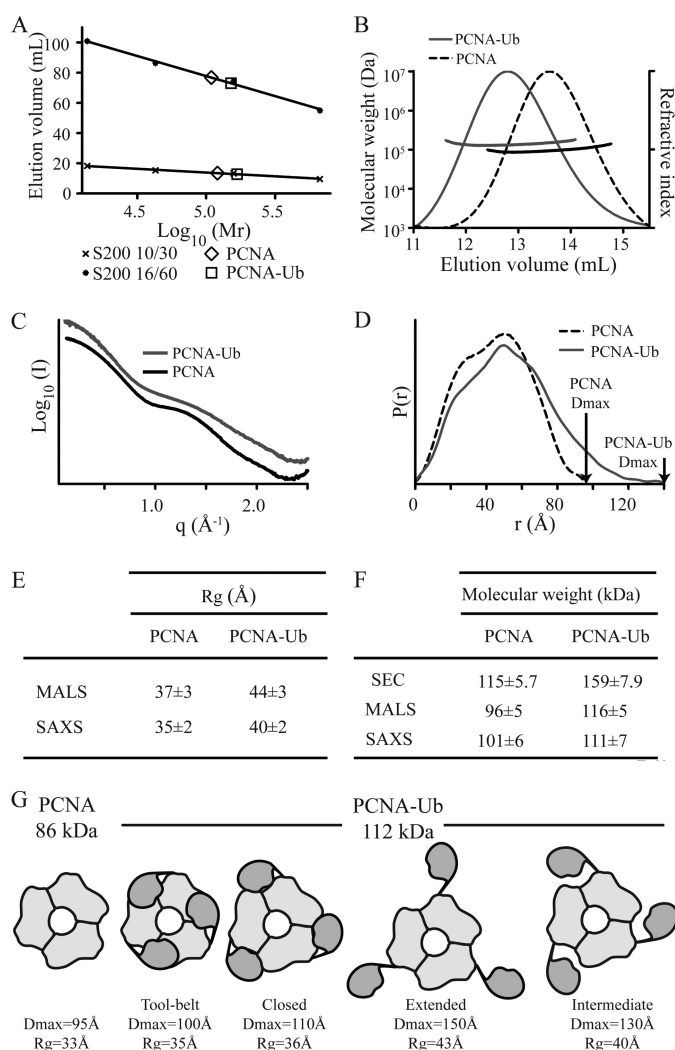


FIGURE 3. Pure PCNA-Ub is monodispersed, but the ubiquitin moieties are flexible with respect to the PCNA. A, SEC analysis of PCNA and PCNA-Ub. The elution volumes of PCNA and PCNA-Ub on S200 10/30 and 16/60 SEC columns preequilibrated with molecular mass standards are shown. B, analysis of PCNA and PCNA-Ub using MALS in-line with SEC. The horizontal scattered light through the protein peak (detected by refractive index measurements) confirms that the samples are monodispersed. C, SAXS scattering curves of PCNA and PCNA-Ub. D, interatomic distance probability distribution ($P(r)$) function of PCNA and PCNA-Ub allowing estimation a longest dimension of PCNA and PCNA-Ub of ~95 and ~140 Å, respectively. E, calculated radii of gyration from MALS and SAXS measurements. F, calculated molecular masses from SEC, MALS, and SAXS measurements. The masses are inferred directly from the data in MALS and are calculated by comparison with molecular mass standards in SEC and SAXS. The SEC-derived molecular masses are averages and differences from the two measurements shown in A. Nonglobularity of the PCNA and PCNA-Ub causes an overestimation of the SEC-derived values. G, schematics of PCNA and PCNA-Ub models, with estimates of physical and hydrodynamic properties. All error measurements are S.D. calculated from three independent measurements, unless otherwise stated. Our hydrodynamic measurements show that the ubiquitin moiety is flexible with respect to PCNA.

larger shift in the PCNA-Ub peak and the resolution of the complex by SDS-PAGE analysis. This demonstrates that the flexible ubiquitin on PCNA-Ub is proficient in Pol η recognition.

DISCUSSION

We describe a protocol to produce pure, monodispersed PCNA-Ub. We characterize the structure of PCNA-Ub in solu-

tion and find the ubiquitins to be flexible and hence able to recognize translesion synthesis polymerases such as Pol η .

Our protocol to produce ubiquitinated PCNA has advantages over existing methods because the yield is greater than Rad6/Rad18/RFC-dependent protocols (19, 20), and the native linkage is structurally identical to the *in vivo* linkage, unlike

chemical mimics of ubiquitinated PCNA (23–25). The protocol is suitable for monoubiquitination of PCNA because UbcH5c retains remarkable specificity for lysine 164. However, the promiscuity of UbcH5c toward other targets may prevent the protocol being generally applicable to monoubiquitination of other substrates (34). Although ubiquitin E2 enzymes do not function independently of E3 ligases *in vivo*, both Rnf8 and CLR4 (Cdt2) can activate UbcH5 toward PCNA (7, 8), so our observed UbcH5 activity may be relevant *in vivo*. The availability of large quantities of native PCNA-Ub will facilitate experiments to study the molecular basis of TLS.

The activity of UbcH5c and Usp1/UAF1 and the NMR analysis confirm that our method to produce PCNA-Ub yields natively folded proteins. We prove that the conjugated ubiquitins are flexible with respect to PCNA, using multiple biophysical techniques. Although we do not rule out transient interactions between the ubiquitin and PCNA molecules, we show that the Ile-44 hydrophobic patch of ubiquitin is predominantly exposed in PCNA-Ub and hence available for polymerase binding (10–12). We propose that flexible states of PCNA-Ub are key intermediates in the TLS pathway. This is consistent with published observations that the attachment site of ubiquitin on PCNA-Ub is not important for polymerase exchange or activity in TLS (13, 23).

A previous SAXS study, using nonnative mimics of PCNA-Ub, used ensembles of conformations to model PCNA-Ub with several ordered structures and only 20–30% of flexible states (26). Although the shapes of the scattering curves are similar, we can fit the data by considering only flexible states and thus avoid the possibility of overfitting that result from ensemble-based methods. We measure a slightly larger PCNA-Ub molecule by SEC, MALS, and SAXS than is reported in that publica-

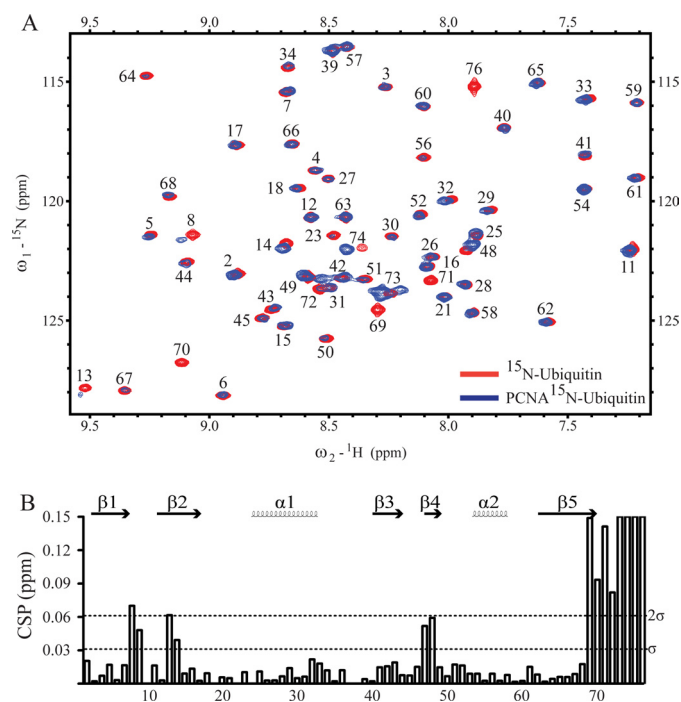


FIGURE 4. NMR analysis of PCNA- ^{15}N -Ub confirms that the conjugated ubiquitin is flexible. *A*, NMR spectra (^1H - ^{15}N HSQC) of free ubiquitin (*red*) and PCNA- ^{15}N -Ub (*blue*). Sharp peaks are observed in both spectra. *B*, magnitude of CSP plotted against residue number. Secondary structure elements of ubiquitin are indicated.

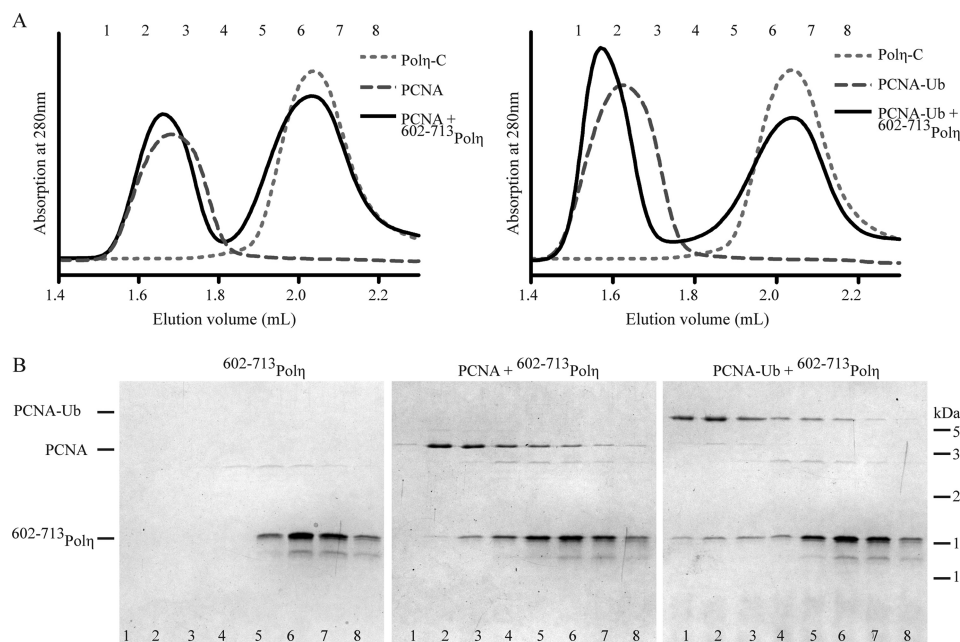


FIGURE 5. Analytical gel filtration analysis of the interaction of $^{602-713}\text{Pol}\eta$ with PCNA and PCNA-Ub. *A*, chromatograms of $^{602-713}\text{Pol}\eta$, PCNA, and PCNA-Ub, or mixtures of $^{602-713}\text{Pol}\eta$ with PCNA/PCNA-Ub. The PCNA peak undergoes a small shift to higher molecular mass upon addition of $^{602-713}\text{Pol}\eta$ (*left*), whereas the PCNA-Ub peak shifts to a larger extent (*right*). *B*, SDS-PAGE analysis of the fractions (*lanes 1–8*) from the analytical gel filtration column. $^{602-713}\text{Pol}\eta$ elutes in earlier fractions upon complex formation with PCNA and PCNA-Ub. Greater amounts of Pol η are observed in the early fractions with PCNA-Ub compared with PCNA. These data show that Pol η will form a transient complex with PCNA and a more stable complex with PCNA-Ub.

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tion. If the differences are significant this would indicate that the nonnative linkages of the mimics cause differences in the conformation of the proteins, favoring more compact states.

Our results provide a methodological framework to study monoubiquitinated proteins and a structural characterization of a prominent target to serve as a benchmark for future studies. Although monoubiquitinated targets are not yet available for structural studies, ubiquitin chains have been characterized. These studies show that Lys-48-linked ubiquitin chains have a compact structure (35), whereas Lys-63- and linear chains are flexible (36). NMR and SAXS are useful tools for studying the structures of ubiquitinated substrates as they become available, to understand whether flexibility is a general feature of ubiquitin conjugation.

Acknowledgments—We thank R. Kleivit, M. Cohn, A. D'Andrea, and D. Sahtoe for plasmids and M. Clerici for UAF1 protein. TAMRA-labeled ubiquitin was provided by R. Merckx and H. Ovaa. SAXS data were collected by F. Groothuizen and A. Perrakis at European Synchrotron Radiation Facility and EMBL outstation at Deutsches Elektronen Synchrotron, NMR spectra at the Utrecht Facility for High Resolution NMR with H. Wienk. P. Ikpa performed pilot experiments. We thank R. Boelens, A. Perrakis, J. Smit, F. Mattioli, and F. Groothuizen for discussion and critical reading of the manuscript and beamline staff for assistance.

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