The inhibitory effect of novel triterpenoid compounds, fomitellic acids, on DNA polymerase β

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We previously found new triterpenoid compounds, designated fomitellic acid A and B, which selectively inhibit the activities of mammalian DNA polymerase α and β *in vitro*. On DNA polymerase β, the fomitellic acids acted by competing with both the substrate and the template primer, but on DNA polymerase α, they acted non-competitively. At least on DNA polymerase β, the evidence suggests that each of the fomitellic acids bind to the active region competing with the substrate and/or template primer, and subsequently inhibits the catalytic activity. We therefore further investigated the enzyme-binding properties by using DNA polymerase β and its proteolytic fragments. The 39 kDa enzyme was proteolytically separated into two fragments of the template-primer-binding domain (8 kDa) and the catalytic domain (31 kDa). The fomitellic acids bound tightly to the 8 kDa fragment, but not to the 31 kDa fragment. The immunoprecipitation by antibodies against the enzyme or each of the fragments also proved the binding. These results suggest that the fomitellic acid molecule competes with the template-primer molecule on its 8 kDa binding site, binds to the site, and the fomitellic acid molecule simultaneously disturbs the substrate incorporation into the template primer.

INTRODUCTION

In mammalian and yeast cells, at least five classes of DNA polymerase, α, β, γ, δ and ϵ, are well known. The biochemical and structural properties of DNA polymerase β have been identified, and even some of their genes have been cloned [1–7]. However, the functions of some polymerases *in vivo*, especially the DNA repair-related enzymes (β and ϵ), remain unclear. We have been investigating the structure and function of the DNA repair-related polymerases, especially the β type [8–14], and have screened the compounds that selectively inhibit the activity of DNA polymerase β. We found four new triterpenoid compounds in a basidiomycete which potently inhibit the activities of mammalian DNA polymerase β, and designated them fomitellic acid (FA) A, B, C and D. The chemical studies concerning their molecular structures will be published elsewhere [15]. The purpose of the present study was to investigate the biochemical effect of the FAs on DNA polymerases, and to obtain new information about the structure and function of DNA polymerase β, the structural studies of which have progressed in eukaryotic DNA polymerases [1–6]. FAs A and B were efficiently produced and collectable, but FAs C and D were minor components and extremely difficult to isolate. We therefore used FAs A and B in the present study.

However, the compounds could also affect the replication polymerases, especially DNA polymerase α: an agent that can potently influence mammalian DNA polymerase β activity and penetrate viable cells was not known in the past. Since there are many tissues in which replication polymerase does not function, the FAs could be a key agent for analyzing the functions *in vivo* of the DNA repair-related enzyme in those tissues. In the present study, we investigated the general biochemical effects of the FAs on DNA-metabolizing enzymes and their mode of inhibiting mammalian DNA polymerase β. The amino acid sequence and the tertiary structure of mammalian DNA polymerase β have been studied extensively [1–7]. As the binding targets, we used not only monomeric recombinant rat DNA polymerase β but also the two distinct domains described by Kumar et al. [16,17], the N-terminal 'template binding' domain (8 kDa) and the C-terminal 'catalytic' domain (31 kDa). We also prepared antibodies against each of their fragments, which can neutralize each of the activities. The FAs were observed to bind to the 8 kDa domain fragment. Based on these results, the inhibition mechanism of the FAs and its relation to the enzyme structure of mammalian DNA polymerase β is discussed.

EXPERIMENTAL

Materials

Protein-G Sepharose, CNBr-activated Sepharose 4B, nucleotides and chemically synthesized template primers, such as poly(dA), poly(rA), poly(rC), oligo(dT)$_{12–18}$ and oligo(dT)$_{16}$, were purchased from Pharmacia (Uppsala, Sweden). [^{3}H]dTTP (43 Ci/mmol) and [α-^{32}P]dTTP (3000 Ci/mmol) were purchased from New England Nuclear Co. (Boston, MA, U.S.A.). Alkaline phosphatase-conjugated forms of goat anti-rabbit IgG and normal goat anti-rabbit IgG were purchased from Vector Laboratories (Burlingame, CA, U.S.A.). PVDF membrane (Immobilon-P) were purchased from Bio-Rad (Hercules, CA, U.S.A.).

Abbreviations used: NBT/BCIP, Nitro Blue Tetrazolium/5-bromo-4-chloro-3-indolyl phosphate; HIV-RT, HIV type-1 reverse transcriptase; TdT, terminal deoxynucleotidyl transferase; FA, fomitellic acid; FA-A, fomitellic acid A; FA-B, fomitellic acid B; NP-40, Nonidet P-40; I/E, inhibitor-to-enzyme ratio.

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NBT/BCIP (Nitro Blue Tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) was purchased from Kirkegaard and Perry Laboratories (MD, U.S.A.). Protein markers were obtained from Pharmacia and were as follows: phosphorylase b, 94 kDa; BSA, 67 kDa; ovalbumin, 43 kDa carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20.1 kDa; a-lactalbumin, 14.4 kDa. M13 DNA was purchased from Takara (Tokyo, Japan). All other reagents were of analytical grade and were purchased from Wako Ltd. (Osaka, Japan).

Enzymes

DNA polymerase α was purified from the calf thymus by immuno-affinity-column chromatography as described previously [18]. Recombinant rat DNA polymerase β was purified from *Escherichia coli* JMpβ5 as described by Date et al. [19]. Fragments of 8 and 31 kDa were prepared by controlled proteolysis at 25 °C with trypsin (1500:1, w/w) for 90 min. Fragments were purified as previously described by Kumar et al. [17]. DNA polymerase II (β like) from a higher plant, cauliflower inflorescence, was purified according to the methods outlined by Sakaguchi et al. [11]. HIV type-1 reverse transcriptase (HIV-RT, recombinant) and the Klenow fragment of DNA polymerase I (from *E. coli*) were purchased from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.). T4 DNA polymerase and Taq DNA polymerase were purchased from Takara. Calf-thymus terminal deoxynucleotidyl transferase (TdT) and bovine pancreas DNase I were purchased from Stratagene Cloning Systems (LaJolla, CA, U.S.A.).

DNA polymerase assays

The standard reaction mixture for polymerase α and Taq polymerase (24 μl final volume) contained 50 mM Tris/HCl, pH 7.5, 1 mM dithiothreitol, 1 mM $MgCl_2$, 5 μM poly(dA)/oligo$(dT)_{12-18}$ (2:1) of 3′-OH molarity, 10 μM [^{3}H]dTTP (100 cpm/pmol), 15 % (v/v) glycerol and 8 μl of an inhibitor–enzyme mixture solution. The standard reaction mixtures for polymerase β and plant polymerase II were the same except that it also contained 150 mM KCl. The reaction mixture for HIV-RT contained 50 mM Tris/HCl, pH 8.3, 3 mM dithiothreitol, 10 mM $MgCl_2$, 5 μM poly(rA)/oligo$(dT)_{12-18}$ (2:1), 10 μM [^{3}H]dTTP (100 cpm/pmol), 15 % (v/v) glycerol, 50 mM KCl and 8 μl of an inhibitor–enzyme mixture solution. The reaction mixture for polymerase I contained 10 mM Tris/HCl, pH 7.5, 0.1 mM dithiothreitol, 7 mM $MgCl_2$, 5 μM poly(dA)/oligo$(dT)_{12-18}$ (2:1), 10 μM [^{3}H]dTTP (100 cpm/pmol), 15 % (v/v) glycerol and 8 μl of an inhibitor–enzyme mixture solution. The reaction mixture for T4 polymerase contained 33 mM Tris–acetate, pH 7.9, 0.5 mM dithiothreitol, 10 mM magnesium acetate, 5 μM poly(dA)/oligo$(dT)_{12-18}$ (2:1), 10 μM [^{3}H]dTTP (100 cpm/pmol), 15 % (v/v) glycerol, 66 mM potassium acetate and 8 μl of an inhibitor–enzyme mixture solution. The reaction mixture for TdT contained 100 mM Tris/HCl, pH 7.2, 0.1 mM dithiothreitol, 1 mM $CoCl_2$, 5 μM oligo$(dT)_{12-18}$, 10 μM [^{3}H]dTTP (100 cpm/pmol), 15 % (v/v) glycerol, and 8 μl of an inhibitor–enzyme mixture solution. After incubation at 37 °C for 60 min, except Taq polymerase which was incubated at 74 °C for 60 min, the radioactive DNA product was collected on a DEAE-cellulose paper (DE81) disc as described by Lindahl et al. [20], and the radioactivity was measured in a scintillation counter. One unit of enzyme activity was defined as the amount that catalyzes the incorporation of 1 nmol of dNTP into synthetic template primers (i.e. poly(dA)/oligo$(dT)_{12-18}$, A:T = 2:1) in 60 min under the normal reaction conditions for each enzyme. DNase I activity was measured as described previously [21].

The effect of FAs on DNA polymerases

FAs were dissolved in DMSO at various concentrations and sonicated for 30 s. 4 μl of sonicated sample was mixed with 16 μl of each enzyme (final 0.05 units) in 50 mM Tris/HCl, pH 7.5, containing 1 mM dithiothreitol, 50 % glycerol and 0.1 mM EDTA, and kept at 0 °C for 10 min. These inhibitor–enzyme mixtures (8 μl) were added to 16 μl of each of the enzyme's standard reaction mixtures, and incubation was carried out at 37 °C for 60 min, except Taq polymerase, which was incubated at 74 °C for 60 min. The activity without an inhibitor was considered to be 100 %, and the remaining activity at each concentration of inhibitor was determined as the percentage of this value.

Gel mobility-shift assay

The gel mobility-shift assay was carried out as described by Casas-Finet et al. [22]. The binding mixture (a final volume of 20 μl) contained 20 mM Tris/HCl, pH 7.5, 40 mM KCl, 50 mg/ml BSA, 10 % DMSO, 2 mM EDTA, 2.2 nmol M13 DNA (single-strand and singly primed), and intact or a fragment of purified DNA polymerase β. Various concentrations of fomitellic acid A (FA-A) and fomitelle acid B (FA-B) were added to the binding mixture. The mixture was incubated at 25 °C for 30 min. Samples were run on a 1.2 % agarose gel in 0.1 M Tris/acetate, pH 8.3, containing 5 mM EDTA at 50 V for 2 h.

DNA synthesis *in vitro* on poly(dA)/oligo(dT)

For DNA synthesis, the reaction mixture (20 μl) contained 50 mM Tris/HCl, pH 7.5, 3 mM $MgCl_2$, 5 mM dithiothreitol, 10 % methanol, 20 μM poly(dA)/oligo$(dT)_{16}$ (2:1), 20 μM [α-^{32}P]-dTTP (60 Ci/mmol), and intact or fragments of purified DNA polymerase β. Various concentrations of FA-A or FA-B were dissolved in 100 % methyl alcohol and then added to the above reaction mixture. The mixture was then incubated at 37 °C for 10 min. The products were precipitated with 100 % ethanol and then washed with 70 % ethanol. A dye mixture in Bromophenol Blue was added to the precipitate, which was then loaded onto a 15 % polyacrylamide/7 M urea gel (40 cm × 20 cm × 0.4 mm) in a buffer containing 6.7 mM Tris/borate, pH 7.5 and 1 mM EDTA [23]. The gel was pre-run for 1 h at 2000 V, and electrophoresis was performed at 2000 V. After electrophoresis, the gel was dried and then exposed to imaging plates for 30 min and scanned with a Bio Imaging Analyzer BAS 2000 system (Fuji Film, Tokyo).

Neutralization of DNA polymerase β by anti-DNA polymerase β polyclonal antibodies

The preparation of the anti-rat DNA polymerase β and its domain fragments of 31 kDa and 8 kDa antisera was described previously [24]. The IgG fraction was purified by CNBr-activated Sepharose 4B-conjugated DNA polymerase β affinity-column chromatography. To neutralize the DNA polymerase β activity with anti-DNA polymerase β polyclonal antibodies or normal IgG, DNA polymerase β (0.05 units) was mixed with the diluted antibodies in 50 mM Tris/HCl, pH 7.5, 1 mM dithiothreitol, 0.15 M KCl, 15 % glycerol and 1 mg/ml BSA. After the first incubation for 1 h at 37 °C, the standard reaction mixture for DNA polymerase β was added, and the reaction was incubated for 1 h at 37 °C.

Western-blot analysis

The electrophoretic transfer of proteins from polyacrylamide

gels to PVDF membranes for immunoblotting was performed basically according to the method described by Winston et al. [25]. The sole difference was that the alkaline phosphatase-conjugated secondary antibody was incubated and then detection was carried out using an NBT/BCIP substrate.

Immuno-precipitation of DNA polymerase β and its fragments

The mixture of DNA polymerase β and its fragments (each 0.5 μg, total 1.5 μg) was mixed with FAs (1 nmol, about 10-fold molar of protein) in 50 mM Tris/HCl, pH 7.5, 1 mM dithiothreitol, 0.15 M KCl, 15 % glycerol and 1 mg/ml BSA. Incubation was carried out for 30 min at 37 °C, and then 0.25 μg anti-DNA polymerase β antibody was added, and the reactions were incubated for 1 h at 37 °C. For the immuno-precipitation experiments, 5 μg of protein-G Sepharose was added to the inhibitor-enzyme-antibody mixtures. After additional incubation for 30 min at 37 °C, the immuno-complex was removed by centrifugation. Then the precipitates were used for SDS/PAGE, and the Western-blot analysis was performed.

Other methods

The protein concentrations were determined by the method of Bradford [26] using BSA as the standard. SDS/PAGE of the proteins was performed with a 15 % gel using the standard method of Laemmli [27].

RESULTS AND DISCUSSION

Effect of FAs on the activities of mammalian DNA polymerase α and β, and the other enzymes

FAs A–D were produced from a basidiomycete, *Fomitella fraxinea*. FA-A and FA-B were used in the present experiments because only these two agents were collectable. The chemical structures FA-A and FA-B are shown in Figure 1. The chemical studies which identified the structures will soon be published elsewhere [15].

As shown in Figure 2(A), both FA-A and FA-B at 100 μM strongly inhibited the activities of calf-thymus DNA polymerase α and rat DNA polymerase β, and DNA polymerase II (plant β-like enzyme) from a higher plant, cauliflower, and HIV-RT were slightly influenced at the same concentration (Figure 2A). At this concentration, FA-A and FA-B had almost no inhibitory effect on the prokaryotic DNA polymerases such as the Klenow fragment of *E. coli* DNA polymerase I, Taq DNA polymerase and T4 DNA polymerase, and the other DNA-metabolizing enzymes such as terminal transferase and DNase I (Figure 2A). Both FA-A and FA-B thus appeared to be selective inhibitors of the mammalian (calf and rat) polymerases. Figures 2(B) and 2(C) show the inhibition-dose curves of FA-A and FA-B to these mammalian polymerases. The inhibition by each of the FAs was dose-dependent. Both FA-A and FA-B were slightly more effective at inhibiting DNA polymerase α, with 50 % inhibition by FA-A and FA-B observed at doses of 60 and 25 μM, and almost complete inhibition was achieved at 100 and 40 μM, respectively (Figures 2B and 2C). For DNA polymerase β, 80 μM of FA-A and 40 μM of FA-B were required to achieve 50 % inhibition. FA-B inhibited the activities of DNA polymerases α and β more potently than did FA-A (Figures 2A, B and C). Since aphidicolin, a potent inhibitor of mammalian DNA polymerase α, inhibits it at 20 μM, the FA-B effect on DNA polymerase α is relatively strong. Both FA-A and FA-B not only influenced the activity of the repair-related polymerase (especially DNA polymerase β), but also penetrated to Hela cells and inhibited their growth (unpublished data), suggesting that these DNA polymerase β inhibitors have not been observed in the past; these inhibitors could be a key agent for analyzing the functions of the repair-related polymerase *in vivo*.

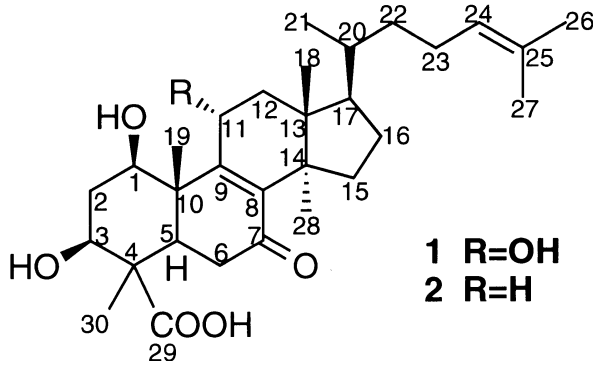

Figure 1 Structures of FA-A (1) and FA-B (2)

Effects of the reaction conditions on DNA polymerase inhibition

To determine the effect of a non-ionic detergent on the binding of FA-A and FA-B (100 μM each) to polymerases, Nonidet P-40 (NP-40) was added to the reaction mixture at a concentration of 0.05 % (Table 1). The DNA polymerase β inhibition by FA-A and FA-B was largely reversed by the addition of NP-40 to the reaction mixture, but the DNA polymerase α activity was not. NP-40 resolved only the binding to DNA polymerase β, indicating that FA-A (Table 1) or FA-B (Table 1) may bind to and interact with the hydrophobic region of the DNA polymerase β protein. The binding to DNA polymerase α is thus thought to be much tighter. We also tested whether an excessive amount of poly(rC) (40 μM) or BSA (80 μg/ml) could prevent the inhibitory effect of FAs (Table 1) to determine whether the effect of the FAs resulted from the non-specific adhesion of FAs to the enzymes, or bound selectively to special sites. Poly(rC) and BSA had little or not influence on the effect of FAs, suggesting that the binding to DNA polymerases occurs selectively (Table 1).

Mode of DNA polymerase α and β inhibition by FA-A and FA-B

Next, to elucidate the inhibition mechanism, the extent of inhibition as a function of DNA template primer or dTTP substrate concentrations was studied (Figure 3). FA-B influenced the activities more strongly than did FA-A; Figure 3 shows the kinetic analyses of FA-B. We also analyzed the kinetics of FA-A, and obtained similar results (data not shown). In the kinetic analysis, poly(dA)/oligo(dT)$_{12-18}$ and dTTP were used as the DNA template primer and substrate, respectively. Double reciprocal plots of the results show that the FA inhibition of DNA polymerase α activity was non-competitive with the DNA template and the substrate (Figure 3A and 3B). In the case of the DNA template, the apparent Michaelis constant (K_m) was unchanged at 14 μM, whereas 80 % and 40 % decreases in V_{max} were observed in the presence of 10 and 25 μM FA-B, respectively (Figure 3A). The K_m for the substrate (dTTP) was 3.3 μM, and the V_{max} for the substrate decreased from 125 to 50 pmol/h in the presence of 25 μM FA-B (Figure 3B). In contrast, the inhibition of DNA polymerase β by FA-B was competitive with the DNA template, since there was no change in the apparent V_{max} (50 pmol/h), while the K_m increased from 5 to 25 μM template

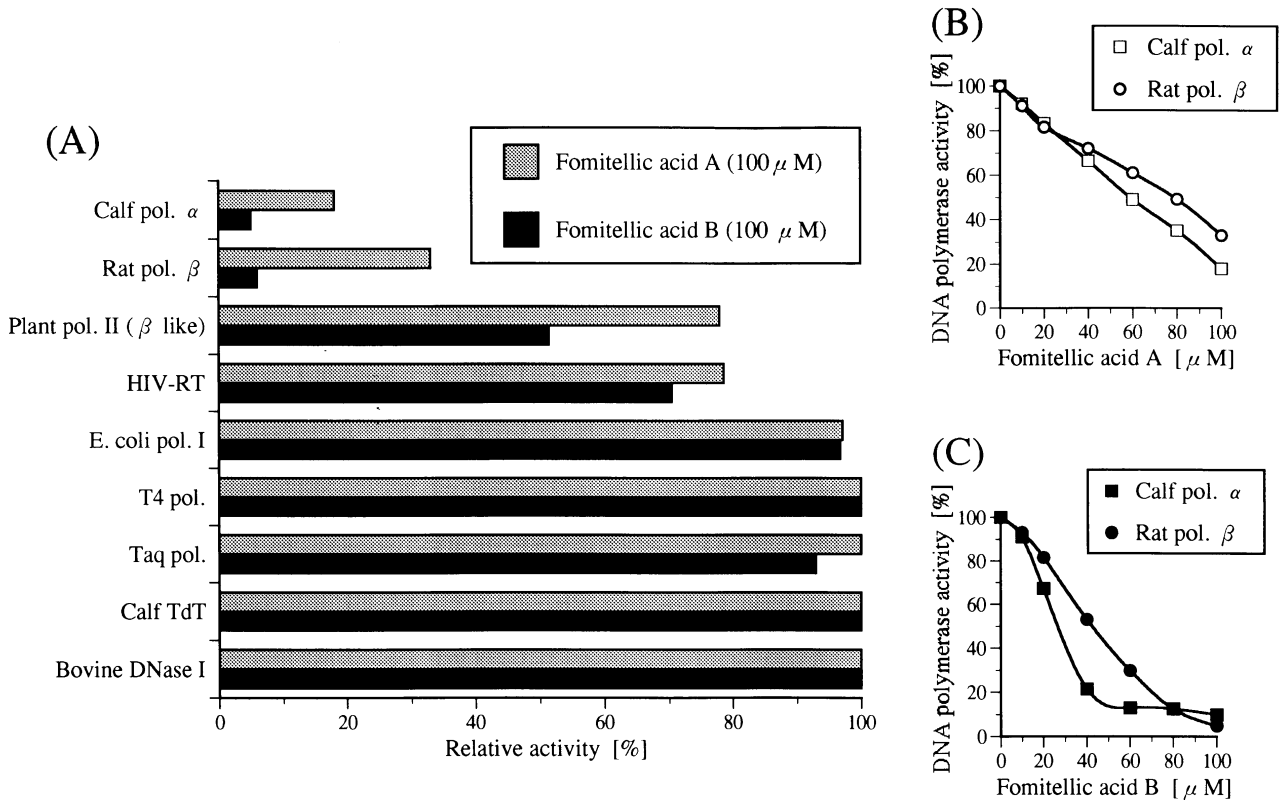

Figure 2 Inhibitory effects of FA-A and FA-B

(**A**) Inhibitory effects of FA-A and B on the activities of various DNA polymerases and other enzymes (0.05 units each). The enzymic activity was measured as described in the text. Enzyme activity in the absence of FA was taken as 100%. Effect on calf-thymus DNA polymerase α (□, ■) and rat DNA polymerase β (○, ●) activities by FA-A (**B**) or FA-B (**C**). Pol, polymerase.

Table 1 Effect of detergents on the inhibition of DNA polymerase α and β activities (0.05 units each) by 100 μM FA-A and 100 μM FA-B

40 μM poly(rC) and 80 μg/ml BSA or NP-40 (0.05%) were added to the reaction mixture.

Compounds added to the reaction mixture	DNA polymerase activity (%)	
	Calf polymerase α	Rat polymerase β
FA-A		
100 μM FA-A	18	33
100 μM FA-A + 40 μM poly (rC)	18	31
100 μM FA-A + 80 μg/ml BSA	23	38
100 μM FA-A + 0.05% NP-40	21	99
FA-B		
100 μM FA-B	10	5
100 μM FA-B + 40 μM poly (rC)	10	5
100 μM FA-B + 80 μg/ml BSA	12	11
100 μM FA-B + 0.05% NP-40	15	96

DNA, in the presence of 0–60 μM FA-B (Figure 3C). Similarly, the apparent V_{max} for the substrate dTTP was unchanged at 85 pmol/h, whereas a 10-fold increase in the K_m was observed in the presence of 50 μM FA-B (Figure 3D). The FA-B inhibition was, therefore, competitive with respect to the substrate dTTP. The inhibition constant (K_i) values, obtained from Dixon plots, were found to be 12 μM and 13 μM for the template DNA and substrate dTTP, respectively (Figures 3E and 3F). Similar results were observed with FA-A, and the DNA polymerase α inhibition was non-competitive (the K_m was unchanged at a concentration of 16 μM DNA template and 2 μM substrate), but the DNA polymerase β inhibition was competitive (the V_{max} for the DNA template and substrate were unchanged at 85 and 100 pmol/h, respectively). The inhibition of DNA polymerase β by FA-A was also competitive with both the DNA template and the substrate, and the K_i values for DNA template and dTTP were 22 and 30 μM, respectively (data not shown). The FAs may interact with or affect both of the binding sites on DNA polymerase β, thereby decreasing its affinity for the DNA template and substrate, whereas they may bind or interact with a domain distinct from the template or substrate binding sites on DNA polymerase α. Since the FAs bear no structural resemblance to either the DNA template or the substrate, we suggest that the structure of both binding sites on DNA polymerase β may incorporate the FA molecules. Namely, regarding DNA polymerase β, the FAs bind to the enzymic active region in competition with substrate and/or template primer, and subsequently inhibit the catalytic activity. We also think that the binding sites on DNA polymerase α and β are structurally different from each other, or an FA binding site on DNA polymerase α, distinct from the DNA template and substrate-binding sites, is more influential than these competitive sites with respect to enzymic inhibition. At least, the binding to DNA polymerase β could be dissociated with NP-40 (Table 1), the enzyme-binding properties of the FAs to DNA polymerase β should be more precisely investigated. The remainder of this report is devoted to an analysis of the action mode of the FAs on DNA polymerase β.

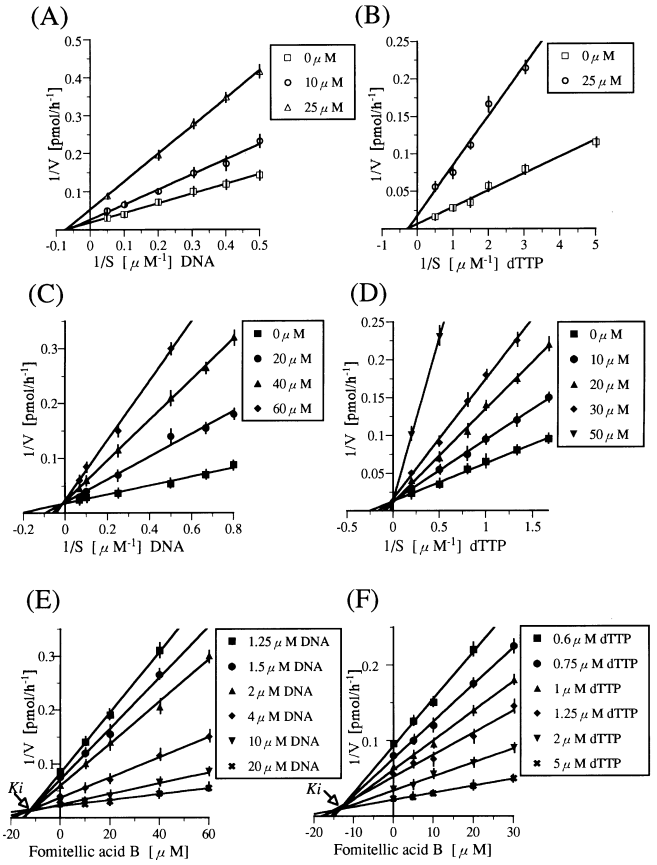

Figure 3 Kinetic analysis of FA-B inhibition of DNA polymerase α and β (0.05 units each)

(**A**) DNA polymerase α activity was measured in he absence (□) or presence of 10 (○) or 25 μM (△) FA-B using the indicated concentrations of the DNA template primer. (**B**) DNA polymerase α activity was assayed with the indicated concentrations of the dTTP substrate in the presence of 25 μM FA-B (○) or in the absence of FA-B (□). (**C**) DNA polymerase β activity was measured in the absence (■) or presence of 20 (●), 40 (▲) or 60 μM (◆) FA-B using the indicated concentrations of the DNA template primer. (**D**) DNA polymerase β activity was assayed with the indicated concentrations of the dTTP substrate in the presence of 10 (●), 20 (▲), 30 (◆) or 50 μM (▼) FA-B or in the absence of FA-B (■). (**E**, **F**) The inhibition constant (K_i) was obtained at 12 and 13 μM from a Dixon plot made on the basis of the same data for **C** and **D**, respectively.

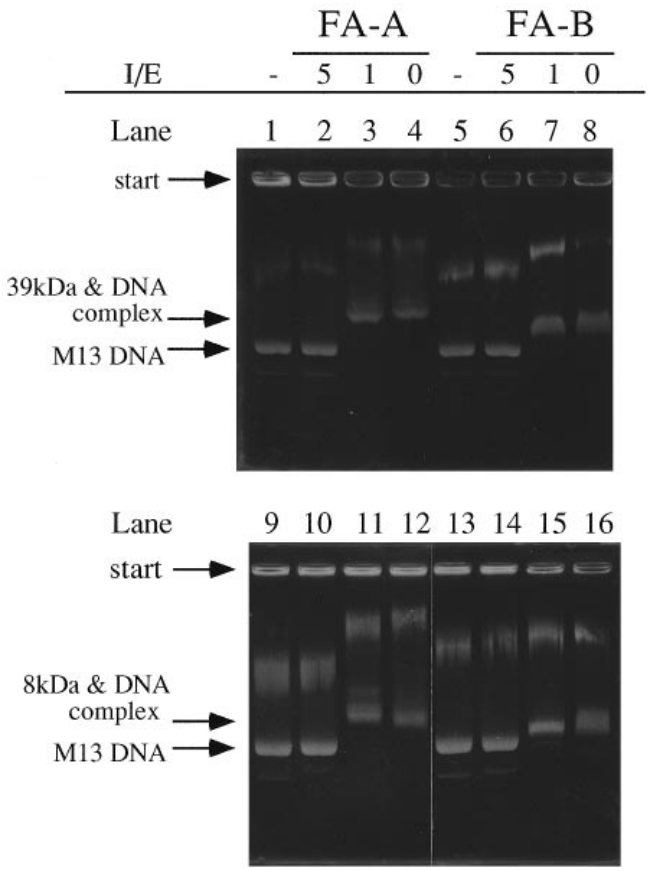

Figure 4 Gel mobility-shift analysis

Gel shift analysis of binding between M13 DNA and DNA polymerase β. M13 DNA (2.2 nmol; nucleotide) was mixed with purified proteins and FAs. Lanes 2–4 and 6–8 contained purified DNA polymerase β (39 kDa) at a concentration of 0.15 nmol; lanes 10–12 and 14–16 contained purified 8 kDa fragment at a concentration of 0.15 nmol; lanes 1, 5, 9 and 13 contained no enzyme. Lanes 1 and 9, lanes 2 and 10, lanes 3 and 11 and lanes 4 and 12 were each mixed with decreasing concentrations of FA-A: 0, 0.75, 0.15, or 0 nmol, respectively. Lanes 5 and 13, 6 and 14, 7 and 15, and lanes 8 and 16 were each mixed with decreasing concentrations of FA-B: 0, 0.75, 0.15 or 0 nmol, respectively. Samples were run on a 1.2% agarose gel in 0.1 M Tris/acetate, pH 8.3, containing 5 mM EDTA at 50 V for 2 h. A photograph of an ethidium bromide-stained gel is shown. I/E, inhibitor-to-enzyme ratio.

Analysis of the binding between FAs and DNA polymerase β

The rat DNA polymerase β used in this study has been extensively studied, including its amino acid sequence and its secondary and tertiary structures [1–7]. The enzyme can be divided into two domain fragments of 8 and 31 kDa polypeptides using controlled proteolysis [17]: an 8 kDa N-terminal fragment and a 31 kDa C-terminal fragment. The 31 kDa domain is the catalytic part involved in DNA polymerization, and the 8 kDa domain is the DNA template primer-binding domain [16]. We prepared the whole enzyme of DNA polymerase β with a molecular mass of 39 kDa, and two domain fragments of 8 kDa and 31 kDa polypeptides. Both fragments were obtained by the controlled proteolysis described by Kumar et al. [17], and purified through FPLC Superose 12 chromatography to near homogeneity (see Figure 4 in [28]. The template primer-binding-protein activity and the DNA polymerization activity were analyzed by a gel mobility-shift assay and by analyzing the products of poly(dA)/oligo(dT)$_{16}$ used as the template primer, respectively.

Figure 4 shows the gel mobility-shift assay of the M13 DNA 39 kDa DNA polymerase β-binding complex (lanes 4 and 8) and M13 DNA 8 kDa domain fragment-binding complex (lanes 12 and 16). The 39 kDa DNA polymerase β and 8 kDa domain fragment bound to M13 DNA and was shifted in the gel, but the 31 kDa fragment, the catalytic domain without a DNA-binding site, was not shifted (see Figure 5A in [28]). In the binding, M13 DNA at 2.2 nmol (nucleotide) was added with 0.15 nmol of the enzyme or the fragment. The M13 DNA used was often separated into a major band and a faint band (lanes 5, 9 and 13). FA-A and FA-B interfered with the complex formations between M13 DNA and DNA polymerase β (upper panel) and between M13 DNA and the 8 kDa fragment (lower panel) to the same extent. The molecular ratios of the FA and the enzyme (or the fragment) are shown as the inhibitor-to-enzyme ratio (I/E) in Figure 4. In the interference by FA-A, the I/E ratios in lanes 2, 3 and 4 for 39 kDa DNA polymerase β and lanes 10, 11 and 12 for the 8 kDa domain fragment were 5, 1 and 0, respectively. At the I/E ratio of 5, the interference by FA-A was nearly perfect, and at the ratio

of 1, it disappeared, suggesting that a few molecules, maybe one molecule, of the FA competes with one molecule of M13 DNA and subsequently interferes with the binding of DNA to the 39 kDa DNA polymerase β or the 8 kDa domain fragment. FA-B interfered at an I/E ratio of 1 in almost the same manner as did FA-A (lanes 5–8 and lanes 13–16). Although the minimum inhibitory concentration of FA-B was much lower, both the I/E ratios of FA-A and FA-B were the same.

Immunological response to the antibodies against DNA polymerase β and its fragments

As described above, the FAs are thought to interfere with the DNA polymerase β activity by binding to the 8 kDa domain fragment in competition with the template primer. We tested this phenomenon using antibodies against DNA polymerase β and each of the domain fragments. We prepared polyclonal antibodies that can neutralize each of the activities. The IgG fraction was purified through CNBr-activated Sepharose 4B-conjugated DNA polymerase β affinity-column chromatography, and the immunoprecipitation procedure for DNA polymerase β and its fragments described in the Experimental section.

DNA polymerase β was incubated with the purified antibodies against the 39 kDa (p39), the 31 kDa (p31) or the 8 kDa peptide (p8) and then the polymerase activity was assayed. As shown in Figure 5A, all the antibodies were able to neutralize the polymerase activity. The strongest neutralization was achieved by the anti-p39 antibody, the second by the anti-p8 antibody and the weakest by anti-p31 antibody (Figure 5A). Figure 5(B) shows the Western-blot analyses of purified proteins of the 39 kDa DNA polymerase β (lanes 1–3), the 31 kDa domain fragment (lanes 4–6) and the 8 kDa domain fragment (lanes 7–9). The anti-p39 antibody was able to make each of the proteins react, indicating that the anti-p39 polyclonal antibody fraction contains at least two species of antibodies which individually recognize the 31 kDa and 8 kDa proteins (lanes 1–3). In contrast, the anti-p31 and the anti-p8 antibodies recognized the 39 kDa DNA polymerase β and the respective antigen proteins, and did not react with the 8 kDa or 31 kDa proteins, respectively. Under the same conditions used in Figure 5B, we added each of the FAs in the immuno-precipitation reaction mixture, and tested their effect on the immuno-precipitation. The Western-blot analysis results after the immuno-precipitation are shown in Figure 5C. Each of the FAs prevented only the immuno-precipitation of the 8 kDa domain fragment by the anti-p39 antibody, and had no influence on those of the 39 kDa DNA polymerase β or the 31 kDa domain fragment. The phenomenon became clearer in the tests using the anti-p8 antibody. The site where FA-A or FA-B binds to the 8 kDa domain fragment is thus thought to be a major site of the epitopes for the antibodies, and the FAs must compete with it. However, neither FA-A nor FA-B could prevent the immunoprecipitation by the anti-p31 antibody of the 39 kDa DNA polymerase β and the 31 kDa domain fragment proteins. From these results, the hypothesis described above that the FA competes with M13 DNA and subsequently interferes with the binding of DNA to the 8 kDa domain fragment appears to be correct. The FA-A or FA-B binding to the template primer-binding domain subsequently inhibits the catalytic activity outwardly in competition with the substrate.

Product analysis after synthesis on poly(dA)/oligo(dT)

The question thus arose as to whether the FAs have no direct influence on the catalytic site. We tested whether the catalytic activity on the 31 kDa domain fragment is inhibited with the FAs. The catalytic domain fragment (31 kDa protein) can bind to the DNA template primer (although weakly), and polymerize the DNA [16].

We used poly(dA)/oligo$(dT)_{16}$ as the template primer, and analyzed newly synthesized DNA fragments produced by the 31 kDa protein (Figure 6). The reaction products *in vitro* were investigated by using denaturing PAGE. In the experiment shown in Figure 6, 25 ng of DNA polymerase β was used, resulting in the re-initiation of nascent chains during the synthesis period. Figure 6 shows the products formed by the enzyme (lanes 1–8) or the 31 kDa domain fragment (lanes 9–11). It is known that DNA polymerase β is a distributive enzyme [29], which adds a single nucleotide and then dissociates from the template–product complex, and the 31 kDa fragment can replicate DNA, as does the whole enzyme. Within a 10 min incubation period, most of the primers were elongated (lanes 1 and 5). With 50 ng of the 31 kDa fragment, DNA replication was also observed (lane 9). The 8 kDa domain fragment was incapable of replicating DNA (see Figure 6A in [28]). At an I/E ratio of more than 5, both FA-A (lanes 1–4) and FA-B (lanes 5–8) completely suppressed the DNA polymerization by the whole enzyme. The 31 kDa domain fragment synthesized DNA without interference from FA-A or FA-B. When the I/E ratio for the enzyme was 1, the DNA synthesis occurred. However, the DNA was synthesized even when the I/E ratio for the 31 kDa catalytic fragment was 50. When the I/E ratio was more than 10000, the FAs barely inhibited the catalytic activity (data not shown). At the range of the FA concentrations that influence the template primer-binding site on the 8 kDa domain, the FAs are thus thought to indirectly inhibit the DNA polymerization on the 31 kDa catalytic site as a result of a lack of template primer, and to outwardly compete with the substrate.

As described in the Introduction, we have been interested in determining the structure and function of the DNA-repair-related polymerases, especially DNA polymerase β. We demonstrated in the present study that on the DNA polymerase β protein, one molecule of the FA competes with one molecule of the template primer and subsequently interferes with the binding of the template primer to the 8 kDa domain, and that the FA binding indirectly inhibits the catalytic activity on the 31 kDa domain. The action of FAs was quite similar to the effect by long-chain fatty acids reported previously [28], although the triterpenoids and the fatty acids are not related structurally at all. The carboxyl group (hydrophilic region of the triterpenoid) at the position of carbon 3 in the FAs is thought to be important for the inhibition of DNA polymerase β, because a semi-synthetic compound without the carboxyl group could not inhibit the activity (data not shown). Compared with FA-A, FA-B has no hydroxyl group at the position of carbon 11, and is a stronger inhibitor than FA-A. FA-B appears to be more hydrophobic than FA-A. The structure without the hydroxyl group was also thought to be important for the inhibition. Both hydrophilic and hydrophobic sites in the fatty acids also played crucial roles in the inhibition [30].

According to the structural studies of DNA polymerase β, the active site is like a crevice between two of the 'thumbs', i.e. the 31 and 8 kDa domains [1–6]; the 8 kDa domain is composed of four α-helices, and these helices are amphipathic with the hydrophobic region in packing [31,32], which may be important for the catalytic activity [16]. Therefore, we speculate that the inhibition process, based on the present results and the previous fatty-acid findings [28], is as follows: initially one FA molecule becomes inserted into the crevice between the template primer-binding site on the 8 kDa domain and the catalytic site on the 31 kDa domain, and then the hydrophobic area of the FAs binds

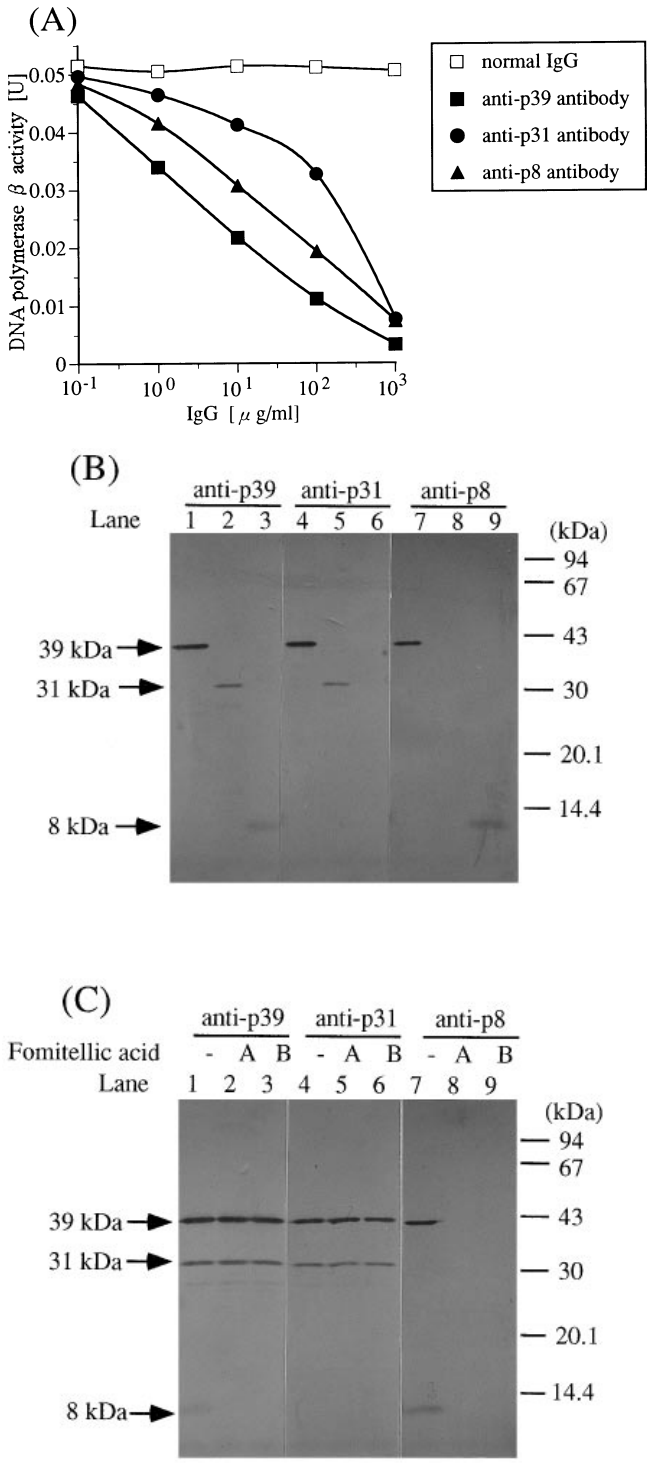

Figure 5 Anti-DNA polymerase β antibodies experiments

(**A**) Neutralization assay. DNA polymerase β (0.05 units) was incubated with the indicated amounts of anti-p39 antibody (■), anti-p31 antibody (●), anti-p8 antibody (▲) and normal IgG as the control (□). (**B**, **C**) Western-blot analysis of 39 kDa DNA polymerase β, 31 kDa domain fragment and 8 kDa domain fragment on a blotted membrane. (**B**) Lanes 1, 4 and 7 were 0.5 μg of purified DNA polymerase β; lanes 2, 5 and 8 were 0.5 μg of purified 31 kDa domain fragment; lanes 3, 6 and 9 were 0.5 μg of purified 8 kDa domain fragment. The transferred membrane was immuno-stained with anti-p39 antibody (lanes 1–3), anti-p31 antibody (lanes 4–6) and anti-p8 antibody (lanes 7–9). Standard markers are indicated by arrows to the left of the panel (in kDa). (**C**) A mixture of purified DNA polymerase β protein of 39 kDa, 31 kDa fragment and 8 kDa fragment (0.5 μg each, total 1.5 μg) was incubated with 1 nmol of FA-A (lanes 2, 5 and 8), 1 nmol of FA-B (lanes 3, 6 and 9) or none (lanes 1, 4 and 7) and precipitated by the antibody against 39 kDa DNA polymerase β (lanes 1–3), 31 kDa domain (lanes 4–6) and 8 kDa (lanes 7–9), respectively. The membrane was incubated with the same antibodies as those described in Figure 5B.

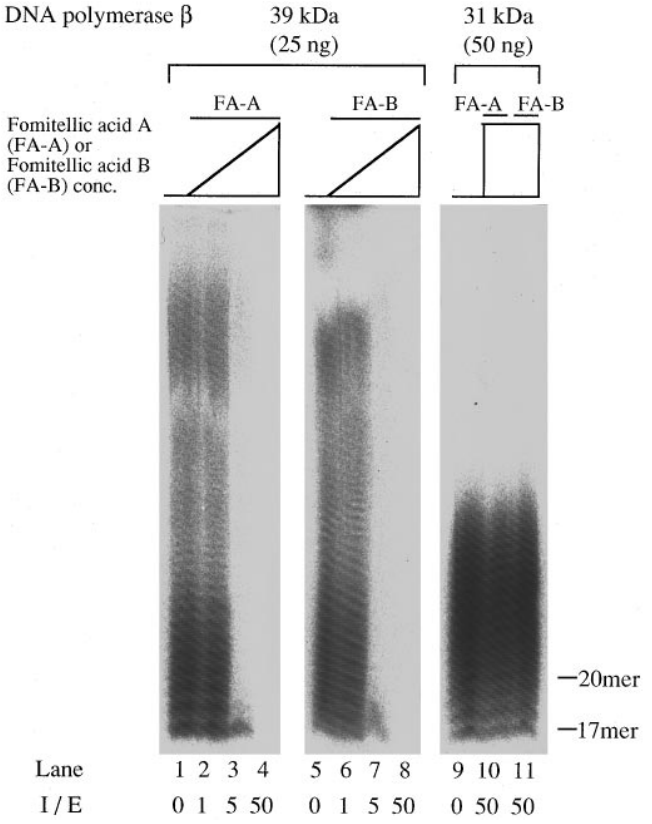

Figure 6 Analysis of the poly(dA)/oligo(dT) template primer synthesis products

DNA-synthesis reactions were carried out with 20 μM of poly(dA)/oligo(dT)$_{16}$ (2:1) and 20 μM of [α-^{32}P]dTTP (60 Ci/mmol), and the products were examined by gel electrophoresis and imaging analysis as described in the Experimental section. The enzyme concentrations were as follows: lanes 1–8, 25 ng (0.64 pmol) of the 39 kDa intact DNA polymerase β; lanes 9–11, 50 ng of the 31 kDa fragment. FA-A concentrations were as follows: lanes 1–4 and 10 were 0, 0.64, 3.2, 32 and 32 pmol, respectively. FA-B concentrations were as follows: lanes 5–8 and 11 were 0, 0.64, 3.2, 32 and 32 pmol, respectively. Lane 9 was without FAs. Markers indicate the positions of the extended primer. Conc, concentration; I/E, inhibitor-to-enzyme ratio.

competitively to some of the hydrophobic portions near the template primer-binding site on the 8 kDa domain. The carboxyl group at the position of carbon 3 (the free hydrophilic group) disturbs the DNA-template access to the hydrophilic amino-acid site involved in DNA binding, and simultaneously it influences the 31 kDa domain-catalytic region on the opposite bank. Since such compounds influencing the DNA polymerase β activity and penetrating viable cells have never been reported before, and since there are many tissues in which the replication polymerase does not function, the FAs could be a key agent for analyzing the functions *in vivo* of the DNA repair-related enzymes such as DNA polymerase β in these tissues.

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