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DNA Replication-Coupled PCNA Mono-Ubiquitination and Polymerase Switching in a Human *In Vitro* System

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Translesion DNA synthesis is a mechanism of DNA damage tolerance, and mono-ubiquitination of proliferating cell nuclear antigen (PCNA) is considered to play a key role in regulating the switch from replicative to translesion DNA polymerases (pols). In this study, we analyzed effects of a replicative pol δ on PCNA mono-ubiquitination with the ubiquitin-conjugating enzyme and ligase UBE2A/HHR6A/RAD6A–RAD18. The results revealed that PCNA interacting with pol δ is a better target for ubiquitination, and PCNA mono-ubiquitination could be coupled with DNA replication. Consequently, we could reconstitute replication-coupled switching between pol δ and a translesion pol, pol η, on an ultraviolet-light-irradiated template. With this system, we obtained direct evidence that polymerase switching reactions are stimulated by mono-ubiquitination of PCNA, depending on a function of the ubiquitin binding zinc finger domain of pol η. This study provides a framework for detailed analyses of molecular mechanisms of human pol switching and regulation of translesion DNA synthesis.

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Introduction

In living cells, DNA is exposed continuously to attack by endogenous reactive species, including oxygen radicals and metabolic intermediates, as well as environmental agents such as ionizing radiation, ultraviolet light (UV), and a variety of chemicals. Although resultant replication-blocking lesions are removed by nucleotide and base excision repair, significant numbers persist due to the balance between generation and excision. Therefore, cells need to be able to tolerate DNA damage during DNA replication.[1] The DNA damage tolerance pathway seems to act on single-stranded gaps[1,2] and therefore also is referred to as post-replication repair (PRR).[3] This process is separable into translesion DNA synthesis (TLS) and template switching (TS). In the TLS pathway, a number of nonessential DNA polymerases (pols) rescue stalled replication by extending the 3′-ends beyond the lesions. This process is essentially error-prone because of its utilization of a damaged template. With TS, the damage is bypassed by a copy choice mechanism using the newly synthesized sister chromatid as the template. For this reason, this process is deemed relatively error-free.[3]

In eukaryotes, a significant fraction of PRR is initiated by RAD6 (*Saccharomyces cerevisiae*)/UBE2A and UBE2B (in humans)- and RAD18-dependent ubiquitination at the lysine 164 residue of proliferating cell nuclear antigen (PCNA).[4–6] UBE2A and UBE2B, also known as HHR6A/RAD6A and HHR6B/RAD6B, respectively, are hereafter referred to as RAD6, applying the name of the yeast gene. RAD6, a ubiquitin-conjugating E2 enzyme, forms a tight complex with RAD18, a ubiquitin protein E3 ligase.[6–12] Mono-ubiquitination by the complex appears to be limited to PCNA already loaded onto DNA by replication factor C (RFC), demonstrated in *S. cerevisiae*[9,10] and humans.[13] It has been shown that Y-family translesion pols, which contain ubiquitin

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Abbreviations used: E1, ubiquitin-activating enzyme E1; PCNA, proliferating cell nuclear antigen; pol, DNA polymerase; PRR, post-replication repair; RFC, replication factor C; RF, replication factor; RPA, replication protein A; ssDNA, single-stranded DNA; TLS, translesion DNA synthesis; TS, template switching; UE, ubiquitin enzyme; UBZ, ubiquitin binding zinc finger; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid.

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binding motifs or ubiquitin binding zinc fingers (UBZs), are recruited by interaction with mono-ubiquitinated PCNA to allow TLS through damage sites.[14–18] Mono-ubiquitination can also be followed by poly-ubiquitination by the UBC13–MMS2 complex, an E2 ubiquitin-conjugating enzyme, and RAD5 (in *S. cerevisiae*)/SHPRH and HLTF (in humans) E3 ubiquitin ligases, leading to TS.[3,13,19–21]

Mono-ubiquitination of PCNA is considered to play a key role in regulation of PRR. In yeast, most TLS depends on PCNA mono-ubiquitination. However, in chicken DT40 cells, mono-ubiquitination of PCNA seems crucial, but not essential, for translesion synthesis.[22,23] The majority of the remaining TLS in the PCNA$^{(K164R)}$ background depends on REV1. The presence of distinct pathways in higher eukaryotes is very clear in PCNA$^{(K164R)}$ knock-in mice, in which only reduction of mutations at template A/T and a compensatory increase at G/C in immunoglobulin genes have been observed.[24] This phenotype is similar to that with pol η-deficient cells,[25–27] suggesting that PCNA mono-ubiquitination is closely linked to functions of pol η.

Mono-ubiquitinated PCNA accumulates on treatment of cells with DNA-damaging agents.[4,6,14,17,28,29] Recent reports have provided evidence that a significant proportion of PCNA mono-ubiquitination is constitutive, since accumulation is observed on inactivation of a de-ubiquitinating enzyme, USP1.[29–31] Importantly, in chicken DT40 cells, elevated levels of mono-ubiquitinated PCNA, which result from disruption of the *USP1* gene, do not increase mutagenesis at endogenously created DNA damage in the immunoglobulin locus.[31] This indicates that ubiquitination of PCNA is not sufficient for activating mutagenic translesion synthesis. In yeasts, it has been shown that PCNA is constitutively ubiquitinated during normal S phase.[28,32] For these reasons, the role of the RAD6–RAD18 complex in mono-ubiquitination of PCNA, its dependence on template damage, and its role in switching polymerases at stalled replication forks are not clear.

To address the molecular mechanisms underlying polymerase switching and its dependence on PCNA mono-ubiquitination by RAD6–RAD18 in mammals, we have established an *in vitro* reconstituted system. This system uses purified recombinant proteins, including pol δ, RFC, PCNA, replication protein A (RPA), ubiquitin-activating enzyme E1 (E1), the RAD6A–RAD18 complex, ubiquitin, and pol η. In this report, we described protein actions with this *in vitro* system.

Results

Reconstitution of PCNA mono-ubiquitination with recombinant human proteins *in vitro*

It has been suggested that mono-ubiquitinated PCNA mediates polymerase switching from replicative to translesion pols.[2] To address molecular mechanisms underlying mono-ubiquitination of PCNA and polymerase switching *in vitro*, we first established methods to obtain highly purified recombinant human replication factors (RFs, including RFC, PCNA, and RPA), ubiquitin enzymes (UEs, including E1, RAD6A–RAD18 complex, and ubiquitin), pol δ, and pol η (Fig. 1a)[33] from overproducing *Escherichia coli* cells by conventional column chromatography. The elution profile for each protein gave a sharp and symmetric peak from the gel-filtration column, demonstrating a quality sufficient for enzyme assays (data not shown).

Reactions for PCNA mono-ubiquitination *in vitro* have been established in *S. cerevisiae* and human systems.[9,10,13] Here, the reactions were reproduced using RPA-coated, singly primed mp18 single-stranded DNA (ssDNA) (Fig. 1b). The reaction condition was originally optimized for DNA replication,[33] and UEs were introduced to give saturated amounts with respect to mono-ubiquitination of PCNA. After

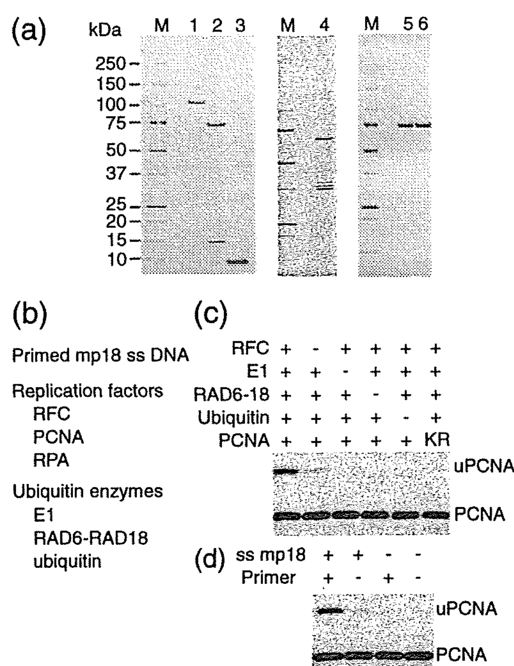

Fig. 1. Reconstitution of PCNA mono-ubiquitination. (a) Purified proteins in this study. Purified recombinant proteins, 500 ng (4.2 pmol) of E1 (lane 1), 650 ng (8.8 pmol) of RAD6A–RAD18 (lane 2), 500 ng (58 pmol) of ubiquitin (lane 3), 800 ng (3.6 pmol) of RFC$^{(P140N555)}$ (lane 4), 500 ng (6.4 pmol) of pol η (wild type) (lane 5), and 500 ng of pol η$^{(D652A)}$ (lane 6) were loaded on an SDS 5–20% gradient polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. (b) Summary of the assay system for PCNA mono-ubiquitination. (c) Requirement of protein components for PCNA mono-ubiquitination. KR represents a mutant of PCNA, PCNA$^{(K164R)}$, in place of the wild type. (d) Requirement of DNA components for PCNA mono-ubiquitination. The reactions were carried out for 30 min. The indicated components were omitted from the reactions, and reaction products were analyzed by Western blotting using anti-PCNA antibodies. uPCNA represents mono-ubiquitinated PCNA.

incubation for 30 min at 30 °C, PCNA molecules were visualized by Western blotting. This assay detected a slower migrating band, the size corresponding to the mono-ubiquitinated PCNA (Fig. 1c and d), which reacted with anti-ubiquitin antibody (data not shown), indicating it to be mono-ubiquitinated PCNA. The PCNA ubiquitination depended on the protein components, RFC and UEs (Fig. 1c), and singly primed mp18 ssDNA (Fig. 1d). Inefficient ubiquitination was detected without RFC (Fig. 1c) or the primer (Fig. 1d), probably due to nonspecific interactions of the proteins with mp18 DNA forming a secondary structure. When PCNA was replaced with a mutant, $PCNA^{(K164R)}$, no products were detected (Fig. 1c). Biochemical activity of the mutant was essentially identical with that of wild type with respect to DNA replication with pol δ (Supplementary Fig. S1), indicating that the mutation did not affect the integrity of the molecule. These results suggested ubiquitin to be specifically conjugated to the lysine 164 residue in these reactions, as detected earlier *in vivo*[4] and *in vitro*.[9,21]

Mono-ubiquitination of PCNA interacting with pol δ

Since pol δ forms a complex with PCNA at the 3′-end during elongation reaction, we asked the question of whether such PCNA could act as a target for ubiquitination. To address this question, we used poly(dA)–oligo(dT) as a DNA source. It is well established that poly(dA)–oligo(dT) is an excellent substrate for pol δ in PCNA-dependent and RFC-independent reactions,[34–42] since PCNA molecules spontaneously are loaded onto DNA from the ends without RFC[43] as illustrated in Fig. 2a. Indeed, we confirmed powerful stimulation of DNA synthesis of pol δ by only the addition of PCNA without RFC and RPA under our assay conditions (Supplementary Fig. S2), suggesting functional interactions between PCNA and pol δ on the poly(dA)–oligo(dT) without RFC. Then, we analyzed PCNA mono-ubiquitination in the presence of UEs. As shown in Fig. 2a, we surprisingly found that mono-ubiquitination of PCNA was strongly stimulated by only the addition of pol δ as well as RFC, but not pol β (Fig. 2b). Importantly, the required amount of pol δ for ubiquitination was stoichiometric, rather than catalytic, to that for PCNA (1 pmol as trimers was present in the reaction mixture) (Fig. 2b), suggesting the possibility that PCNA interacting with either RFC or pol δ is able to be a target for ubiquitination.

To obtain additional evidence, we used 5′-biotinylated oligo(dT) for the assay. First, loading of PCNA was monitored by stimulation of DNA synthesis (Fig. 2c). Neither the biotin moiety at the 5′-end nor streptavidin itself affects polymerase reactions (Fig. 2c, lanes 2 and 3, data not shown). When streptavidin was preincubated with template DNA, the stimulatory effect of PCNA was canceled (Fig. 2c, lanes 4 and 5), and it was partially restored by addition of RFC (Fig. 2c, lanes 5 and 6). Importantly, the results were identical with those when PCNA was preincubated with the template DNA before addition of streptavidin (Fig. 2c, lanes 7–12), indicating the amount of PCNA molecules on DNA without pol δ to be negligible. These results suggested that spontaneously loaded PCNA is basically unstable but can remain on the DNA by interaction with pol δ. Next, mono-ubiquitination of PCNA was analyzed by addition of UEs (Fig. 2d). The results showed that stimulation of PCNA mono-ubiquitination by pol δ was markedly reduced by addition of streptavidin (Fig. 2d, lanes 3 and 4) and was partially restored by addition of RFC (Fig. 2d, lanes 4 and 5). Identical results were obtained when PCNA was preincubated with the template DNA before addition of streptavidin (Fig. 2d, lanes 6–10). These results supported the possibility that PCNA molecules, interacting with pol δ, are able to be ubiquitinated.

To determine whether the interaction between pol δ and PCNA affected efficiency of mono-ubiquitination, we performed the following experiments with isolated encircled PCNA molecules on plasmid DNA as the substrate (Fig. 2e). After loading reactions, DNA–PCNA complexes were separated from DNA-free PCNA by gel filtration and then fractions containing DNA–PCNA complexes were subjected to ubiquitination assays using nicked circular plasmids, since they well support PCNA mono-ubiquitination[13] (data not shown). For PCNA loading, a mutant RFC, $RFC^{(p140N555)}$, which was formed with a truncated RFC1 subunit that lacked a nonspecific DNA binding domain,[44] was employed. The benefit of using $RFC^{(p140N555)}$ for loading reactions is prevention of contamination due to nonspecific interactions between the DNA binding domain of RFC and DNA on subsequent gel filtration.[45] When purified fractions were reacted with UEs, we detected a small amount of ubiquitinated PCNA (Fig. 2f, lanes 2 and 9), suggesting that encircled PCNA is capable of ubiquitination without pol δ or RFC. To see the effects of RFC or pol δ, we then introduced these proteins into the reactions. Here, we detected increased products depending on the amounts of RFC (Fig. 2f, lanes 2–7) and pol δ (Fig. 2f, lanes 9–14), suggesting that both have the ability to stimulate the reaction. In this experiment, it was critical to confirm that the PCNA–DNA complex is stable under our assay conditions, as an important control. If a significant fraction of PCNA dissociated spontaneously during incubation, the stimulation might be a consequence of stabilization of PCNA by interaction with pol δ. To assess the stability of encircled PCNA, we divided the purified complexes into three. One sample was immediately reacted with UEs for 30 min; others were preincubated for 30 min without RAD6A–RAD18 and E1 in the presence or absence of a restriction enzyme, HincII, for linearization of the plasmid, and then the reaction was carried out for a further 30 min by introduction of RAD6A–RAD18 and E1 (Supplementary Fig. S3a). The results of Western blotting showed that the capacities for ubiquitination were not changed before and after the incubation for 30 min but halted by linearization of DNA

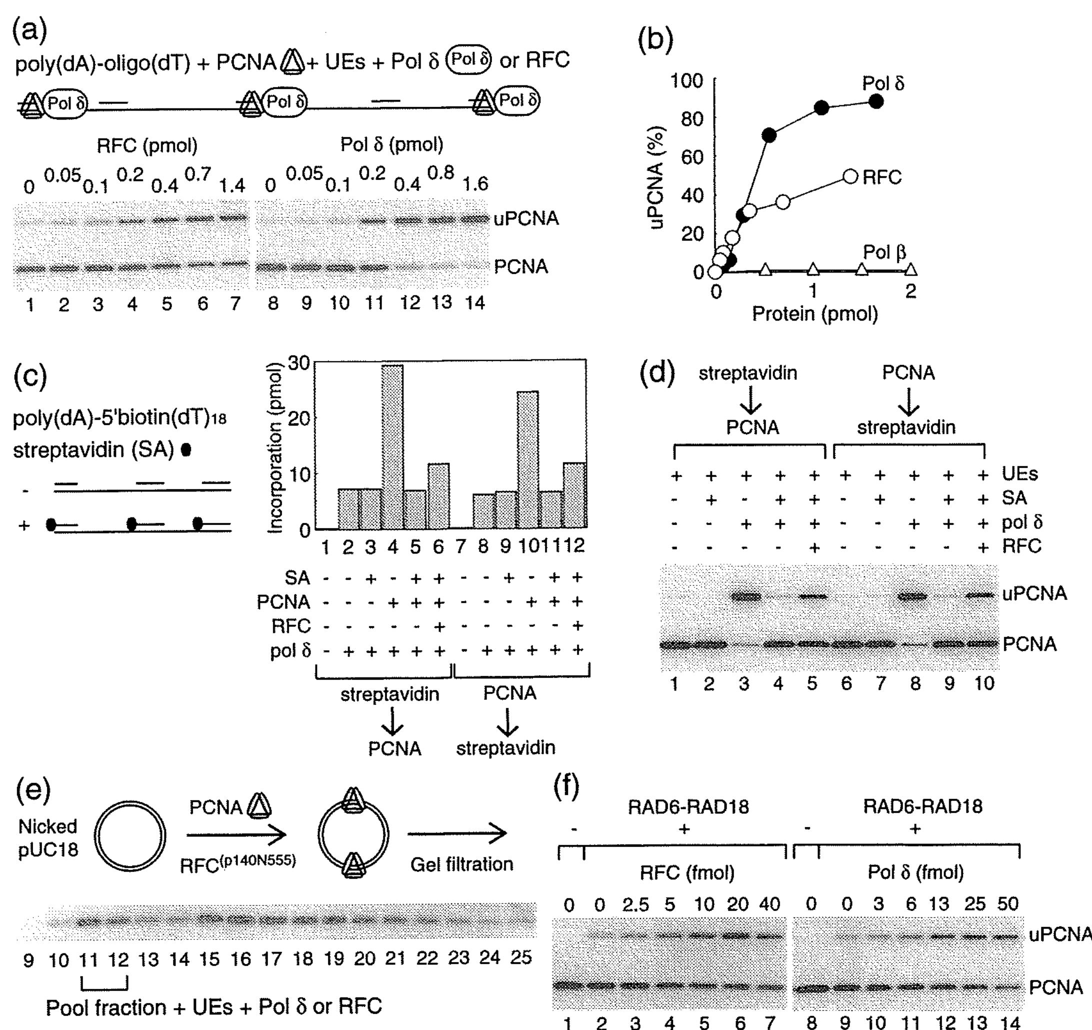

Fig. 2. Mono-ubiquitination of PCNA interacting with pol δ. (a) Titration of pol δ and RFC in the PCNA mono-ubiquitination reaction with poly(dA)–oligo(dT) as the DNA source. The experimental design is shown in the upper part. In this reaction, poly(dA)–oligo(dT) and PCNA were incubated with UEs. Amounts of oligo(dT) as primer termini were 0.9 pmol in the reaction. Indicated amounts of RFC or pol δ were introduced into the reaction mixtures followed by incubation for 30 min. Reaction products were analyzed by Western blotting using anti-PCNA antibodies. (b) Quantification of mono-ubiquitinated PCNA shown in (a), and with pol β. Titration of pol β was performed as for pol δ described in (a). The signal intensity detected by a CCD camera was quantified and plotted as the relative amount of the mono-ubiquitinated form. (c) DNA synthesis on poly(dA)–5′ biotinylated oligo(dT). The 5′ biotinylated oligo(dT), 18-mer, was annealed with poly(dA). In lanes 1 to 6, DNA was incubated with streptavidin (SA) on ice for 10 h and then mixed with PCNA. In lanes 7 to 12, DNA was incubated with PCNA on ice for 1 h and then incubated with streptavidin on ice for 10 h. Replication reactions were carried out for 10 min under standard reaction conditions with pol δ (220 fmol) and RFC (1.4 pmol) as indicated. (d) PCNA mono-ubiquitination assays with poly(dA)–5′ biotinylated oligo(dT). The substrate DNA was prepared as described in (c). Reactions were carried out for 10 min under standard reaction conditions with pol δ (840 fmol) and RFC (1.4 pmol) as indicated. Reaction products were analyzed by Western blotting using anti-PCNA antibodies. (e) Purification of PCNA assembled on nicked circular DNA by gel-filtration chromatography. A schematic representation of the experimental design is shown in the upper part. After loading PCNA on nicked circular DNA by $RFC^{(p140N555)}$, the products were passed through a gel-filtration column. Indicated fractions were analyzed by Western blotting. Encircled PCNA on DNA eluting in the void volume of the gel filtration in fractions 11 and 12 was subjected to ubiquitination reactions (f). (f) PCNA mono-ubiquitination assays with purified PCNA–DNA complexes. Encircled PCNA (e) was incubated with the indicated amounts of RFC or pol δ in the presence of UEs. Reaction products were analyzed by Western blotting using anti-PCNA antibodies. uPCNA represents mono-ubiquitinated PCNA.

(Supplementary Fig. S3b), indicating that encircled PCNA is the target for ubiquitination and spontaneous dissociation of PCNA was negligible under the reaction conditions. The result could also rule out the possibility of contamination of $RFC^{(p140N555)}$. Because $RFC^{(p140N555)}$ has the potential to unload encircled PCNA,[46] if it were present in the reactions, the result would be reduction of the capacity for

ubiquitination by preincubation for 30 min. Taking these results together, we suggest that PCNA interacting with either pol δ or RFC on DNA is a better target of RAD6A–RAD18, rather than PCNA just encircled on DNA.

PCNA mono-ubiquitination can be coupled to DNA replication

Next, we asked whether the PCNA molecules in the replication machinery consisting of pol δ and RFC during elongation reactions[33] are able to be a target. To address this question, we reconstituted the DNA replication reaction with pol δ in the presence of UEs under the ubiquitin assay conditions described in Fig. 1b. DNA synthesis was monitored in the additional presence of [α-^{32}P] dTTP (Fig. 3). The time course was analyzed by alkaline agarose gel electrophoresis of the products (Fig. 3a and b), and incorporation of radioactivity was determined (Fig. 3c). The results demonstrated that UEs exhibited no influence on the size of the product (Fig. 3a and b) and total amounts of DNA synthesis (Fig. 3c). This is consistent with findings in a yeast system.[9]

Then, mono-ubiquitination of PCNA was monitored by Western blotting under the same conditions as shown in Fig. 3b. The results demonstrated that PCNA molecules interacting functionally with pol δ during elongation were able to be ubiquitinated (Fig. 4a and b). In the absence of dCTP, further stimulation by addition of pol δ was not observed (Fig. 4a and b), suggesting the levels of stimulation by each of RFC and pol δ to be equivalent and not additive for the following reasons. In the reaction without pol δ, RFC forms complex with PCNA at apparently all the 3′-ends.[33] Such PCNA molecules interacting with RFC could be ubiquitinated efficiently (Fig. 2f). By addition of pol δ, the same number of PCNA molecules now could make complexes with pol δ at the 3′-ends. Those PCNA molecules interacting with pol δ could be again better targets for ubiquitination (Fig. 2f). In spite of addition of pol δ, the number of PCNA molecules interacting with either RFC or pol δ should be constant. Therefore, it was very surprising that the ubiquitination of PCNA was stimulated by DNA synthesis (Fig. 4a and b), since the number of PCNA molecules interacting with pol δ must be constant during elongation reactions in consideration of the fact that pol δ should interact with PCNA at only the 3′-ends.

To address why the elongation reaction stimulated the ubiquitination, we analyzed the status of PCNA molecules on the DNA by a previously established method to isolate PCNA–DNA complexes in reaction mixtures for DNA replication (Fig. 4c).[33] A primer containing an extended 5′ tail with one biotin molecule was annealed to mp18 ssDNA (Fig. 4c). The 5′ tail of the primer did not exert any influence on DNA synthesis (data not shown).[33] The primed mp18 ssDNA molecules were attached to magnetic beads, and then DNA replication reactions with RFs, UEs, and pol δ were carried out as described in Fig. 4c. After reactions for 10 min, the beads were washed and bound PCNA was detected by Western blotting (Fig. 4d). Chemiluminescence signals detected with a CCD camera were quantified with reference to a standard curve for PCNA in the same blot (Fig. 4e). We have demonstrated previously that the assay detects PCNA molecules that are loaded on DNA in an RFC-dependent manner.[33]

First, general properties of loaded PCNA in DNA replication in the absence of RAD6A–RAD18 were analyzed (Fig. 4d and e, lanes 1–4).[33] The amount of PCNA detected in reactions with RFC alone was 54 (±7) fmol (Fig. 4d and e, lane 2). Since the background signal, which is the amount of PCNA detected after linearization of DNA with a restriction enzyme, HincII, was 29 (±4) fmol (Fig. 4d and e, lane 4), we estimated that the net amount of loaded PCNA could be about 25 fmol, equivalent to the amount of the primer template (33 fmol) (Fig.

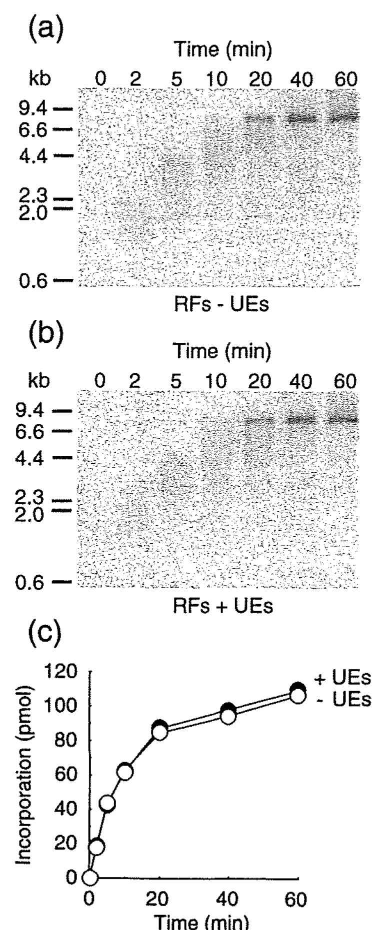

Fig. 3. Pol δ holoenzyme assays in the presence or absence of UEs. (a and b) Time courses of DNA synthesis in the presence (b) or absence (a) of UEs. Reactions were carried out for the indicated times under standard assay conditions with pol δ (380 fmol). Products were analyzed by 0.7% alkaline agarose gel electrophoresis. (c) Incorporation of dNMP was measured as described in Materials and Methods.

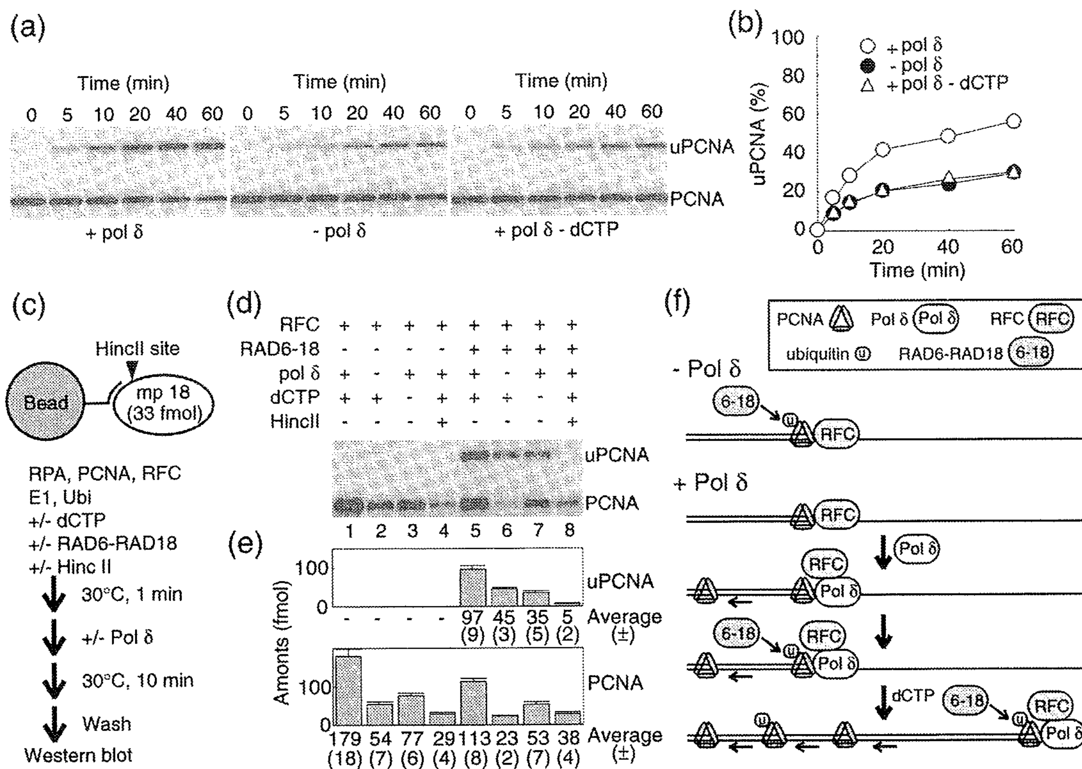

Fig. 4. PCNA mono-ubiquitination coupled with DNA replication. (a and b) Time courses of reactions for PCNA mono-ubiquitination in the presence or absence of pol δ (380 fmol) and/or dCTP. The reactions were carried out under the conditions described in Figs. 1b and 3b and in Materials and Methods. Reaction products were analyzed by Western blotting using anti-PCNA antibodies (a). The signal intensity detected by a CCD camera was quantified, and averages of three to five independent experiments were plotted as the relative amount of the mono-ubiquitinated form (b). Error bars are smaller than their symbols. (c) Outline of the assay to determine amounts of PCNA loaded on DNA. DNA was attached to magnetic beads via biotin–streptavidin linkage. The reactions were carried out for 10 min with the indicated factors in the presence or absence of pol δ (380 fmol). After termination of the reactions, the beads were washed and bound PCNA was analyzed by Western blotting (d and e). (d) A representative image of the Western analysis. uPCNA represents mono-ubiquitinated PCNA. (e) Chemiluminescence signals detected with a CCD camera were quantified with reference to a standard curve for PCNA in the same blot. Averages of four independent experiments with SD in parentheses were shown with graphs. (f) Conceivable protein actions based on the model proposed previously.[33] After loading by RFC, PCNA in the complex was ubiquitinated during 10 min incubation. Addition of pol δ induced dynamic actions so that additional PCNA molecules were loaded on DNA. Probably, the PCNA molecules interacting with pol δ were preferentially ubiquitinated as shown in Fig. 2f. Primer extension by addition of dCTP facilitated further accumulation of PCNA on DNA. PCNA molecules that were released behind the 3′-end before ubiquitination were not better substrates for ubiquitination, resulting in coexistence of ubiquitinated and ubiquitin-free PCNA molecules in a mosaic fashion.

4e, note the difference between lanes 2 and 4) with approximately one PCNA molecule loaded onto template DNA.[33,47,48] Addition of pol δ in the absence of dCTP raised the amount of PCNA slightly (Fig. 4d and e, lane 3). Consequently, approximately 48 fmol of PCNA was detected on the 90-mer primer (Fig. 4e, the difference between lanes 3 and 4) corresponding to one to two molecules on DNA, as illustrated in Fig. 4f.[33] Primer extension by addition of dCTP facilitated further accumulation of PCNA on DNA to about 150 fmol of the net amount of PCNA (Fig. 4e, lane 1, note the difference between lanes 1 and 4) corresponding to four to five molecules on DNA, as illustrated in Fig. 4f.[33,48]

Then, we analyzed ubiquitinated PCNA by introduction of RAD6A–RAD18 into the reaction mixture (Fig. 4d and e, lanes 5–8). In the absence of pol δ, almost all PCNA molecules were detected as ubiquitinated forms (Fig. 4d, lane 6). Introduction of pol δ in the absence of dCTP did not appreciably affect the amount of ubiquitinated PCNA (the slight reduction might be attributed to pull-down efficiencies of the experiment, which could be affected slightly by respective protein factors), even though the total amount was increased (Fig. 4d, lanes 3 and 7). Consequently, the significant fraction of PCNA on DNA persisted without ubiquitination, even in the presence of excess amounts of UEs. Primer extension with dCTP further increased the amount of ubiquitinated PCNA, but significant fractions of PCNA remained without ubiquitination (Fig. 4d, lane 5). These results are consistent with the observation in Fig.

2f, which is that the PCNA molecules, just encircled on DNA, are not efficient targets for ubiquitination, but rather PCNA molecules, proximately located at the 3′-end and making complex with pol δ as illustrated in Fig. 4f. We suggest that stimulation of ubiquitination by DNA synthesis was mainly a consequence of continuous loading of PCNA onto the DNA and subsequent ubiquitination of the newly loaded PCNA molecules.

Reconstitution of DNA replication on a damaged template with polymerase switching between pol δ and pol η

It is critical to ask whether polymerase switching reactions between pol δ and translesion pols are observed in this *in vitro* system. To reconstitute polymerase switching, we used UV-irradiated mp18 ssDNA as a template. First, mp18 ssDNA was irradiated with different doses of UV (50–1600 J/m^2) after which its capacity as a template for replication with pol δ was examined (Supplementary Fig. S4). The result showed DNA synthesis to be inhibited in a UV dose-dependent manner. Under these reactions, ubiquitination of PCNA was similarly reduced and reached a level equivalent to that without dCTP on the template irradiated with the highest dose (1600 J/m^2), at which most of DNA synthesis was blocked just like that without dCTP (Supplementary Fig. S4a–c). By determining the status of PCNA mono-ubiquitination on UV-irradiated DNA by the method as shown in Fig. 4c, we concluded that the reduction of PCNA ubiquitination with UV-irradiated templates could be attributed to the reduced amounts of PCNA on DNA during elongation reactions (Supplementary Fig. S4d and e).

In the following experiments, we chose a template irradiated at 100 J/m^2, with which significant reduction of the full-length products was observed (Supplementary Fig. S4a). Since the lesions inhibited elongation with pol δ (Supplementary Fig. S4a), we could detect further elongation by addition of pol η when polymerase switching occurred.[49,50] After reactions in the presence of increasing amounts of pol η without RAD6A–RAD18, the products were analyzed by alkaline agarose gel electrophoresis (Fig. 5a). The results showed that the average size of the products was increased in a manner dependent on the amount of pol η up to 0.2 pmol (Fig. 5a, lanes 1–8 and graphs). However, further addition of excess pol η resulted in a decreased size of products, suggesting inhibitory effects on elongation (Fig. 5a, lanes 8–11 and graphs). When the template was replaced with intact mp18 DNA, inhibitory effects

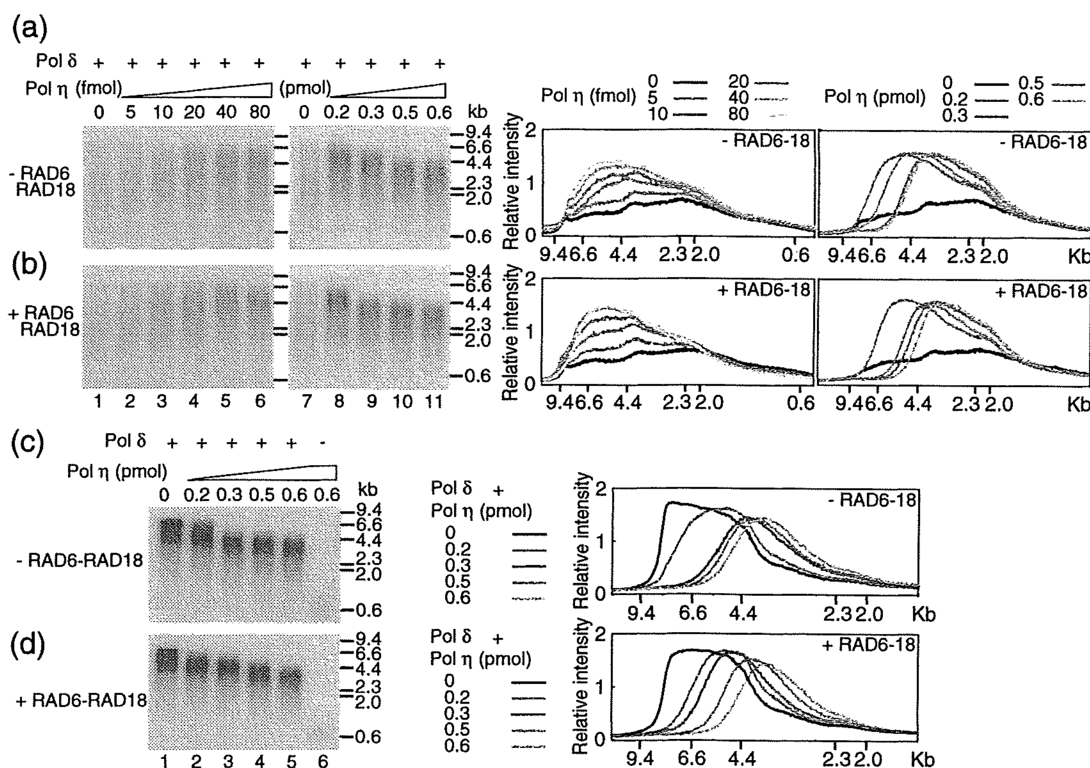

Fig. 5. Reconstitution of PCNA mono-ubiquitination-independent polymerase switching on UV-irradiated templates. Titration of pol η under standard reaction conditions containing RFs and UEs in the presence of pol δ (380 fmol). Intact (c and d) and UV-irradiated mp18 DNA (100 J/m^2) (a and b) were used as templates. Reactions were carried out for 10 min with the indicated amounts of pol η, in the presence (b and d) or absence (a and c) of RAD6A–RAD18. Products were analyzed by 0.7% alkaline agarose gel electrophoresis. The gel images were analyzed by Multi Gauge software (FUJIFILM), and the relative intensity of each lane is shown as a function of the product size in graphs.

on the elongation were also observed (Fig. 5c). By monitoring polymerase activity of pol η itself, we detected products around 0.6 kb with the maximum amount of pol η (Fig. 5c, lane 6), suggesting a much lower capacity for replication than with pol δ. We consider that the inhibition could be due to competitive association of pol η at 3′-ends. Such competition was not detected even when large amounts of pol β were introduced into the reaction mixture (Supplementary Fig. S5). These results indicated that pol η mechanistically has the potential to access 3′-ends without ubiquitination of PCNA and that polymerase switching between pol δ and pol η occurs frequently and spontaneously during DNA synthesis in this *in vitro* system.

Then, RAD6A–RAD18 was introduced to assess the effect of PCNA ubiquitination (Fig. 5b). The result showed that the size of products was also increased by addition of pol η, although we could not detect a clear difference from reactions without RAD6A–RAD18 using a wide range of concentrations of pol η (Fig. 5a and b). Furthermore, stimulation of pol η itself was also not clear (Fig. 5c and d, lane 6), which might be due to resolution of the products in this gel system. We considered the possibility that the concentration of template was sufficiently high for pol η to access 3′-ends without interaction with ubiquitinated PCNA.[51–53] To test this possibility, we reduced the concentration of the template as far as possible (Fig. 6). Again, the control

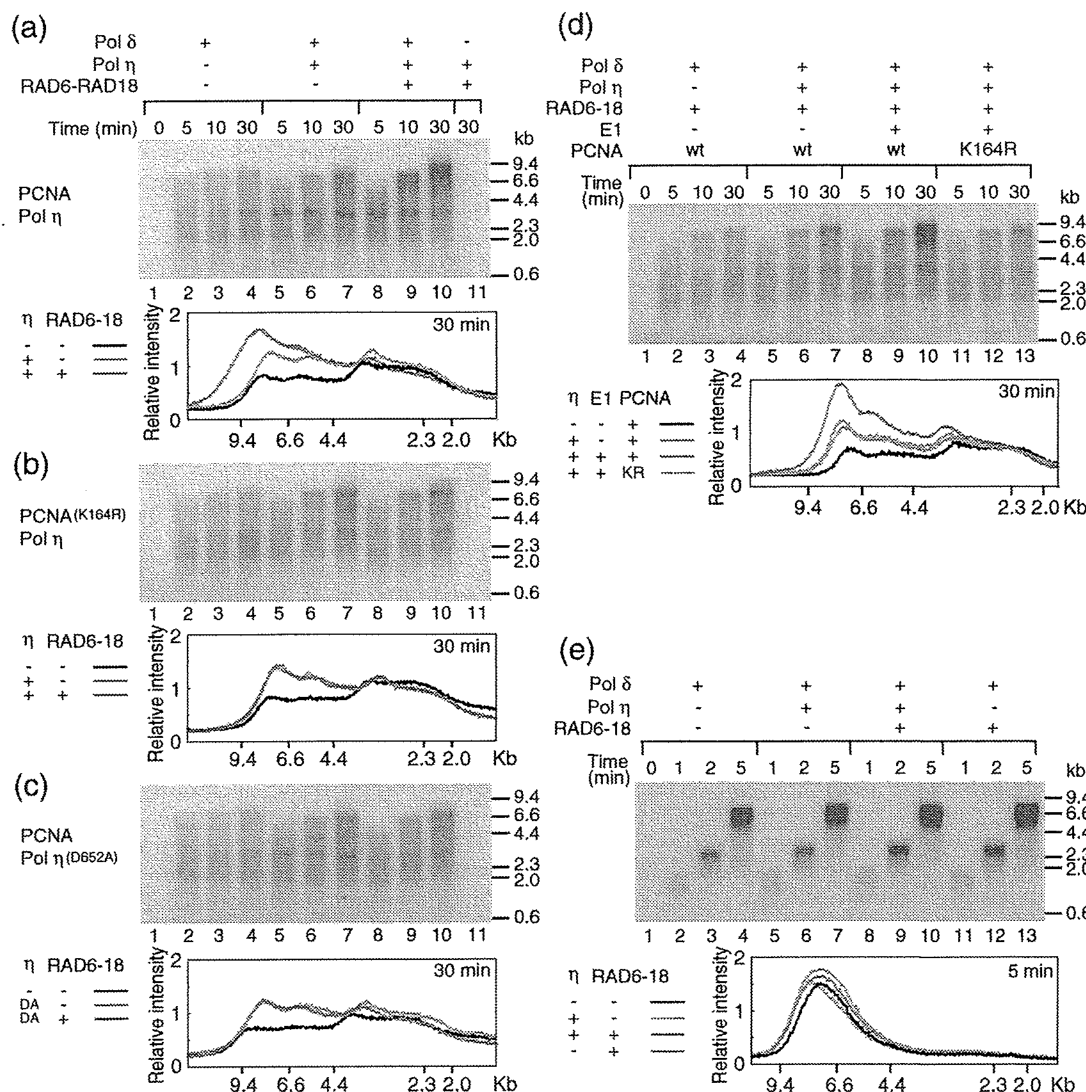

Fig. 6. Reconstitution of PCNA mono-ubiquitination-dependent polymerase switching on UV-irradiated templates. Intact (e) or UV-irradiated mp18 DNAs (100 J/m^2) (a–d) were used as templates. Reactions containing RFs, UEs, and indicated pols were carried out for the indicated times under the conditions described in Materials and Methods. For (b)–(d), wild-type proteins were replaced with the indicated mutants. Products were analyzed by 0.7% alkaline agarose gel electrophoresis. The gel images were analyzed by Multi Gauge software (FUJIFILM), and the relative intensity of each lane at 30 min (for UV-irradiated templates) or 5 min (for intact templates) is shown as a function of the product size in graphs.

reaction with pol δ alone showed a smear size distribution of the products, which was due to stalling of DNA replication (Fig. 6a, lanes 2–4 and graph). When pol η was introduced (Fig. 6a, lanes 5–7 and graph), an increase in the products (around 7 to 4 kb) was observed, demonstrating again ubiquitination-independent polymerase switching. Moreover, we now detected further accumulation of full-length products on addition of RAD6A–RAD18 (Fig. 6a, lanes 8–10 and graph). Under these reaction conditions, pol η seemed to be hardly contributing to gross DNA replication because no products were detectable after 30 min reaction with pol η in the absence of pol δ (Fig. 6a, lane 11). Notably, when the template was replaced with intact DNA, the replication finished after 5 min, and RAD6A–RAD18 and pol η were without effect (Fig. 6e).

It has been proposed that RAD6A–RAD18 has two biochemical functions for polymerase switching. One is mono-ubiquitination of PCNA. The other is targeting of pol η to 3′-ends by direct interaction.[6] To distinguish the respective contributions of the two functions of RAD6A–RAD18 in this *in vitro* system, we used a PCNA mutant, $PCNA^{(K164R)}$ (Fig. 6b). In the RAD6A–RAD18-independent reactions (Fig. 6b, lanes 5–7), an increase in the products of around 7 to 4 kb was observed to a similar extent as with wild-type PCNA (Fig. 6a). When RAD6A–RAD18 was introduced, no further accumulation of such products was observed (Fig. 6b, lanes 8–10 and graph). As a complementary experiment, E1 was omitted to prevent ubiquitination of PCNA in the presence of RAD6A–RAD18 (Fig. 6d). The results also demonstrated that omitting E1 (Fig. 6d, lanes 5–7) and replacement of PCNA with the mutant, $PCNA^{(K164R)}$ (Fig. 6d, lanes 11–13), reduced the amounts of such products to the same levels with each other, as compared with complete reactions (Fig. 6d, lanes 8–10 and graph). These results suggested that RAD6A–RAD18 itself could not stimulate recruitment of pol η without PCNA ubiquitination in this *in vitro* system.

To address the roles of UBZ[15] of pol η for polymerase switching, we replaced pol η with a UBZ defective mutant, pol $\eta^{(D652A)}$.[15] We detected clear accumulation of products around 7 to 4 kb with the mutant in the absence of RAD6A–RAD18 (Fig. 6c, lanes 5–7 and graph), indicating that the mutation did not affect either polymerase activity or the potential to access 3′-ends. When RAD6A–RAD18 was introduced, further accumulation of such products was not observed (Fig. 6c, lanes 8–10 and graph), demonstrating a crucial role of UBZ in stimulation of polymerase switching *in vitro*.

Discussion

In this work, we demonstrated that PCNA interacting with pol δ can act as a target for ubiquitination, and therefore, PCNA mono-ubiquitination could be coupled with DNA replication. Consequently, we could reconstitute replication-coupled switching between pol δ and pol η on a UV-irradiated template, while the results demonstrated discrepancies with currently accepted models, which have been proposed based on *in vivo* evidence, suggesting that regulatory factors could be missing in this *in vitro* system. Our results do allow us to discuss possible regulatory mechanisms of human pol switching.

Dynamic property of PCNA during the elongation phase of DNA synthesis

In this study, we demonstrated that PCNA accumulated on newly synthesized DNA during elongation. This is consistent with our previous report.[33] We have suggested that it could be a consequence of dynamic properties of pol δ previously.[33] Such a property of pol δ has been also described in other reports for mammalian[38,54] and yeast pol δ.[48,55] The general conclusion from these studies could be that pol δ is spontaneously and frequently dissociated from and associated to the 3′-ends during elongation. In our previous studies, we provided evidence that PCNA is released behind the 3′-end on such dissociation of pol δ. This is quite reasonable, because PCNA is tethered at the 3′-end by interactions with pol δ. We have also found that subsequent reloading of PCNA plays a crucial role in efficient elongation.[33] Therefore, it is rational and practical to attribute the accumulation of PCNA to its reloading during elongation. Notably, in yeast pol δ, accumulation of PCNA during DNA synthesis has been also reported.[48] However, several studies demonstrating opposite results also exist in the literature. Einolf and Guengerich have reported that processivity of mammalian pol δ calculated from kinetic parameters is quite high.[56] Recently, two studies have demonstrated stable pol δ–PCNA complexes at the 3′-ends in yeast.[57,58] The discrepancy might be attributed to a possibility that any modification(s) and/or accessory factor(s) in the respective preparations could differently affect the stability of pol δ.

Mechanisms of PCNA mono-ubiquitination

In this *in vitro* system, PCNA interacting with pol δ on DNA could be ubiquitinated efficiently so that PCNA mono-ubiquitination occurred coupled with DNA replication. In this reaction, RAD6–RAD18 probably accessed PCNA at the reverse side to the interaction with pol δ and RFC. This conclusion is supported by the finding that excess amounts of pol δ and RFC did not inhibit the ubiquitination reaction. Indeed, the Lys164 residue is found on the opposite side of the pol δ-interacting surface of PCNA.[59,60] A possible mechanism for the stimulation by pol δ and RFC could be that these proteins act to dispose PCNA on DNA with the proper geometry for catalysis. Indeed, it is known that the β-clamp of *E. coli*, the counterpart of PCNA, is tilted on DNA.[61] Tilted PCNA probably does not fit the catalytic site of RAD6–RAD18.

Our results showed that PCNA mono-ubiquitination is further stimulated by pol δ if the polymerase is actively replicating. This observation is consistent with findings in a cell-free system.[62] Importantly, the stimulation was not due to direct activation of ubiquitination reactions, but rather a consequence of multiple loading of PCNA molecules on the template, which was coupled with elongation.[33,48] Consequently, inhibition of elongation reduced the amounts of PCNA on the DNA, which also resulted in a decrease in the amount of ubiquitinated PCNA. Thus, growing 3′-ends and two conditions with stalled DNA synthesis, due to exhaustion of dNTP or DNA lesions, all appeared indistinguishable from one another in terms of biochemical actions of RFs, UEs, and pol δ. These findings indicate that factors that can recognize stalled replication itself and prevent association of RAD6A–RAD18 to moving replisome are missing in this *in vitro* system.

Mechanisms of polymerase switching

In this study, we reconstituted polymerase switching between pol δ and pol η, revealing two pathways *in vitro*. One is dependent on PCNA mono-ubiquitination and the other is independent of PCNA mono-ubiquitination, which predominates at higher concentrations of template DNA. This result suggested an intrinsic ability of pol η to access 3′-ends during replication, consistent with a previous report of a cell-free system.[63] Although our *in vitro* system revealed a mechanistic potential of pol η to access 3′-ends during replication, the PCNA mono-ubiquitination-independent pathway must be negligible *in vivo*, since genetic evidence has demonstrated the essential requirement of PCNA mono-ubiquitination for recruitment of pol η.[5,15,22,24,64,65] Importantly, Ouchida *et al.* have proposed the presence of a negative regulatory mechanism preventing inappropriate association of pol η with 3′-ends.[66] The discrepancy could be attributed to lack of protein factors involved in such a mechanism in this *in vitro* system.

The PCNA mono-ubiquitination-dependent reaction appeared at a very low concentration of 3′-end (130 pM, approximately 40 molecules/nucleus in human cells) and featured a crucial role for UBZ of pol η. The results suggest that interaction between UBZ and mono-ubiquitinated PCNA is essential for efficient bypass of one stalled 3′-end in the nucleus. Recent reports concerning yeast pol η in bypass reactions with mono-ubiquitinated PCNA have been contradictory. Polymerase activity of pol η was stimulated by mono-ubiquitinated PCNA in some but not all cases.[9,10] From our results, we consider that differences might be attributable to the assay conditions *in vitro*, depending on which of the two pathways is predominant.

Interaction between RAD6–RAD18 and pol η has been demonstrated,[6,12] and this might be responsible for recruitment to 3′-ends.[6] In the present study, however, polymerase switching was not stimulated by RAD6A–RAD18 itself when PCNA mono-ubiquitination was absent, suggesting that targeting of pol η by RAD6A–RAD18 could be coupled with PCNA ubiquitination.

Conceivable protein actions for human pol switching between pol δ and pol η in this *in vitro* system are shown in Fig. 7. The elongation complex consists of RFC, pol δ, and PCNA,[33] which could be a target for RAD6–RAD18. Usually, pol δ is not retained continuously at 3′-ends as a stable complex with PCNA; rather, it undergoes repeated association and dissociation cycles.[33,38,54] During this cycling,

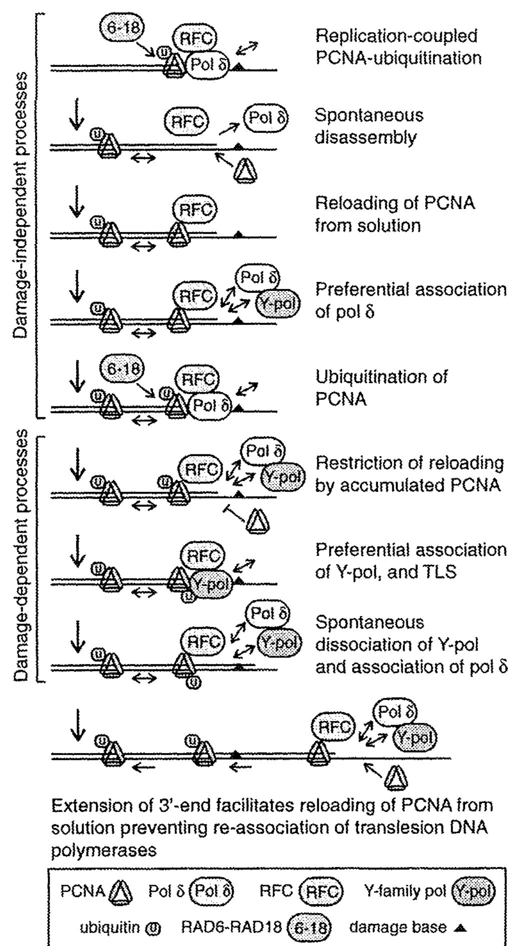

Fig. 7. Conceivable protein actions for PCNA mono-ubiquitination and polymerase switching in the present human *in vitro* system. The elongation complex consists of RFC, PCNA, and pol δ.[33] RAD6A–RAD18 preferentially ubiquitinates PCNA interacting with pol δ at a 3′-end. During association and dissociation cycles of pol δ in the growing 3′-end, the PCNA is released from the 3′-end of DNA, and new PCNA molecules are loaded from solution, preventing the pol η from association. At stalled 3′-ends, in contrast, PCNA accumulates and saturates on DNA during association and dissociation cycles of pol δ; consequently, the same PCNA molecules are repeatedly recruited to the 3′-end. Once the PCNA is ubiquitinated, it persists until association of pol η through interaction with the ubiquitinated PCNA. After DNA synthesis is restored, a fresh PCNA molecule is loaded from solution, reducing the probability of pol η access.

PCNA is released and left behind, and RFC incorporates PCNA that slides back to the 3′-end or reloads PCNA from solution.[33] These are dynamic and stochastic events. When PCNA molecules accumulate on DNA, the probability of reutilization is much higher than that of reloading from solution.[33] Thus, at growing ends, ubiquitin-free PCNA molecules normally tend to be loaded from solution. Under these circumstances, ubiquitinated PCNA molecules are not retained proximately to the 3′-ends so that pol η is restricted from association. At a stalled 3′-end, PCNA molecules are quickly accumulated onto DNA until reaching saturation. Consequently, the same PCNA molecule stays proximately to the stalled 3′-end. Once the PCNA molecule is ubiquitinated, it persists until pol η associates through interaction with the mono-ubiquitinated PCNA and extends the 3′-end. Once DNA synthesis is restored, PCNA molecules on DNA are diluted, facilitating reloading of fresh PCNA from solution and reducing the probability of pol η access.

Importantly, our results in the human system are quite different from some recent reports with a yeast system,[57,58] but not all.[48,55] In those reports,[57,58] it seems that pol δ is stably associated with PCNA during elongation but destabilizes with stalling replication. Such destabilization is more significant when PCNA is mono-ubiquitinated. Once pol η binds to ubiquitinated PCNA, association of pol δ is prohibited. Therefore, it is possible that some factors, which can stabilize pol δ–PCNA and pol η–PCNA interactions, could be missing in this human *in vitro* system.[33,37,38,54] Our system could thus be useful to address such missing factors for further understanding of molecular mechanisms of polymerase switching in humans.

Materials and Methods

Proteins

Recombinant human proteins were overproduced in *E. coli* cells and purified by conventional column chromatography. Detailed procedures for plasmid construction and protein purification are described in the Supplementary Data.

PCNA mono-ubiquitination assays

The standard reaction mixture (25 μl) contained 20 mM Hepes–NaOH (pH 7.5); 50 mM NaCl; 0.2 mg/ml bovine serum albumin (BSA); 1 mM DTT; 10 mM $MgCl_2$; 1 mM ATP; 0.1 mM each of dGTP, dATP, dCTP, and dTTP; 33 fmol of singly primed mp18 ssDNA (with the 90-mer primer, CTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGCCAAGCTTGCATGCCTGCAGG); RPA (9 pmol); PCNA (1 pmol); RFC (88 fmol); E1 (850 fmol); RAD6A–RAD18 complex (950 fmol); and ubiquitin (170 pmol). Reaction mixtures were prepared on ice and then incubated at 30 °C for the indicated times. After termination of reactions with addition of 2 μl of 300 mM ethylenediaminetetraacetic acid (EDTA), the mixtures were immediately chilled on ice. Ubiquitination of PCNA was measured by Western analysis with an anti-PCNA antibody (Santa Cruz, sc-7907). Detection was carried out using an ECL chemiluminescence kit (GE Healthcare, Tokyo, Japan) and a CCD camera.

For ubiquitination assays with poly(dA)–oligo(dT), 100 ng of DNA including 900 fmol of oligo(dT) (GE Healthcare), instead of 33 fmol of mp18 DNA, was mixed under the standard reaction conditions except for the omission of dNTPs, RPA and RFC.

DNA replication assays

The standard reaction mixtures with [α-^{32}P]dTTP (25 μl) were preincubated at 30 °C for 1 min, and then reactions were started by addition of pol δ (380 fmol). After incubation at 30 °C for the indicated times, reactions were terminated with 2 μl of 300 mM EDTA, and the mixtures were immediately chilled on ice. Products of DNA synthesis were analyzed as described earlier.[33] Gel images of autoradiography were analyzed by Multi Gauge software Version 3.0 (FUJIFILM, Tokyo, Japan).

For replication assays with poly(dA)–oligo(dT), 100 ng of DNA including 900 fmol of oligo(dT) (GE Healthcare), instead of 33 fmol of mp18 DNA, was mixed under the standard reaction conditions including [α-^{32}P]dTTP, but not other dNTPs, RPA and RFC.

PCNA mono-ubiquitination of DNA–PCNA complexes isolated by gel filtration

For the introduction of nicks, plasmid pUC18 was reacted with N.BstNBI (New England BioLabs, Tokyo, Japan) at 55 °C for 60 min. Then, DNA was extracted with phenol/chloroform and precipitated with ethanol. PCNA (8 pmol), RFC$^{(p140N555)}$ (36 fmol), and the nicked plasmid pUC18 (133 fmol) were incubated at 30 °C for 15 min in 100 μl of buffer containing 20 mM Hepes–NaOH (pH 7.5), 100 mM NaCl, 0.2 mg/ml BSA, 1 mM DTT, 10 mM $MgCl_2$, and 1 mM ATP. The mixture was then immediately applied at room temperature to a 2-ml column of 4% agarose beads (A-1040-S, Agarose Beads Technologies, Madrid, Spain) equilibrated in buffer containing 20 mM Hepes–NaOH (pH 7.5), 1 mM DTT, 10 mM $MgCl_2$, and 50 mM NaCl, and fractions of three drops each were collected on ice. The fractions eluted in void volume containing DNA were pooled, and then 12.5-μl aliquots were incubated with E1 (850 fmol), RAD6A–RAD18 complex (38 fmol), and ubiquitin (170 pmol) in the presence of the indicated amounts of RFC or pol δ at 30 °C for 30 min in 25-μl reaction mixtures [20 mM Hepes–NaOH (pH 7.5), 50 mM NaCl, 0.2 mg/ml BSA, 1 mM DTT, 10 mM $MgCl_2$, 1 mM ATP, and 0.1 mM each of dGTP, dATP, and dTTP].

Isolation of PCNA on DNA bound to magnetic beads

The 5′-biotinylated primer (TCTCTCTCTCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGCCAAGCTTGCATGCCTGCAGG) was annealed to 33 fmol mp18 ssDNA and immobilized onto a 10-μl suspension of streptavidin magnetic beads, Dynabeads M280 (Life Technologies, Tokyo, Japan), as described previously.[33] Assays were carried out under standard reaction conditions as described for Fig. 4c. After termination of the reactions with 2 μl of 300 mM EDTA, the beads were washed and analyzed.[33]

Assay for PCNA mono-ubiquitination-dependent polymerase switching

RPA (900 fmol), PCNA (1 pmol), RFC (26 fmol), E1 (850 fmol), RAD6A–RAD18 complex (950 fmol), ubiquitin (170 pmol), and 3.3 fmol of singly primed mp18 ssDNA (with the 36-mer primer, CAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGG) were mixed under standard reaction conditions with [α-^{32}P]dTTP in the presence or absence of pol η (10 fmol). After incubation at 30 °C for 1 min, pol δ (750 fmol) was added and the mixture was further incubated for the indicated times. Reaction products (10 μl) were analyzed by 0.7% alkaline agarose gel electrophoresis.[33] Gel images of autoradiography were analyzed by Multi Gauge software Version 3.0 (FUJIFILM).

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2010.01.003

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Specific amino acid residues are involved in substrate discrimination and template binding of human REV1 protein

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ABSTRACT

REV1 is a member of the Y-family DNA polymerases, but is atypical in utilizing only dCTP with a preference for guanine (G) as the template. Crystallography of the REV1–DNA–dCTP ternary complex has revealed a unique mechanism by which template G is evicted from the DNA helix and incoming dCTP is recognized by an arginine residue in an α-loop, termed the N-digit. To better understand functions of its individual amino acid residues, we made a series of mutant human REV1 proteins. We found that R357 and L358 play vital roles in template binding. Furthermore, extensive mutation analysis revealed a novel function of R357 for substrate discrimination, in addition to previously proposed specific interaction with incoming dCTP. We found that the binding pocket for dCTP of REV1 has also significant but latent affinity for dGTP. The results suggest that the positive charge on R357 could prevent interaction with dGTP. We propose that both direct and indirect mechanisms mediated by R357 ensure specificity for dCTP.

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Introduction

Accurate DNA replication is crucial for living organisms. Replicases, which are responsible for genomic duplication, are high-fidelity DNA polymerases whose fidelity is achieved by two characteristics [1]. One is high nucleotide selectivity. The correct Watson–Crick base pair is able to enter into binding pockets without steric clashes. The other is proof reading activity catalyzed by intrinsic 3′ exonuclease. As a consequence of these properties, replicases can not extend primer termini beyond damage bases, resulting in replication blocks [1].

Cells also have another class of DNA polymerases [2], the Y-family members pol η, ι, and κ, which are able to extend primer ends beyond damage bases [3,4]. Consequently, stalled DNA synthesis can be restored. The molecular mechanism allowing incorporation of a deoxyribonucleotide opposite a damaged template is of great interest and there is evidence that a lack of proofreading activity and lower nucleotide selectivity are involved [1], even though DNA polymerase η an κ also utilize Watson–Crick base pairs for nucleotide selection [4]. DNA polymerase ι has an additional property, which is utilization of Hoogsteen base pairs for nucleotide selection, to bypass damage bases [4].

REV1 is a well-conserved Y-family polymerase in eukaryotes. Despite belonging with polymerase family, this enzyme is able to utilize only dCTP as the dNTP source for its deoxycytidyl transferase activity [5]. dCMP is thereby incorporated opposite template G, various DNA lesions, and also opposite templates A, T, and C [6]. Steady-state kinetic studies have demonstrated that templates G and apurinic/apyrimidinic (AP) site are good substrates for the transferase reactions [7–10] and crystallography of yeast and human REV1 has revealed unique mechanisms for recognition of template G and incoming dCTP [11,12]. REV1 has a loose α-loop structure, termed the N-digit, and another structure, named the G-loop, which are conserved in the REV1 family, but not other Y polymerase members. The template G and incoming dCTP do not pair with each other. Instead, the template G is evicted from the DNA helix and interacts with amino acids in the G-loop, and incoming dCTP forms a hydrogen bond with an arginine residue in the N-digit [11,12].

In the present study, we made a series of mutant human REV1 proteins to analyse which amino acid residues are involved in substrate discrimination and template binding. The results suggested that the conserved amino acid residues in the N-digit play crucial roles in bypass synthesis of damaged templates. Together with information from the crystal structure, we discuss novel functions of the N-digit for substrate discrimination and template binding of human REV1 protein.

Abbreviations: AP, apurinic/apyrimidinic; F, tetrahydrofuran; 8oxG, 8-oxoguanine.

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Materials and methods

Proteins. Truncated human *REV1* genes were made by PCR with introduction of an NdeI site at the initiation codon, and inserted into the NdeI site of pET15b. In some cases, the coding region of the resulting plasmids was subcloned into an XbaI site of pBAD22A in which gene expression was induced by arabinose [13]. All proteins were purified as his-tagged fusion proteins, as described previously [13,14] by sequential chromatography using HiTrap Chelating and Superdex 200 columns (GE Healthcare). Protein concentrations were determined by BIO-RAD protein assay using BSA (BIO-RAD) as the standard.

Transferase assay. Oligonucleotide templates 5′-CTCGTCAGCAT CTTCAXCATACAGTCAGTG-3′ [X = G, A, T, C, tetrahydrofuran (F) and 8-oxoguanine (8oxG)] and the primer 5′-CACTGACTGTATG-3′ were purchased. The latter was labeled using polynucleotide kinase (New England BioLabs) and [γ-^{32}P] ATP (GE Healthcare), and annealed to the templates. The standard reaction mixture (25 μl) contained 50 mM Tris-HCl buffer, pH 8.0, 2 mM $MgCl_2$, 25 mM $(NH_4)_2SO_4$, 0.1 mg/ml BSA, 5 mM dithiothreitol, 0.1 mM dNTP, 100 nM primer-template and 1 μl of protein sample, diluted with buffer (50 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]-NaOH, pH 7.5, 10% glycerol, 10 mM β-mercaptoethanol, 500 mM NaCl, and 0.1 mg/ml BSA) as indicated. After incubation at 30 °C for the indicated time, reactions were terminated with 10 μl of stop solution (30 mM EDTA/94% formamide/0.05% bromophenol blue/0.05% xylene cyanole) and products were resolved on 20% polyacrylamide gels containing 8 M urea and autoradiographed at −80 °C. The amount of DNA present in each band was quantified using a Bio-Imaging Analyzer BAS2000 (Fuji Photo Film Co., Ltd.).

Results

To determine the minimum region required for the deoxycytidyl transferase activity of REV1 protein, we made and purified a series of deletion mutants as his-tagged fusion proteins at the N-termini. The transferase activities of the truncated proteins were measured by the standard assay containing 0.1 mM dCTP and 100 nM primer-template with template G. The results revealed that the minimum region exhibiting equivalent activity with full length REV1 was the portion between amino acid residues 341 and 829 (Fig. 1A). Further truncation of 29 amino acid residues at the N-terminal or 10 amino acid residues at the C-terminal reduced the transferase activity to less than 1% of the wild-type value. The kinetic parameters of the deletion mutant consisting of the minimum region, REV1$^{(341-829)}$ indicated that the activity was in fact slightly higher than that of the full-length protein (Table 1). We considered that the difference is due to the instability of the full-length protein [13] but the properties were found to be essentially identical (Table 1). We concluded that the REV1$^{(341-829)}$ is the necessary and sufficient region for the transferase activity, which is slightly shorter than that previously reported [12].

The N-terminal 29 amino acid residues of REV1$^{(341-829)}$, which is the portion of the α-helix structure named the N-digit [12], proved essential for transferase activity (Fig. 1A). The region is well conserved in REV1 proteins of various species (Fig. 1B). To determine the functional role of the conserved amino acid residues, we first replaced each of the most conserved five amino acid residues of REV1$^{(341-829)}$ with alanines (Fig. 1B). The mutant proteins F348A, S356A, R357A, L358A, and H359A were produced in *E. coli*, and purified with the same qualities (Fig. 2).

Then, we determined kinetic parameters of these mutants using template G, and 8oxG and F as model substrates for damaged bases. The replacement of F348 with alanine did not greatly affect the dCMP transferase activity (Table 2). In contrast, replacements in the conserved SRLH motif markedly reduced the activity

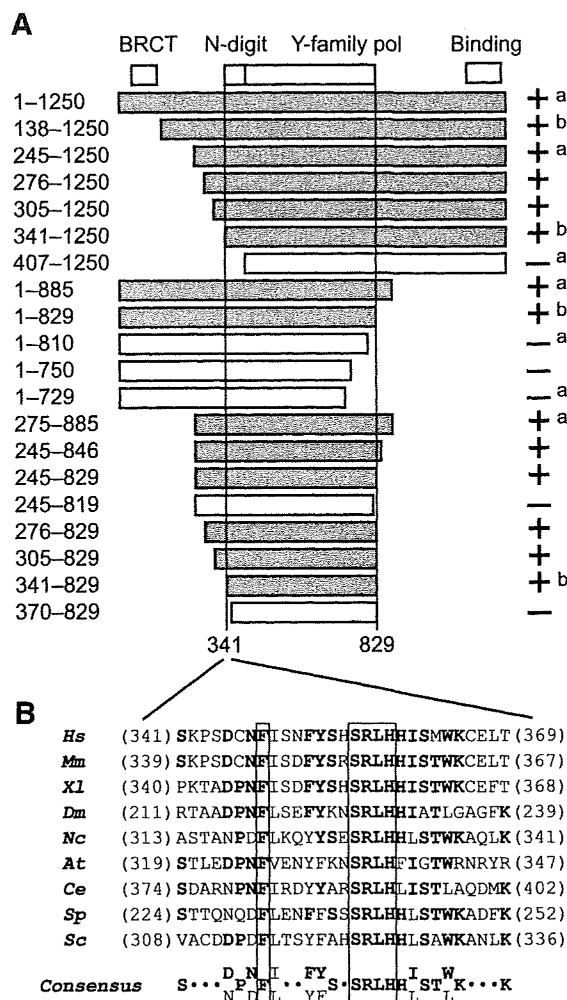

Fig. 1. Deletion analysis of human REV1 protein. (A) Schematic representation of deletion mutants of REV1 protein with their activities. Numbers indicate corresponding amino acid residues of deletion mutants. BRCT, BRCA1 C terminal (BRCT) domain; Y-family pol, conserved region among the family. Binding, binding domain with REV7 and other Y-family DNA polymerases, polη, ι, and κ. + and gray boxes represent activity more than 50% of the full length level. – and open boxes represent transferase activity less than 1% of the full length level. a, data from Ref. [13]. b, data from Ref. [14]. (B) Alignment of amino acid sequences between 341–369 of human REV1 (*Hs*) and corresponding regions of *Mm* (*Mus musculus*), *Xl* (*Xenopus laevis*), *Dm* (*Drosophila melanogaster*), *Nc* (*Neurospora crassa*), *At* (*Arabidopsis thaliana*), *Ce* (*Caenorhabditis elegans*), *Sp* (*Schizosaccharomyces pombe*), and *Sc* (*Saccharomyces cerevisiae*). The conserved amino acids residues are shown in bold letters and the most conserved ones are boxed.

(Table 2). In particular, replacement of R357 and L358 strongly reduced k_{cat} and increased K_M values with the three templates (Table 2). To test the possibility that the defect might be attributed, at least in part, to lowered affinities to template DNA, we determined K_M values. Expectedly, the result showed that K_M values for the three templates with these mutants were much higher than that for wild-type (Table 3), suggesting vital roles of R357 and L358 residues in DNA binding. Interestingly, with L358A the defects in template G and template 8oxG were much more severe than that for the template AP site, whereas with R357A the K_M values were similarly increased with all the three templates (Table 3). This suggested that R357 and L358 residues play distinct roles in DNA binding (see Discussion).

We found that one mutant, R357A exhibited a drastic change for dNTP discrimination on reactions with template G (Fig. 3),

Table 1
Kinetic analysis of REV1$^{(341-829)}$ for dCMP incorporation with various DNA templates. Kinetic assays were performed for 5 min in 25 µl reaction mixtures using 23 fmol REV1$^{(341-829)}$ and 2.5 pmol of the indicated primer-templates. The AP template contained a tetrahydrofuran as an AP site analog. To determine K_M values for dCTP, its concentrations ranged from 1 to 1000 µM. K_M and k_{cat} were evaluated from the plot of the initial velocity versus the dCTP concentration using a hyperbolic curve-fitting program. Data from two to four independent experiments were plotted together and the correlation coefficients (R^2) were more than 0.97.

Template	REV1[a]			REV1$^{(341-829)}$		
	k_{cat} (s^{-1})	K_M (µM)	k_{cat}/K_M ($s^{-1}M^{-1}$)	k_{cat} (s^{-1})	K_M (µM)	k_{cat}/K_M ($s^{-1}M^{-1}$)
G	0.050	0.54	93000	0.073	0.33	220000
A	0.052	23	2300	0.080	29	2800
T	0.027	180	150	0.062	100	610
C	0.030	170	180	0.058	100	560
AP	0.082	7.6	11000	0.13	11	12000

[a] Data from a Ref. [9].

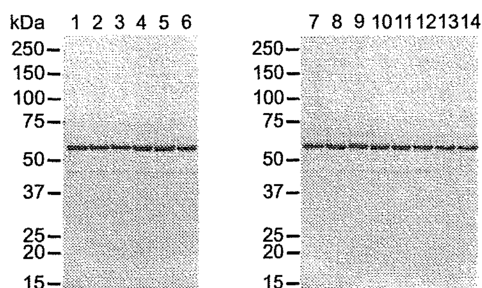

Fig. 2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of purified REV1 mutants. The purified proteins (1 µg) were loaded on an 4–20% gradient polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. lane 1, REV1$^{(341-829)}$; lane 2, F348A; lane 3, H359A; lane 4, S356A; lane 5, R357A; lane 6, L358A; lane 7, REV1$^{(341-829)}$; lane 8, R357A; lane 9, R357K; lane 10, R357S; lane 11, R357G; lane 12, R357T; lane 13, R357Q; lane 14, R357M.

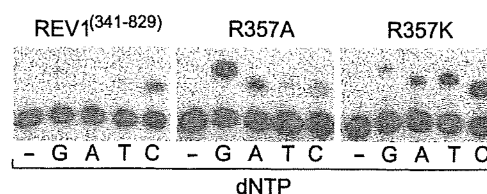

Fig. 3. Altered substrate specificity of R357A and R357K mutants. REV1$^{(341-829)}$ (23 fmol) R357A (26 fmol) or R357K (44 fmol) were incubated under standard reaction conditions with 100 nM of template G and 0.1 mM of each dNTP at 30 °C for 10 min. The reaction products were resolved in 20% polyacrylamide gels containing 8 M urea, and autoradiographed at −80 °C.

the K_M for dCTP being increased 240-fold (Table 4). Surprisingly, the K_M for dGTP, an inappropriate nucleotide, was decreased to 23 µM from 300 µM for the wild-type (Table 4). The efficiencies of dGMP incorporation opposite template G and template 8oxG of the mutant were similar to that for dCTP incorporation opposite template 8oxG of the wild-type (Table 4).

For further analysis of mechanisms of dNTP discrimination by R357, we replaced the arginine residue to lysine, serine, glycine, threonine, glutamine, and methionine. The mutant proteins were purified with equivalent qualities (Fig. 2). Regarding substrate specificity of these mutants under standard assay conditions we found that the properties of R357S, R357G, R357T, R357Q, and R357M were similar to that of R357A having preference for dGTP, while R357K was unique (Fig. 3, data not shown). Consequently, we selected R357K and R357S for further kinetic analysis. The kinetic parameters of the mutant R357S demonstrated similarity to those for R357A, exhibiting preference for dGTP (Table 4). In contrast, R357K retained the preference for dCTP and exhibited slightly higher activities for incorporation of dATP and dTTP on the template G (Table 4).

Table 2
Kinetic analysis of REV1$^{(341-829)}$ and mutants for dCMP incorporation with various DNA templates. Kinetic assays were performed for 5 min in 25 µl reaction mixtures using 2.5 pmol of the indicated primer-templates. The enzyme concentrations ranged from 44 fmol to 1.8 pmol. To determine K_M values for dCTP, its concentrations ranged from 1 to 1000 µM. K_M and k_{cat} were evaluated from the plot of the initial velocity versus the dCTP concentration using a hyperbolic curve-fitting program. Fold reduction of k_{cat} values is shown in parenthesis. Data from two to four independent experiments were plotted together and the correlation coefficients (R^2) were more than 0.97.

Protein	Template G		Template 8oxG		Template AP	
	k_{cat} (s^{-1})	K_M (µM)	k_{cat} (s^{-1})	K_M (µM)	k_{cat} (s^{-1})	K_M (µM)
REV1$^{(341-829)}$	0.073 (1.0)	0.33	0.032 (1.0)	9.1	0.13 (1.0)	11
F348A	0.065 (1.1)	0.79	0.027 (1.1)	3.1	0.053 (2.5)	32
S356A	0.068 (1.1)	10	0.033 (1.0)	7.7	0.014 (9.5)	78
R357A	0.025 (3.0)	79	0.017 (1.9)	57	0.0023 (57)	920
L358A	0.022 (3.4)	71	0.0042 (7.6)	29	0.0032 (42)	490
H359A	0.080 (1.0)	35	0.028 (1.1)	8.1	0.015 (9.2)	160

Table 3
Kinetic analysis of REV1$^{(341-829)}$ and mutants, R357A and L358A, for various DNA templates. Kinetic assays were performed for 5 min in 25 µl reaction mixtures using indicated templates with 88 fmol of REV1$^{(341-829)}$, 1.8 pmol of R357A and L358A. To determine K_M values for the templates, the concentrations ranged from 10 nM to 100 nM. The dCTP concentration was fixed at 100 µM. K_M and k_{cat} values were evaluated from the plot of the initial velocity versus the template concentrations using a hyperbolic curve-fitting program. Data from two to four independent experiments were plotted together and the correlation coefficients (R^2) were more than 0.94.

Protein	Template G K_M (nM)	Template 8oxG K_M (nM)	Template AP K_M (nM)
REV1$^{(341-829)}$	28	40	45
R357A	120	230	190
L358A	110	360	78

Table 4
Kinetic analysis of REV1[(341–829)] R357 mutants for dNMP incorporation with various DNA templates. Kinetic assays were performed for 5 min in 25 μl reaction solutions using 2.5 pmol of the indicated primer-templates. The enzyme concentrations ranged from 44 fmol to 88 fmol. To determine K_M values for dNTPs, the concentrations ranged from 1 to 1000 μM. K_M and k_{cat} were evaluated from the plot of the initial velocity versus the dCTP concentration using a hyperbolic curve-fitting program. Data from two to four independent experiments were plotted together and the correlation coefficients (R^2) were more than 0.95.

dNTP	Template G		Template 8oxG		Template AP	
	k_{cat} (s^{-1})	K_M (μM)	k_{cat} (s^{-1})	K_M (μM)	k_{cat} (s^{-1})	K_M (μM)
REV1[(341–829)]						
G	0.097	300	0.014	72	0.0027	260
A	0.0037	420	0.00075	95	0.00035	680
T	0.048	200	0.014	79	0.0033	410
C	0.073	0.33	0.032	9.1	0.13	11
R357A						
G	0.097	23	0.043	27	0.014	190
A	0.080	48	0.028	56	0.0032	130
T	0.0058	100	0.0098	68	0.0020	580
C	0.025	79	0.017	57	0.0023	920
R357K						
G	0.014	340	0.0075	310	0.00042	380
A	0.027	210	0.012	88	0.0010	240
T	0.028	100	0.015	84	0.0015	270
C	0.042	31	0.045	82	0.0032	380
R357S						
G	0.088	25	0.037	9.2	0.014	170
A	0.047	130	0.023	44	0.0023	450
T	0.013	68	0.0078	35	0.0022	770
C	0.035	84	0.014	21	0.0025	960

Discussion

Although REV1 is a member of Y-family DNA polymerases and the amino acid sequence is well conserved among the members, its catalytic activity is restricted to utilization of dCTP as the dNTP source with a preference for a guanine residue as the template. Deletion analyses here revealed that an extra domain named the N-digit is essential for dCMP transferase activity of REV1, as reported previously [11,12]. This domain is conserved only in the REV1 family but not other Y-family polymerases, suggesting a crucial role for dCMP transferase activity. In this study, we determined for the first time the K_M values for templates. When each of R357 and L358 residues was replaced with alanine, the K_M values were increased. Interestingly, the defect of L358A was not as severe with template F as with the templates G and 8oxG, suggesting that the leucine residue might be crucial for binding to template G and damaged bases, but not the AP site. This is consistent with the model from the crystal structure and suggests that the leucine residue evicts template G from the DNA helix, then taking up much of the vacated space [12]. L358 could thus play a positive role in pushing out the base moiety. In this situation, R357 forms a hydrogen bond with the 5′ phosphate of the ejected template G [12]. Lack of this interaction on the R357A mutant could result in lowered affinity for template DNA.

When the R357 residue was replaced with others, the specificity for dCTP was altered. The kinetic analysis demonstrated that the arginine residue has a crucial function for selective utilization of dCTP. This result also is in good agreement with the model suggested by the crystal structure that the arginine residue forms hydrogen bonds with the cytidine residue of an incoming dCTP [12]. Besides, other amino acid residues surrounding the nucleotide binding pocket could also interact with the cytidine residue, since R357K mutant still retained a significant selectivity for dCTP. Furthermore, our analysis using mutants revealed that the nucleotide binding pocket has a significant but latent affinity for dGTP. When the R357 residue was replaced with other amino acids, in most of the cases, the enzyme lost affinity for dCTP, whereas an affinity for dGTP was obtained. Only in the case of the lysine was the affinity for dGTP rather reduced. It has been established that utilization of dGTP is an intrinsic property of wild-type REV1 [7–10,15]. The results suggest that surrounding amino acid residues in the nucleotide binding pocket except for the R357 residue probably have the potential to stabilize incoming dGTP. A positive charge of arginine or lysine could prevent such interaction with dGTP. Therefore, we suggest that R357 has two functions, which are specific interaction with dCTP and prevention of dGTP binding. We propose that both direct and indirect mechanisms ensure specificity for dCTP.

Our results also demonstrated significance of the SRLH motif for reaction with template AP sites. Mutations not only in R357 and L358 residues, but also S356 and H359, much reduced the transferase activity. Especially, the L358 residue could optimize the binding to a template AP site by occupying the empty space. A SRLH motif could be required for accurate arrangement of the template AP site and dCTP with the active site of REV1.

Many studies have demonstrated in yeast that the deoxycytidyl transferase activity of Rev1 is responsible for bypass synthesis of AP sites in different experimental systems, in which a gapped plasmid [16,17] or oligonucleotides containing an AP site [18–20] were transfected into yeast cells. Furthermore, expression of altered human uracil-DNA glycosylases that remove undamaged cytosines or thymines, was found to result in Rev1 dependent incorporation of dCMP opposite template AP site, generated by the glycosylases in yeast cells [21]. In spite of the elegant mechanisms of the enzyme to ensure incorporation of dCTP [11,12] and accumulating *in vivo* evidence, biological significance for insertion of dCMP is still obscure. In this study, we found novel mutants, which can incorporate dGMP with higher efficiency than dCMP. These could be useful for further analysis *in vivo* to address the biological significance for insertion of dCMP.

In conclusion, our experimental data suggest functional roles of conserved amino acid residues involved in substrate discrimination and template binding of the human REV1 protein. The proposed molecular mechanisms are supported by the model reported recently from crystallography. Furthermore, the mutants obtained in this study should facilitate further analysis *in vivo* to address the biological significance for insertion of dCMP by REV1.

Conflict of interest

None.

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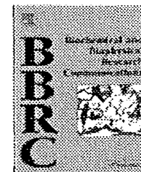

Roles of POLD4, smallest subunit of DNA polymerase δ, in nuclear structures and genomic stability of human cells

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ABSTRACT

Mammalian DNA polymerase δ (pol δ) is essential for DNA replication, though the functions of this smallest subunit of POLD4 have been elusive. We investigated pol δ activities *in vitro* and found that it was less active in the absence of POLD4, irrespective of the presence of the accessory protein PCNA. shRNA-mediated reduction of POLD4 resulted in a marked decrease in colony formation activity by Calu6, ACC-LC-319, and PC-10 cells. We also found that POLD4 reduction was associated with an increased population of karyomere-like cells, which may be an indication of DNA replication stress and/or DNA damage. The karyomere-like cells retained an ability to progress through the cell cycle, suggesting that POLD4 reduction induces modest genomic instability, while allowing cells to grow until DNA damage reaches an intolerant level. Our results indicate that POLD4 is required for the *in vitro* pol δ activity, and that it functions in cell proliferation and maintenance of genomic stability of human cells.

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Introduction

Eukaryotic DNA polymerase δ (pol δ), a key enzyme that participates in DNA replication and repair, consists of four subunits; POLD1 (catalytic subunit, alternatively called p125), POLD2 (p50), POLD3 (p68), and POLD4 (p12) [1,2]. Among those, POLD4 binds to POLD1, POLD2, and an accessory protein of PCNA, which allows pol δ to exhibit its full activity [1,3].

A previous study showed that the *POLD4* ortholog of *Cdm1* in *Schizosaccharomyces pombe* is a non-essential gene related to cell growth, division, and sensitivity to DNA damaging reagents [4]. *Saccharomyces cerevisiae* does not have a POLD4 counterpart, indicating that POLD4 is dispensable in lower eukaryotic cells. In contrast, siRNA-mediated knockdown of POLD4 caused a significant decrease in the proliferation rate of FGF2-activated mouse-endothelial cells [5]. However, it remains unknown whether POLD4 is required for other types of mammalian cells, such as those related to human cancer, or if it has additional functions in mammalian cells.

In the present study, we analyzed the roles of POLD4 for cell proliferation in human lung cancer cell lines. Our findings indicate that POLD4 is required for maintaining the proper nuclear structures and suggest that the pathological structures reflect elevated DNA damage in chromosomes.

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Materials and methods

Antibodies. The antibodies used in this study were anti-POLD4 (POLD4 subunit of pol δ) ascites (2B11, Abnova, Taipei City, Taiwan), anti-lamin B (c-20) (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-γ-tubulin (Sigma–Aldrich, St. Louis, MO).

In vitro pol δ activity. Three- and 4-subunit DNA from pol δ were expressed in *Escherichia coli*, and purified as described previously [6]. pol δ activity was determined in a reaction mixture (25 μl) containing 20 mM HEPES–NaOH (pH 7.5), 50 mM NaCl, 0.2 mg/ml BSA, 1 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM ATP, 0.1 mM each of dGTP, dATP, dCTP, and [α-^{32}P]dTTP, 100 ng poly dA-oligo dT (GE Healthcare, Piscataway, NJ), 86 ng (1.0 pmol as a trimer) of PCNA, and 11–88 ng (46–372 fmol) of pol δ at 30 °C for 10 min. Following incubation, the reactions were terminated with 2 μl of 300 mM EDTA. pol activity was determined with reference to the incorporation of [α-^{32}P]dTMP, as previously described [6].

Colony formation assay. To assess cell proliferation, colony formation assays were performed as previously described [7]. In order to rule out the off-target effect, we designed two independent DNA sequences as follows: MS543F, 5′-GATCCCCagtctctggcatctctatcAT TCAAGAGATgatagagatgccagagactTTTTTGGAAA-3; MS544R, 5′-AG CTTTTCCAAAAAagtctctggcatctctatcATCTCTTGAATgatagagatgccagagactGGG-3; MS551F, 5′-GATCCCCgcatctctatcccctatgaATTCAAGAGATtcataggggatagagatgcTTTTTGGAAA-3; and MS552R, 5′-AGCTT TTCCAAAAAgcatctctatcccctatgaATCTCTTGAATtcataggggatagagatgc GGG-3, in which the targeting sequences are indicated in lowercase letters. To construct shRNA vectors, MS543F and MS544R