The 3'–5' exonuclease of human DNA polymerase δ (pol δ) is regulated by pol δ accessory factors and deoxyribonucleoside triphosphates

Suk-Hee Lee

Department of Virology and Molecular Biology, St Jude Children's Research Hospital, Memphis, TN 38101, USA

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ABSTRACT

Human DNA polymerase δ (pol δ) is required for the synthesis of leading strand of simian virus 40 (SV40) DNA replication *in vitro*. Pol δ requires the accessory factors, proliferating cell nuclear antigen (PCNA), activator 1 (A1; also known as replication factor C [RF-C]), human single-stranded DNA binding protein (HSSB; also known as replication protein A [RP-A]) for the elongation of primed template DNA. Since pol δ has an associated 3'–5' exonuclease activity, the effect of pol δ accessory factors on the exonuclease activity was examined. The 3'–5' exonuclease activity was stimulated 8–10 fold by the addition of HSSB, and this stimulatory effect was preferential to HSSB since other SSBs from *E.coli*, T4 or adenovirus, had a little or no effect. The stimulatory effect of HSSB was markedly inhibited by the combined action of A1 and PCNA. Furthermore, the addition of deoxyribonucleoside triphosphates (dNTPs) completely abolished the effect of HSSB on the 3'–5' exonuclease activity even in the absence of pol δ accessory factors. These results suggest that accessory factors and dNTPs regulate both the polymerase and the 3'–5' exonuclease activities.

INTRODUCTION

The simian virus 40 (SV40) DNA replication system *in vitro* has been an excellent model in understanding the enzymology of eukaryotic DNA replication. In the SV40 DNA replication system, both leading and lagging strands are initiated by DNA pol α -primase complex (1, 2). After initiation, pol α falls off the template DNA, and pol δ takes over to synthesize leading strand DNA. During the polymerase switch, accessory factors for pol δ , binding at the 3'-end of primers, not only facilitate pol δ to recognize the end but also prevent pol α to reutilize it (3–7).

For the elongation of primed template DNA, pol δ requires accessory factors, activator 1 (A1; also known as replication factor C [RF-C]), proliferating cell nuclear antigen (PCNA), human single-stranded DNA binding protein (HSSB, also known as replication protein A [RP-A]) and ATP. Multiprotein complex A1 preferentially binds at the primer template junction, and

recruits PCNA to form a PCNA-A1 complex at the 3'-end of the primer in the presence of ATP (4, 5). Upon hydrolysis of ATP by the ATPase activity of A1 complex, pol δ can then bind to the A1-PCNA–DNA complex for the elongation of the primer. HSSB is required for pol δ activity, however, it can be substituted by any other SSB, such as *Escherichia coli* (*E.coli*) single-stranded DNA binding protein (SSB), bacteriophage T4 gene 32 protein (T4 g32) or adenovirus DNA binding protein (Ad DBP) suggesting the role of SSB in pol δ activity might be the sequestration of single-stranded DNA to facilitate A1 to recognize primer ends more efficiently (8).

Highly purified human DNA pol δ contains at least two subunits of 125 and 48 kDa (9) and the 125 kDa subunit has been demonstrated to be the catalytic polypeptide (10). Human pol δ has associated 3'–5' exonuclease activity (11), and this exonuclease activity is likely to be an intrinsic property since, in *Saccharomyces cerevisiae*, both activities reside in the same subunit (12). However, very little is known about the regulation of exonuclease activity of pol δ in DNA replication. Therefore, the effect of pol δ accessory factors on the 3'–5' exonuclease activity of pol δ was examined. The 3'–5' exonuclease activity of pol δ was markedly stimulated by HSSB, and this effect was relatively specific to HSSB since other SSBs has very little or no effect on it. The effect of HSSB on the 3'–5' exonuclease activity was inhibited by the A1 and PCNA complex at the primer ends. The addition of deoxyribonucleoside triphosphate (dNTPs) completely inhibited the effect of HSSB on the 3'–5' exonuclease activity even in the absence of pol δ accessory factors suggesting the 3'–5' exonuclease activity is controlled by the pol δ accessory factors and dNTPs in a way to accommodate two activities of pol δ complex.

MATERIALS AND METHODS

DNAs, proteins and other reagents

The following reagents were obtained commercially: unlabeled nucleotides and aphidicolin were from Boehringer Mannheim, pol(dA)₄₅₀₀ from Life Sciences, oligo(dT)_{12–18}, T4 g32 and *E.coli* SSB from Pharmacia, radioactive nucleotides [α -³²P] dATP (3000 Ci/mmol) and [³H] dTTP (40 Ci/mmol) from New England Nuclear, and single-stranded circular (ssc) ϕ x174 DNA from New England Biolab. The Klenow fragment of *E.coli* DNA

polymerase I and *E. coli* exonuclease III were purchased from Boehringer Mannheim. DNA primer of the ϕ x174 DNA [30-mer, nucleotides (nt) complementary to ϕ x174 (ssc) #5127–5156] was synthesized using an Applied Biosystems 380A DNA/RNA synthesizer. *N*²-(*p*-n-butylphenyl)-2-deoxyguanosine 5'-triphosphate (BuPdGTP) was kindly provided by Dr George Wright (University of Massachusetts, MA). AdDBP was kindly provided by Dr Mark K. Kenny (University of Dundee, UK).

Preparation of multisubunit complex, A1 (4), PCNA (4), and HSSB (13) have been described. Human pol δ was purified by the published procedure (14) with the following modification; hydroxylapatite pool of pol δ (0.15 mg of protein/ml; 14 ml; 3.0×10^5 units) was dialyzed against buffer A [25 mM Tris-HCl (pH 7.5), 10% glycerol, 1 mM DTT, 1.0 mM EDTA, 0.05% (v/v) Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 200 μ g/liter antipain, 100 μ g/liter leupeptin] containing 50 mM KCl and loaded onto a heparin-sepharose column (1 ml of bed volume) and the proteins were eluted with a linear gradient of KCl (50–600 mM). Pol δ eluted at 0.38 M KCl from heparin-sepharose column (0.05 mg/ml; 8 ml; 1.8×10^5 unit) was dialyzed against buffer A containing 50 mM KCl for 3 hrs and loaded onto FPLC-mono Q column and the proteins were eluted with a linear gradient of KCl (50–500 mM). Pol δ fractions (10 μ g/ml; 1.2 ml; 4.0×10^4 unit) were pooled and stored at -70°C . One unit of pol δ activity supported the incorporation of 1 nmol of dTMP in the presence of PCNA, SSB, A1, ATP, and poly(dA)₄₅₀₀: oligo(dT)_{12–18} after 1 hr incubation at 37°C .

Figure 1. Coelution of pol δ and the 3'–5' exonuclease activities from the FPLC mono Q column chromatography. The FPLC mono Q fractions as described in 'Materials and Methods' were assayed for pol δ and the 3'–5' exonuclease activities. One μ l of each designated fraction was used for each assay. Panel A, the standard pol δ assay was performed as described under 'Materials and Methods'. Panel B, the 3'–5' exonuclease assay of the fractions. The assay was carried out as described in 'Materials and Methods' except 10 fmol of 3'-end labeled singly-primed ϕ x174 (2,800 cpm/fmol) was used for the reaction. Lane A, no protein was added; B, 0.05 unit of *E. coli* exonuclease III was added. The numbers at the top of the lanes indicate the fractions of the mono Q column analyzed.

Pol δ assay

Pol δ activity was measured in a reaction mixture (30 μ l) containing 40 mM Tris-HCl (pH 7.8), 7 mM MgCl₂, 1 mM DTT, 150 μ g BSA, 0.1 μ g of poly(dA)₄₅₀₀: oligo(dT)_{12–18} (at a nucleotide ratio of 20:1), 33.3 μ M [³H]dTTP (300 cpm/pmol), 2 mM ATP, 1.0 μ g of HSSB (or T4 g32), 0.1 μ g of PCNA and 0.05 μ g of A1. The incubation was for 30 min at 37°C , and the amount of acid-insoluble dTMP was determined.

Preparation of the 3'–5' exonuclease substrate

The substrate for the 3'–5' exonuclease assay was prepared by annealing of 80 pmol of ϕ x174 DNA primer (30-mer, nucleotides #5127–5156) to ϕ x174 (ssc) DNA (9.2 pmol). A reaction mixture (150 μ l) containing the DNA in 50 mM Hepes/KOH (pH 8.0), 0.8 M NaCl, 1 mM EDTA, was heated to 95°C for 3 min, followed by incubation at 60°C for 30 min. The mixture was then cooled slowly for 30 min to room temperature. The annealed substrate was then 3'-end labeled by the incorporation of two residues of [³²P]dAMP. For this purpose, a reaction mixture (80 μ l), containing 50 mM of Hepes/KOH (pH 8.0), 50 mM NaCl, 10 mM Mg(OAc)₂, 10 mM DTT, bovine serum albumin (BSA; 0.1 mg/ml), 10 μ Ci of [α -³²P]dATP (3,000 Ci/mmol), 2 pmol (as molecule) of primed ϕ x174 DNA, and 5 units of the Klenow fragment of *E. coli* DNA polymerase I, was incubated at 30°C for 30 min. Unlabeled dATP was then added to 100 μ M and the

Figure 2. Effect of HSSB on the associated 3'–5' exonuclease activity of pol δ . The substrate used for the assay of 3'–5' exonuclease was prepared by annealing 160 pmol of ϕ x174 oligomer (30-mer, nt 5127–5156) to ϕ x174 ss circular DNA (1.8 pmol) and 3'-end labeling using a ³²P-dATP and the Klenow enzyme as described. The reaction mixture (30 μ l) contained 100 fmol of 3'-end labeled singly-primed ϕ x174 DNA (250 cpm/fmol of DNA molecule), 50 mM Hepes, pH 7.5, 50 μ g/ml of BSA, 2.5 mM of MgCl₂, 0.5 mM DTT, and 1.0 mM of ATP. Where indicated, 0.1 unit of purified pol δ and 1.0 μ g of purified HSSB were added. Reactions were for 2, 5, 10 and 30 min at 37°C , followed by the addition of 25 mM of EDTA and half of the sample was loaded on 8%-PAGE containing 7M urea. A, The upper band indicates the position of 32-mer and the lower bands represent ³²P-dAMP released by 3'–5' exonuclease. B represents the quantitation of the amount of ³²P-dAMP released by the 3'–5' exonuclease in a reaction.

incubation was continued for an additional 15 min and the reaction was terminated by the addition of 0.5 M EDTA (pH 8.0) to 10 mM. After the phenol extraction, unincorporated dATP and unhybridized primers were removed by the spin column (sepharose 4B) procedure (14).

The 3'–5' exonuclease assay

The exonuclease activity was measured in a reaction mixture (30 μ l) containing 100 fmol of 3'-end labeled singly-primed ϕ x174 (250 cpm/fmol of DNA molecule), 50 mM Hepes/KOH (pH 7.5), BSA (50 μ g/ml), 2.5 mM $MgCl_2$, 0.5 mM DTT, and 1.0 mM of ATP. Reaction was for 15 min unless indicated otherwise. The reaction was stopped by the addition of 25 mM of EDTA, and the sample was boiled for 3 min and loaded on the 8% polyacrylamide gel electrophoresis (PAGE) containing 7M urea in TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.3). After the electrophoresis, the gel was dried and exposed on X-ray film. For the quantitation of the 3'–5' exonuclease activity, the bands corresponding to the substrate and products (dAMP) were excised and counted.

RESULTS

Co-elution of the 3'–5' exonuclease activity with pol δ activity

The heparin-sepharose pool of pol δ activity was subjected to mono Q column (FPLC) and the fractions were measured for pol δ and the 3'–5' exonuclease activities. The pol δ activity was measured in the presence of accessory factors, A1, PCNA, and *E. coli* SSB with poly(dA)₄₅₀₀: oligo(dT)_{12–18} as a template DNA (4). Under this assay condition, the pol δ activity was absolutely dependent on the presence of A1 and PCNA. The 3'–5' exonuclease activity was assayed with 3'-end labeled singly-primed ϕ x174 DNA in which 30-mer (ϕ x174 nucleotides #5156–5127) of primer annealed to ϕ x174 ssDNA was extended to 32-mer by incorporation of two $\alpha^{32}P$ -dAMP at the 3'-end of the primer using Klenow enzyme. This exonuclease substrate will allow us to distinguish between 3'–5' and 5'–3'

exonuclease activities. As shown in Figure 1, both pol δ and 3'–5' exonuclease activities were co-eluted as a single-peak from the column and their relative specific activities were almost the same across the fractions indicating our purified pol δ preparation has its associated 3'–5' exonuclease.

HSSB stimulates the 3'–5' exonuclease activity of pol δ

Since HSSB is one of the accessory factors of pol δ for the elongation of primed DNA, the effect of HSSB on the 3'–5' exonuclease activity of pol δ was examined. The peak mono Q (FPLC) fractions of pol δ (fractions 40–48) were pooled and assayed for the exonuclease activity. The assay condition was the same as described in Figure 1, except the use of 10-fold molar excess of the substrate DNA to the 3'–5' exonuclease (100 fmol instead of 10 fmol of 3'-end labeled singly-primed ϕ x174 DNA) in order to see the effect of the accessory factors. As shown in Figure 2, purified HSSB complex contained no detectable exonuclease activity and the 3'–5' exonuclease activity of pol δ utilized less than 10% of the total substrate. When the pol δ and HSSB were added in the reaction, however, the 3'–5' exonuclease activity of pol δ was stimulated 8–10 fold by the addition of HSSB.

The 3'–5' exonuclease of pol δ is preferentially stimulated by HSSB

Pol δ activity is stimulated by the HSSB in the presence of pol δ accessory factors, A1 and PCNA, however, the HSSB action can be replaced by other SSBs such as T4 g32, *E. coli* SSB and Ad DBP, suggesting the role of HSSB in the pol δ activity is not specific. Since HSSB stimulated the 3'–5' exonuclease activity of pol δ , we examined the effect of other SSBs on it to see whether the role of HSSB was confined to the binding of ssDNA. Compared to HSSB, the *E. coli* SSB, T4 g32, or Ad-DBP has a little or no stimulatory effect on the 3'–5' exonuclease activity of pol δ at the level of pol δ used (0.1 unit) (Figure 3). Sub-saturating level of *E. coli* SSB (0.4 μ g) to cover the single-stranded DNA region stimulated the exonuclease activity about 2.5-fold but had lower stimulatory effect at the higher concentration. This suggests that the role of HSSB in stimulation of the 3'–5' exonuclease activity of pol δ may not merely involve in the sequestration of single-stranded DNA.

Pol δ accessory factors, activator 1 and PCNA inhibit 3'–5' exonuclease of pol δ

The pol δ accessory factors, A1 and PCNA form a complex at the 3'-OH end of the primed DNA template in the presence of ATP which can then bind pol δ to elongate (3, 5). Since pol δ forms a complex with A1 and PCNA on the primed DNA template, the effect of these accessory factors on the 3'–5' exonuclease activity of pol δ was examined. As shown in Figure 4, the 3'–5' exonuclease activity was not affected by either PCNA (lane 8) or A1 (lane 9) or a combination of both (lane 12). The stimulatory effect of HSSB on the 3'–5' exonuclease activity of pol δ , however, was markedly inhibited by the combined action of A1 and PCNA (lane 13, Figure 4), but not by either PCNA or A1 (lanes 11 and 12, Figure 4). The inhibitory effect of PCNA and A1 on the 3'–5' exonuclease activity in the presence of HSSB was plateaued at 60% even with excess amount of A1 and PCNA, and this inhibitory effect was dependent on the presence of ATP (data not shown) indicating that the inhibitory effect is due to the A1-PCNA complex formation.

Figure 3. Effect of different SSBs on the 3'–5' exonuclease of pol δ . Reaction conditions were same as described in Figure 2 with the exception of different SSBs which are as indicated. Panel A represents the quantitation of the amount of ^{32}P -dAMP released. Reactions were for 15 min at 37°C and analyzed on 8% PAGE containing 7M urea (Panel B). Lane A, no protein was added; lane B, 0.05 unit of ExoIII was added.

Figure 4. Effect of activator 1 (A1) and PCNA on the 3'-5' exonuclease of pol δ. Reaction conditions were the same as in Figure 2. Where indicated, 0.05 μg of A1, 0.1 μg of PCNA, 1.0 μg of HSSB and 0.1 unit of pol δ were added. Lane 1 contained no protein, lane 2 contained 0.05 unit of *E. coli* exonuclease III.

Figure 5. Effect of deoxyribonucleoside triphosphates (dNTPs-T) on the 3'-5' exonuclease activity of pol δ. Reaction conditions were the same as in Figure 2. Where indicated, 100 μM of dNTPs-T, 0.05 μg of A1, 0.1 μg of PCNA, and 1.0 μg of HSSB were added. Lanes 2 through 13 contained 0.1 unit of pol δ.

The 3'-5' exonuclease activity is inhibited by deoxyribonucleoside triphosphates (dNTPs) but not by ATP

Since pol δ requires dNTPs as well as ATP for the elongation of primed DNA template, the effect of these nucleotides on the 3'-5' exonuclease activity was examined. The stimulatory effect of HSSB on the 3'-5' exonuclease activity was completely inhibited by the addition of the dATP, dGTP, and dCTP (dNTPs-T) as shown in Figure 5 (lane 3 compared to lane 9). The inhibitory effect of dNTP on the 3'-5' exonuclease activity was not affected by the addition of either A1 or PCNA or both. The addition of dNTPs-T allows the incorporation of three nucleotides at the 3' end of the primer by the pol δ in the lane containing HSSB, A1 and PCNA (lane 12) which resulted in the mobility shift of the labeled primers on the gel. In contrast to dNTPs, ATP had no effect on the 3'-5' exonuclease activity of pol δ and also had no effect on the stimulation of HSSB on the 3'-5' exonuclease activity (Figure 6).

Effect of polymerase inhibitors on the 3'-5' exonuclease activity

The drugs which inhibit pol δ activity were examined for their effects on the 3'-5' exonuclease activity of pol δ. When the 3'-5' exonuclease of pol δ was assayed, both aphidicolin and BuPdGTP inhibited the 3'-5' exonuclease activity of pol δ

Figure 6. Effect of ATP on the 3'-5' exonuclease activity of pol δ. Reaction conditions were as described in Figure 2. Lane 1 contained no protein, and lanes 2 through 7 contained 0.1 unit of pol δ.

Figure 7. Effect of aphidicolin and BuPdGTP on the 3'-5' exonuclease activity of pol δ. The 3'-5' exonuclease activity in the absence of HSSB (○, △) was measured as described in Figure 1. The 3'-5' exonuclease activity in the presence of 1.0 μg of HSSB (●, ▲) was measured as described. The concentration of aphidicolin (circle) and BuPdGTP (triangle) are described in μg and mM, respectively.

(Figure 7). Similar inhibitory effects of aphidicolin and BuPdGTP on 3'-5' exonuclease activity were observed in a reaction containing HSSB.

DISCUSSION

A1, PCNA, and HSSB are absolutely required for the pol δ activity on the elongation of primed DNA template. A1 and PCNA, in the presence of HSSB, form an ATP-dependent complex at the 3' end of the primer, which can then be recognized and elongated by pol δ upon the addition of dNTPs (3, 5).

In this paper, the effect of pol δ accessory factors on the 3'-5' exonuclease activity has been described. Since pol δ has an associated 3'-5' exonuclease activity, which is likely to be an intrinsic property of pol δ polypeptides (16), the effects of A1, PCNA, and HSSB on the 3'-5' exonuclease of pol δ were examined. The 3'-5' exonuclease activity of pol δ was stimulated 8-10 fold by the presence of HSSB (Figure 2). The effect of HSSB on the 3'-5' exonuclease activity was preferential because other SSBs tested (*E. coli* SSB, T4 g32, and Ad DBP) were much

less effective on it. This suggests that the role of HSSB on the 3'-5' exonuclease may be more than just binding to ssDNA. On the other hand, the stimulatory effect of HSSB on the 3'-5' exonuclease activity was significantly inhibited by the A1-PCNA complex but not by either PCNA or A1. This indicates that the A1-PCNA complex at the end of primer not only functions as accessory factors of pol δ for the elongation of the primer but also prevents the 3'-5' exonuclease activity of pol δ . The effect of HSSB on the 3'-5' exonuclease activity was completely inhibited by the addition of dNTPs even in the absence of A1 and PCNA. The above results indicate that the recognition of the primer template between the 3'-5' exonuclease and the polymerase activities of pol δ are different, and therefore their activities are independently regulated by the pol δ accessory factors (HSSB, A1, and PCNA) and dNTPs.

In *E. coli*, the 3'-5' exonuclease subunit (ϵ) of DNA polymerase III is greatly stimulated by its association with the polymerase subunit (α), and this stimulation is mainly due to increased affinity of the ϵ subunit for the 3'-end of the primer (17). Since the 3'-5' exonuclease activity of pol δ is preferentially stimulated by HSSB, it is possible that HSSB interacts with pol δ to increase the affinity of pol δ for the 3'-end of the primer in the absence of A1 and PCNA.

In DNA replication, the 3'-5' exonuclease of pol δ is likely to be involved in the proof-reading activity during the polymerization. Our results, however, indicate that the 3'-5' exonuclease of pol δ may not function during polymerization since the accessory factors and dNTPs inhibit the 3'-5' exonuclease activity. It should be pointed out, however, that the experimental conditions described here may not represent the actual proof-reading activity during the polymerization. It will be interesting to see the effect of the accessory factors on the 3'-5' exonuclease of pol δ with a 3'-mismatched substrate.

Pol δ accessory factors, HSSB and PCNA, has been shown to be involved in UV-induced nucleotide excision repair (18, 19). Shirji et al. (19) and Nichols and Sancar (20) reported that PCNA is not required for the actual excision *per se* but is involved in later repair process. Therefore, it is likely that pol δ and/or pol ϵ is involved in the later stage of DNA repair such as gap filling process (21). It is possible that the 3'-5' exonuclease of pol δ may also be involved in the early excision process by creating the gap in the presence of excision nuclease and HSSB so that the removal of the excised DNA by helicase and the filling-in the gap by polymerase can be done more efficiently.

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