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Interaction between DNA Polymerase λ and RPA during Translesion Synthesis

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Abstract—Replication of damaged DNA (translesion synthesis, TLS) is realized by specialized DNA polymerases. Additional protein factors such as replication protein A (RPA) play important roles in this process. However, details of the interaction are unknown. Here we analyzed the influence of the hRPA and its mutant hABCD lacking domains responsible for protein–protein interactions on ability of DNA polymerase λ to catalyze TLS. The primer–template structures containing varying parts of extended strand (16 and 37 nt) were used as model systems imitating DNA intermediate of first stage of TLS. The 8-oxoguanine disposed in +1 position of the template strand in relation to 3′-end of primer was exploited as damage. It was shown that RPA stimulated TLS DNA synthesis catalyzed by DNA polymerase λ in its globular but not in extended conformation. Moreover, this effect is dependent on the presence of p70N and p32C domains in RPA molecule.

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Damages in DNA appear through the action of exogenous and endogenous factors during the whole cell cycle. In spite of the fact that cells use a wide variety of repair mechanisms, the replication complex is sometimes faced with inevitable DNA synthesis using damaged templates. Unrepaired damages block functioning of the replication machinery. Translesion DNA synthesis (TLS) is one of the main strategies used by cells to overcome such situations. Recent studies have shown that special DNA polymerases catalyze this process [1]. In spite of intensive study of TLS, some problems are still unsolved: how many DNA polymerases can participate in TLS at various stages; which DNA polymerases and protein factors participate in DNA synthesis for particular type of lesion; what are the details of switching of the replication mechanism to the TLS mode?

DNA polymerase λ belongs to the X-family and is one of the DNA polymerases that catalyze TLS. DNA polymerase λ is a single-subunit protein with molecular mass 67 kD, possesses DNA polymerase activity, is capable of template-independent DNA synthesis using single-stranded DNA substrates, and lacks the 3′-5′-exonuclease activity [2]. This enzyme was recently discovered as a DNA-dependent DNA polymerase in cells of eukaryotes [3, 4]. Now it is suspected that DNA polymerase λ participates in base excision repair (BER) [5, 6]. This enzyme participates also in non-homologous end joining of DNA [7]. However, the biological role of DNA polymerase λ is still unknown. It was demonstrated that DNA polymerase λ is able to catalyze DNA synthesis across apurinic/apyrimidinic sites (AP sites) [8] and modified bases [8-10]. The effect of a processivity factor of replication DNA polymerases, PCNA, on "normal" synthesis catalyzed by DNA polymerase λ [8, 11] and on DNA synthesis across a tetrahydrofuran fragment, a synthetic analog of the AP site, was also found [8, 9]. PCNA stabilizes DNA polymerase binding to DNA substrate, increasing its affinity for the 3′-end of the primer and processivity of DNA synthesis, but has no influence on the rate and accuracy of the synthesis [8, 12]. This influence is provided via protein–protein interactions between the enzyme and the processivity factor: a PCNA-binding site has been found in the DNA polymerase λ molecule [8, 12].

Abbreviations: AP sites) apurinic/apyrimidinic sites; BER) base excision repair; nt) nucleotide; PCNA) proliferating cell nuclear antigen; RPA) replication protein A; TLS) translesion DNA synthesis.

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The data indicate that replication protein A (RPA) influences the accuracy of functioning of DNA polymerase λ on an undamaged template [8, 11] and that RPA participates in DNA synthesis across 8-oxoguanine and 2-hydroxyadenine catalyzed by DNA polymerase λ [9, 10]. It is known that RPA influences DNA synthesis catalyzed by DNA polymerase λ exclusively in a complex with fixed stoichiometry between the replication factor and the enzyme [11]. It is also known that the accuracy of synthesis increases, because RPA decreases affinity of DNA polymerase to dNTP on binding to the non-complementary nucleotide [11]. However, the detailed mechanism of the influence of RPA on this process is still unknown.

RPA is a necessary participant in many processes of DNA metabolism and plays coordination role due to its specific interaction with DNA as well as with other proteins. RPA is a stable heterotrimeric complex consisting of polypeptides with molecular masses 14, 32, and 70 kD (p14, p32, and p70, respectively) [13]. Among its functions are stabilization of DNA in the single-stranded state, protection of DNA from the action of DNA-modifying factors, positioning of proteins on assembly of multicomponent protein–nucleic acid complexes, and stimulation of functioning of other protein factors [14-18]. RPA is one of the key participants in initiation and elongation of DNA replication [19, 20]. To fulfill most of these functions, RPA must bind to single-stranded DNA with high affinity. It is considered that RPA can bind to DNA, forming two types of complexes; they differ in stability and structure and also in the binding site area (10 and 30 nt) [13, 21]. In the first type of complexes, RPA has a globular conformation. If the length of the free part of the single-strand is sufficient and there is no excess of RPA concentration over DNA concentration, the protein extends along the DNA, forming additional contacts, and transforming into a more stable form. In this type of RPA–DNA complexes, the protein adopts an extended conformation. Thus, study of the effect of various protein–nucleic acid complexes that are involved in RPA functioning in multicomponent enzyme systems such as TLS is of special interest.

MATERIALS AND METHODS

The following materials and reagents were used: BSA from New England Biolabs (England); phage T4 polynucleotide kinase (5000 U/ml) from SibEnzyme (Russia); [γ-^{32}P]ATP with specific activity 5000 Ci/mmol from the Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences; dNTP, reagents for electrophoresis and the main components of buffers from Sigma (USA). Other reagents and buffer components were of analytically pure and extra pure grade of Russian production. The wild type hRPA, its mutant hABCD, and the yeast analog scRPA were isolated and kindly provided by I. O. Petruseva (Institute of Chemical Biology and Fundamental Medicine), A. Bochkarev from the University of Oklahoma (USA), and M. S. Wold from the University of Iowa (USA). Human recombinant DNA polymerase λ was isolated from *Escherichia coli* BL21(DE3)RP cells as described in [22]. The following oligonucleotides were from GenSet (Switzerland): template strand for 8oxoG$_{16}$ substrate 5′-(d)GGCTTCATCGTTGTC***X***CAGACCTG-GTGGATACCG-3′, template strand for 8oxoG$_{37}$ substrate 5′-(d)GACTACATTTCATCTGGCTTGGGCTT-CATCGTTGTC***X***CAGACCTGGTGGATACCG-3′, and primer strand 5′-(d)CGGTATCCACCAGGTCTG-3′, where ***X*** is an 8-oxo-2′-deoxyguanosine residue (8-oxoguanine in the text).

Preparation of 5′-^{32}P-labeled primers. Radioactive label was inserted into the 5′-end of the primer using phage T4 polynucleotide kinase as described in [23]. The reaction mixture (10 μl) contained 0.5 μM primer, 10 MBq [γ-^{32}P]ATP, and 5 U T4 polynucleotide kinase. The reaction was terminated by addition of 1/5 final volume of the denaturing buffer (0.02% Bromophenol Blue and 0.01% Xylene Cyanol in formamide). Nucleotide material was extracted from the corresponding gel portion localized by autoradiography, precipitated, dried in air, and dissolved in water to the appropriate concentration.

The Michaelis constants and the maximum rates of DNA synthesis for various DNA substrates were determined in two steps. In the first step, kinetics of dNMP incorporation into 8oxoG$_{16}$ and 8oxoG$_{37}$ by DNA polymerase λ was studied. The reaction was performed in 80 μl of mixture containing 0.15 μM DNA polymerase λ, 5 μM dNTP, 0.01 μM ^{32}P-labeled DNA substrate, 0.5 mM $MnCl_2$, TBD buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM dithiothreitol (DTT), and 0.25 mg/ml BSA) at 37°C. Aliquots (10 μl) were taken after selected time periods: 0, 1, 3, 5, 10, 15, 20, and 40 min. The reaction was terminated by addition of 1/5 final volume of the denaturing buffer. The reaction products were separated by electrophoresis in 20% polyacrylamide gel under denaturing conditions as described in [24] and visualized autoradiographically using a FX Molecular Imager from BioRad (USA). The data were collected and analyzed using Quantity One software from BioRad. The data were processed using OriginPro7.5 (Microcal Software, USA). To determine the Michaelis constants and the maximum rates of DNA synthesis catalyzed by DNA polymerase λ, in the second step dNTP concentration was varied from 0.01 to 10 μM. Concentrations of other components of the reaction mixture remained as before. The reaction products were analyzed as described above. The data were processed using the Michaelis–Menten kinetic scheme.

When the effects of hRPA and its mutant hABCD on the polymerization reaction catalyzed by DNA polymerase λ were studied, the reaction mixtures 10 μl in vol-

ume contained 0.15 μM DNA polymerase λ, 5 μM dCTP, 0.01 μM ^{32}P-labeled DNA substrate, 0.5 mM $MnCl_2$, hRPA or its mutant form at final concentrations 0, 1, 2, 5, 10, 12, 15, and 20 nM, and TBD buffer. The reaction was performed at 37°C and terminated by addition of 1/5 final volume of the denaturing buffer. The reaction products were analyzed as described above.

RESULTS AND DISCUSSION

In this work, we studied the effect of hRPA and its mutant form hABCD lacking the domains responsible for protein–protein interactions on the ability of DNA polymerase λ to synthesize DNA using a template strand with an 8-oxoguanine residue. Primer–template systems with various length of 5′-protruding strands—16 nt (8oxoG_{16}) and 37 nt (8oxoG_{37})—were used as model DNA structures imitating the first-stage intermediate of TLS. The lengths were chosen in accordance with various types of RPA binding to single-stranded DNA. The 8-oxoguanine residue located in the +1 position of the template strand in relation to the 3′-end of the primer served as a lesion (see "Materials and Methods").

Kinetic parameters of the reaction of dNMP incorporation catalyzed by DNA polymerase λ using 8-oxoguanine-containing DNA substrate 8oxoG_{37}

	K_m, μM	V_m, pM/sec	$(V_m/K_m) \times 10^6$, sec^{-1}
dAMP	0.057 ± 0.005	40.6 ± 1.3	712.5
dCMP	0.025 ± 0.002	41.3 ± 1.0	1651

Note: Results are presented as the average value of three independent experiments (± standard error). Experiments were performed as described in "Materials and Methods".

8-Oxoguanine appearing in DNA, e.g. under oxidative stress, might cause point mutations in the genome since it can form a Hoogsten base pair with dAMP along with a complementary pair with dCMP [25]. In this work, the efficiency of DNA synthesis on 8oxoG_{37} substrates in the presence of dATP and dCTP was evaluated. Kinetic parameters of the reactions are presented in the table; as shown, the efficiency of incorporation of the "correct" dCMP is two times higher than analogous efficiency of dAMP incorporation. In further experiments dCTP was used as the substrate.

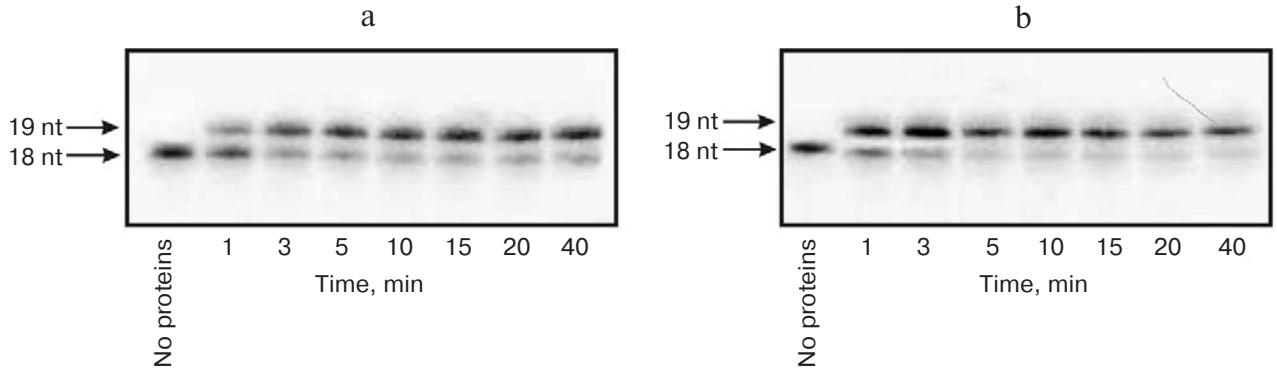

Fig. 1. Kinetics of dCMP incorporation into DNA substrates 8oxoG_{16} (a) and 8oxoG_{37} (b) catalyzed by DNA polymerase λ. The reaction conditions are described in "Materials and Methods".

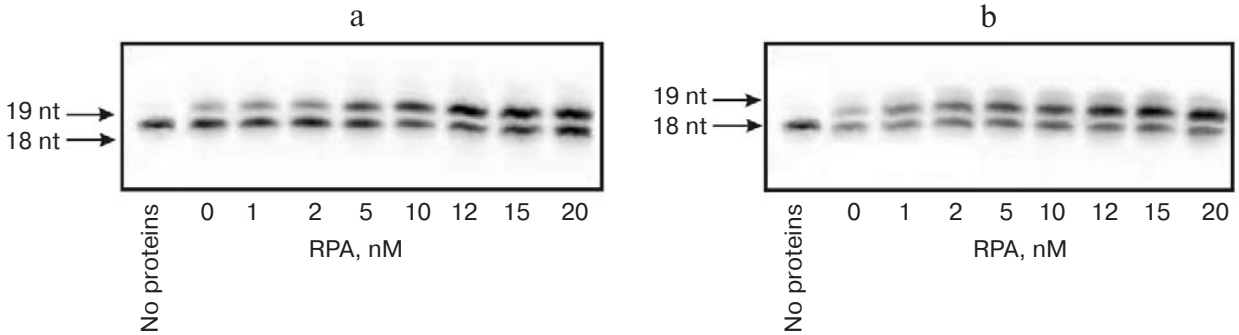

Fig. 2. Effect of hRPA on dCMP incorporation into DNA substrates 8oxoG_{16} (a) and 8oxoG_{37} (b) catalyzed by DNA polymerase λ. The reaction conditions are described in "Materials and Methods".

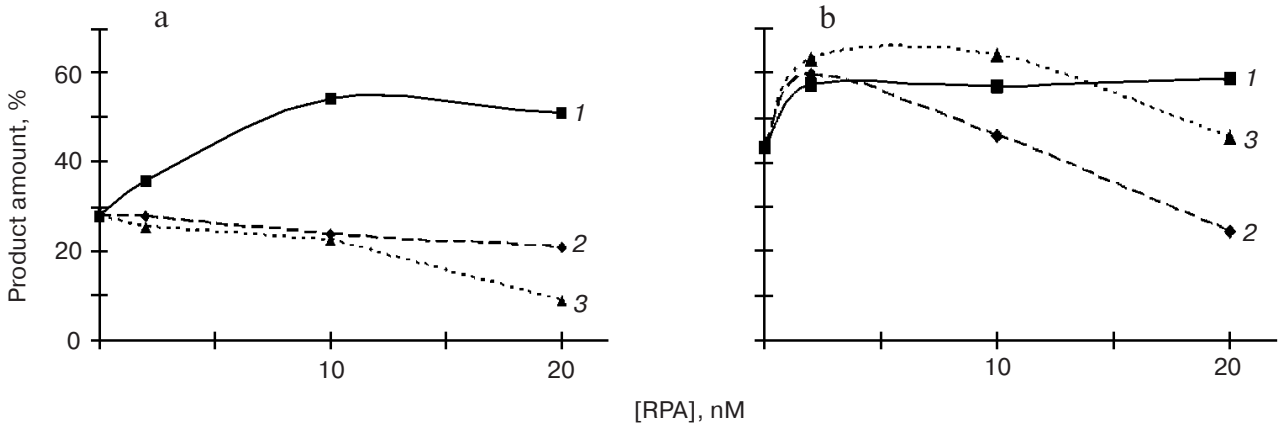

Fig. 3. Effect of hRPA (*1*), its mutant form hABCD (*2*), and yeast scRPA (*3*) on dCMP incorporation into DNA substrates $8oxoG_{16}$ (a) and $8oxoG_{37}$ (b) catalyzed by DNA polymerase λ. Final RPA concentrations in the reaction mixture are indicated on the *x* axis.

The effect of RPA and its mutant was evaluated from the change in the rate of accumulation of the product of the polymerization reaction. Conditions for the reaction of DNA synthesis catalyzed by DNA polymerase λ were chosen so that the yields of products on $8oxoG_{16}$ and $8oxoG_{37}$ substrates in the absence of RPA were 30 and 43%, respectively (Fig. 1). The dependences of the rate of polymerization reaction catalyzed by DNA polymerase λ on the wild type hRPA concentration are presented on Figs. 2 and 3. As shown, when the primer–template structure $8oxoG_{16}$ with protruding strand 16 nt in length is used, the yield of reaction product increases with increase in RPA concentration. However, such effect was not observed when the structure $8oxoG_{37}$ with a long protruding strand was used. Assuming that the types of RPA binding to the chosen DNA substrates are different, we suggest that RPA should have a globular rather than extended conformation for stimulation of TLS catalyzed by DNA polymerase λ.

Nonetheless, the mechanism of this influence is still unclear. Since the mutual influence of proteins is realized via protein–protein contacts, we tried to evaluate the contribution of some RPA domains participating in such interactions. For this, we used the hABCD mutant consisting of four DNA-binding domains A, B, C, D, and the p14 subunit. The absence of the C-terminal domain of the p70 subunit and the N- and C-terminal domains of the p32 subunit, which are responsible for protein–protein interactions, is the special feature of this protein form [21]. In spite of deletion of certain domains, hABCD interacts with DNA as the wild type hRPA does [26]. The data are presented in Fig. 3. As shown, increase in hABCD concentration in the reaction mixture does not cause stimulation of DNA synthesis across 8-oxoguanine catalyzed by DNA polymerase λ if DNA substrates $8oxoG_{16}$ and $8oxoG_{37}$ are used. Replacement of the wild type hRPA by its yeast analog scRPA in the TLS model system gave analogous results (Fig. 3). The data indicate that stimulation of DNA synthesis by RPA is dependent on the N-terminal domain of the p70 subunit and the C-terminal domain of the p32 subunit, that is, mediated by protein–protein interactions between RPA and DNA polymerase λ. The absence of a stimulating effect on hRPA replacement by its yeast analog scRPA indicates that such interactions are species-specific.

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