Domain Topology of the DNA Polymerase D Complex from a Hyperthermophilic Archaeon *Pyrococcus horikoshii*†

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ABSTRACT: Family D DNA polymerase (PolD) is a recently found DNA polymerase extensively existing in Euryarchaeota of Archaea. Here, we report the domain function of PolD in oligomerization and interaction with other proteins, which were characterized with the yeast two-hybrid (Y2H) and surface plasmon resonance (SPR) assays. A proliferating cell nuclear antigen, PhoPCNA, interacted with the N-terminus of the small subunit, DP1(1−200). Specific interaction between the remaining part of the small subunit, DP1(201−622), and the N-terminus of the large subunit, DP2(1−300), was detected by the Y2H assay. The SPR assay also indicated the intrasubunit interaction within the N-terminus, DP2(1−100), and the C-terminus, DP2(792−1163), of the large subunit. A synthetic 21 amino acid peptide corresponding to the sequence from cysteine cluster II, DP2(1290−1310), tightly interacted (a dissociation constant K_D = 4.3 nM) with the N-terminus of the small subunit, DP1(1−200). Since the peptide could increase the 3′−5′ exonuclease activity of DP1 [Shen et al. (2004) *Nucleic Acids Res. 32*, 158], the short region DP2-(1290−1310) seems to play dual roles to form the PhoPolD complex and to regulate the 3′−5′ exonuclease activity of DP1 through interaction with DP1(1−200). Furthermore, DP2(792−1163) containing the catalytic residues for DNA polymerization, Asp1122 and Asp1124, interacted with the intrasubunit domain, DP2-(1−100), and the intersubunit domain, DP1(1−200). DP2(792−1163) probably forms the most important domain deeply involved in both the catalysis of DNA polymerization and stabilization of the PhoPolD complex through these multiple interactions.

DNA polymerases play an important role in DNA replication, recombination, and repair. Family D DNA polymerases (PolDs)[1] are a new type of DNA polymerase recently found in Euryarchaea (*1*, *2*). They have both DNA polymerase activity and 3′−5′ exonuclease activity. PolDs use RNA primers more efficiently and have a higher intrinsic processivity than family B DNA polymerases (*3*). The genes encoding PolD are adjacent to several other genes related to DNA replication, repair, and recombination in the archaea genome. Accordingly, PolD was proposed to be a major DNA replicase in Euryarchaea.

DNA replication is a highly organized process involving a large set of proteins and enzymes to achieve a faithful, fast and timely result. Usually two kinds of proteins confer speed and high processivity to the DNA polymerase involved. One is proliferating cell nuclear antigen (PCNA), which has a ring-shaped form and acts as a sliding DNA clamp. PCNA can encircle duplex DNA in order to tether the associated DNA polymerase to the DNA template, while another protein, replication factor C (RFC), which works as a clamp loader, helps in the loading and unloading of PCNA. The direct interaction between PCNA and DNA polymerase D from *Pyrococcus furiosus* has been studied (*4*). PCNA was found to interact mainly with DP2 and only very weakly with DP1 in *P. furiosus* using immunological procedures and pull-down experiments. The addition of PCNA to *Pfu*PolD stimulated the DNA synthesis of *Pfu*PolD. The RFC from *P. furiosus*, *Pfu*RFC, was found to stimulate *Pfu*PCNA-dependent DNA synthesis of both DNA polymerase B and DNA polymerase D from *P. furiosus* (*5*). The RFC in *Methanobacterium thermoautotrophicum* ΔH, *Mth*RFC, was also found to support the family B DNA polymerase-catalyzed, PCNA-dependent DNA chain elongation (*6*).

The family D DNA polymerase found in *Pyrococcus* has two subunits: the small subunit (DP1) and the large subunit (DP2) (*7*−*9*). The two genes encoding DP1 and DP2 are arranged in tandem in the *Pyrococcus* genome. Though the amino acid sequences of euryarchaeotic DP2 do not have significant similarity to any protein or ORF in the database, there are several conserved regions in known DP2 proteins. PolD from *Pyrococcus horikoshii* (PhoPolD) is a hetero-tetramer L_2S_2 with two large subunits (DP2) and two small

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[1] Abbreviations: BSA, bovine serum albumin; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; DTT, dithiothreitol; DP1, small subunit of the family D DNA polymerase; DP2, large subunit of the family D DNA polymerase; IPTG, isopropyl β-D-thiogalactopyranoside; PCNA, proliferating cell nuclear antigen; PhoPolD, family D DNA polymerase from *P. horikoshii*; PolD, family D DNA polymerase; RFC, replication factor C; RFCL, RFC large subunit; RFCS, RFC small subunit; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SPR or BIAcore, surface plasmon resonance; Y2H, yeast two hybrid.

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subunits (DP1) (*8*). The active sites of DNA polymerization were identified as Asp1122 and Asp1124, both located in the most conserved region of DP2, by site-directed mutagenesis (*8*). The catalytic residues for DNA polymerization must be different from those for 3′−5′ exonuclease activity since the mutants D1122A and D1124A retained their exonuclease activity though their polymerization activity decreased dramatically.

Though the biochemical characterization of PolD has been studied, knowledge about the subunit interaction of PolD is still scarce. Recently, the carboxyl terminus (1255−1332) of DP2 and two regions, 201−260 and 599−622 of DP1, were found to be critical for the formation of complex by making and characterizing various truncated proteins of PhoPolD (*10*). The amino terminus (1−200) of DP1 plays a possible regulatory role in the 3′−5′ exonuclease activity of PhoPolD. The internal region of a putative zinc-finger motif in the COOH terminus of DP2, DP2(1289−1308), is critical for the 3′−5′ exonuclease activity but is dispensable for the DNA polymerization (*11*).

This study focuses on the polymerase D holoenzyme from *P. horikoshii*. In particular, the yeast two-hybrid (Y2H) system was employed to define regions of intermolecular interaction between DP1, DP2, and PCNA. In addition, intra- and intermolecular interactions between these domains were verified by using surface plasmon resonance (SPR).

MATERIALS AND METHODS

Microorganisms, Chemicals, Plasmids, Enzymes, and DNA Manipulation. The *Escherichia coli* strains used in this study were ultracompetent *E. coli* XL2-Blue MRF′ and BL21-CodonPlus (DE3)-RIL competent cells (Stratagene). Both strains were cultured in 2YT medium. Ampicillin was used at a final concentration of 100 μg/mL. Plasmid DNA was purified using a Qiagen plasmid kit (Qiagen). Restriction enzymes and other DNA-modifying enzymes were used as recommended by their manufacturer (Toyobo, Takara). Genomic DNA of *P. horikoshii* OT3 was purified by Dr. H. Higashibata (*12*). The template for the amplification of DNA fragments from DP2 was pGEMEX-L(−), while that for the DNA from DP1 was pET15b-S (*8*). Genomic DNA of *P. horikoshii* OT3 was used as a template to amplify PhoPCNA (PH0665), the small subunit of PhoRFC, PhoRFCS (PH0112), and the large subunit of PhoRFC, PhoRFCL (PH0113). The intein in the PhoRFCS gene was deleted as described in the section Cloning of the Genes and Construction of Expression Vectors. KODPlus (Toyobo) was used as DNA polymerase for PCR. DNA sequencing was performed to confirm that no spurious mutations had been introduced during PCR for all of the recombinant plasmids.

Yeast Two-Hybrid (Y2H) Assay. Matchmaker GAL4 two-hybrid system 3 was purchased from Clontech. The procedures for culture and plates for yeast growth and selection were as described in the instruction manual. Amplified full-length or fragmented DNA was ligated in-frame with the GAL4 DNA-binding domain (amino acids 1−147) or GAL4 activation domain (amino acids 768−881) at the multiple cloning site of the pGBKT7 and pGADT7 vectors, respectively. Simultaneous cotransformation of *Saccharomyces cerevisiae* strain Y187 with two different plasmids was performed using the LiOAc method. The cotransformants were plated on Trp$^-$ Leu$^-$/SD medium. Colonies that appeared were tested for β-galactosidase activity by a colony-lift filter method using X-gal as substrate. β-Galactosidase activity was quantified in a subsequent liquid culture assay using *o*-nitrophenyl β-D-galactopyranoside (ONPG) as substrate. Arbitrary units of activity were calculated as follows: β-galactosidase units $= 1000A_{420}/(tVA_{600})$, where $t =$ minutes of incubation and $V = 0.1$ mL $\times$ concentration factor. All quantitative measurements were made at least four times, and the average was calculated. The murine p53 (pGBKT7-53) and SV40 large T-antigen (pGADT7-T) vectors were used as a positive control, and human lamin C (pGBKT7-lam) and pGADT7-T as a negative control, in the cotransformation and β-galactosidase assay (Clontech).

Cloning of the Genes and Construction of Expression Vectors. DP2(1−100) was amplified using as primers 5′-GGGGGCATATGGTGCTGATGGAGCTTCCAAAG-3′ and 5′-GGGGGGGATCCTTACTTTTCCTTACTTCCCAGATC-3′. The restriction sites for *Nde*I and *Bam*HI are underlined. *Nde*I- and *Bam*HI-digested DNA of DP2(1−100) was inserted into the expression vector pET11a (Novagen). DP2-(792−1163) was amplified using 5′-GGGGGCATATG-ACATCCGGCTGGAAAATG-3′ and 5′-GGGGGGGATCC-TTACGGATCTAAGCGCGTTGT-3′. DP1(1−200) was amplified using 5′-GGGGGCATATGGATGAATTCGTAAA-GGGATTA-3′ and 5′-GGGGGGGATCCTCAATAGTC-TTTTTCTATCGGAACGAC-3′. DP2(792−1163) and DP1-(1−200) were digested with *Nde*I and *Bam*HI and inserted into the expression vector pET15b (Novagen), respectively.

PhoPCNA was amplified using as primers 5′-GGGGG-CATATGCCATTCGAAATAGTCTTTGAGGG-3′ and 5′-GGGGGCTCGAGTCACTCCTCAACCCTTGG-3′. The restriction sites of *Nde*I and *Xho*I are underlined. *Nde*I- and *Xho*I-digested DNA was inserted into a reconstructed expression vector, pET11a with an *Xho*I site, which was derived from pET11a.

The intein in PhoRFCS was removed using the splicing overlap extension strategy (*13*). Briefly, primer I (5′-GGG-GGGCATATGCATAATATGGAAGAGGTTCGCGAGG-3′) and primer III (5′-GCAGGTCCTCCTGGTGTTGGAAA-GACTACAGCAGCTTTAGCCCTCTCA-3′) were used to amplify the N-terminal fragment of PhoRFCS. Primer IV (5′-TGAGAGGGCTAAAGCTGCTGTAGTCTTTCCA-ACACCAGGAGGACCTGC-3′) and primer II (5′-GGG-GGATCCTCACTTCTTCTTTCCAACTAAGGTAAA-3′) were used to amplify the C-terminal fragment of PhoRFCS. The restriction sites of *Nde*I and *Bam*HI are underlined. The two amplified DNAs were annealed, and primers I and II were used for the second PCR. The intein-removed PhoRFCS DNA was digested with *Nde*I and *Bam*HI and inserted into the expression vector pET11a.

Expression and Purification of Proteins from PhoPolD-DP2(1−100), DP2(792−1163), and DP1(1−200). The DP2-(1−100) gene was inserted into the expression vector pET11a. The recombinant plasmid was introduced into competent *E. coli* BL21-CodonPlus (DE3)-RIL cells. When the OD_{600} reached 0.4, IPTG (0.5 mM) was added to induce gene expression. After induction at 37 °C for 4 h, cells were harvested. Cells were resuspended in a buffer containing 50 mM Tris-HCl (pH 8.0), 0.5 M NaCl, and protease inhibitor (Roche Molecular Biochemicals). After sonication and centrifugation (30000*g*, 30 min), the supernatant was heated

at 75 °C for 10 min. It was then applied to an anion-exchange column of HiTrap Q (5 mL) (Amersham Pharmacia) and eluted with a linear gradient of 0−1 M NaCl in 50 mM Tris-HCl (pH 8.0) using a FPLC system (Amersham Pharmacia).

The recombinant plasmid pET15b-DP2(792−1163) was introduced into *E. coli* BL21-CodonPlus (DE3)-RIL competent cells. When the OD_{600} reached 0.5, IPTG (0.5 mM) was added to induce gene expression. After induction at 25 °C overnight, cells were harvested. Cells were resuspended in buffer A (20 mM sodium phosphate, pH 7.4, 0.5 M NaCl, and 10% glycerol) with 6 M urea. After sonication and centrifugation (30000*g*, 30 min), the supernatant was mixed with preloaded nickel resin (Novagen). After being mixed gently for 1 h, the resin was loaded onto a column and washed with a buffer containing 20 mM imidazole and 6 M urea in buffer A. DP2(792−1163) was eluted with the elution buffer containing 1 M imidazole and 6 M urea in buffer A. The eluate was dialyzed against buffer A stepwise with 6, 4, 2, and 0 M urea for at least 4 h and at last dialyzed against a buffer containing 20 mM sodium phosphate (pH 7.4), 50 mM NaCl, and 10% glycerol. After centrifugation at 30000*g* for 30 min, purified refolding DP2(792−1163) was obtained.

The recombinant plasmid pET15b-DP1(1−200) was also introduced into competent *E. coli* BL21-CodonPlus (DE3)-RIL cells. When the OD_{600} reached 0.4, IPTG (0.5 mM) was added to induce gene expression. After induction at 37 °C for 4 h, cells were harvested. Cells were resuspended in a buffer containing 50 mM Tris-HCl (pH 8.0), 0.5 M NaCl, and protease inhibitor. After sonication and centrifugation (30000*g*, 30 min), the supernatant was heated at 75 °C for 10 min. The supernatant was loaded onto a Ni column (Novagen). After being washed with a buffer containing 20 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9, the protein was eluted with a buffer containing 200 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9. The eluate was concentrated by using Centricon YM-10 (Millipore) and dissolved in 50 mM of Tris-HCl. The protein was applied to an anion-exchange column of HiTrap Q (5 mL) (Amersham Pharmacia) and eluted with a linear gradient of 0−1 M NaCl in 50 mM of Tris-HCl (pH 8.0) using a FPLC system (Amersham Pharmacia). DP1(1−200) was eluted at 0.15 M NaCl.

Expression and Purification of PhoPCNA. The recombinant plasmid pET11a-PhoPCNA was introduced into competent *E. coli* BL21-CodonPlus (DE3)-RIL cells. When the OD_{600} reached 0.5, IPTG (0.5 mM) was added to induce gene expression. After induction at 37 °C for 4 h, cells were harvested. The cells were resuspended in a buffer containing 50 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 2 mM 2-mercaptoethanol, 0.1 mM EDTA, and 10% glycerol. After sonication and centrifugation (30000*g*, 20 min), the supernatant was heated at 75 °C for 15 min and centrifuged at 30000*g* for 20 min. The supernatant was then heated at 80 °C for 10 min and centrifuged again. Next, 0.15% poly(ethylenimine) (Sigma) and 0.58 M NaCl were added to the supernatant, and the mixture was stirred at 4 °C for 30 min. After centrifugation at 30000*g* for 20 min, $(NH_4)_2SO_4$ was added to the supernatant until 80% saturation. After 2 h stirring on ice and centrifugation at 30000*g* for 20 min, the precipitate was solved in a buffer of 50 mM Tris-HCl (pH 8.0), 0.05 mM DTT, 0.1 mM EDTA, and 10% glycerol. After dialysis against the same buffer, the sample was applied to an anion-exchange column of HiTrap Q (5 mL) (Amersham Pharmacia) and eluted with a linear gradient of 0−1 M NaCl in 50 mM Tris-HCl (pH 8.0) using a FPLC system (Amersham Pharmacia). PhoPCNA was eluted at 0.5 M NaCl.

Expression and Purification of PhoRFCS. The recombinant plasmid pET11a-PhoRFCS gene was introduced into competent *E. coli* BL21-CodonPlus (DE3)-RIL cells. When the OD_{600} reached 0.5, IPTG (1 mM) was added to induce gene expression. After induction at 25 °C overnight, cells were harvested. The cells were suspended in 50 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 2 mM 2-mercaptoethanol, 0.1 mM EDTA, and 10% glycerol. After sonication and centrifugation (30000*g*, 20 min), the supernatant was heated at 75 °C for 15 min and centrifuged at 30000*g* for 20 min. Then, 0.15% poly(ethylenimine) (Sigma) was added to the supernatant, and the solution was stirred at 4 °C for 30 min. After centrifugation at 30000*g* for 20 min, $(NH_4)_2SO_4$ was added to the supernatant until 80% saturation. After 1 h stirring on ice and centrifugation at 30000*g* for 20 min, buffer B including 10 mM sodium phosphate (pH 6.8), 7 mM 2-mercaptoethanol, 0.05 mM $CaCl_2$, and 10% glycerol was added to solute the precipitate. After dialysis against buffer B and centrifugation (30000*g*, 20 min), the supernatant was applied to a hydroxyapatite column, Econo-Pac CHT-II Cartridge (Bio-Rad), which was preequilibrated with buffer B using a FPLC system (Amersham Pharmacia). The protein was eluted with 0−1 M sodium phosphate (pH 6.8). PhoRFCS was eluted at 0.35 M sodium phosphate. Samples were concentrated using Centriprep YM-10 and then applied to a gel filtration column of Superose 12 HR10/30 (Amersham Pharmacia) with 50 mM Tris-HCl and 0.2 M NaCl (pH 8.0) using the FPLC system.

Protein Concentration Measurement and Protein Analysis. Protein concentration was estimated using the Bio-Rad protein assay system with bovine serum albumin (BSA) as a standard (Bio-Rad). The electrophoresis of proteins was conducted on a 10−15% gradient gel using the Phast system (Amersham Pharmacia). The protein standard was from Bio-Rad and contained 10 protein bands of 10, 15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa. The N-terminal amino acid sequence of the protein was determined by Takara Co. A 21 amino acid peptide corresponding to DP2(1290−1310), VKCNTKFRRPPLDGKCPICGG, and a random peptide of 21 amino acids, MKEGIPSVARFCLGKTPDRFG, were synthesized by Hokkaido System Sciences Co.

ATPase Activity Measurement of PhoRFCS. ATPase activity was measured in a reaction mixture (20 μL) containing 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 0.01% BSA, 7 mM $MgCl_2$, 2 μM ATP, 20 nM [γ-^{32}P]ATP, single-strand M13 DNA, and 0.6 μg of purified PhoRFCS. After incubation at 65 or 75 °C for 30 min, the reaction was terminated by adding 2 μL of 50 mM Na_2EDTA (pH 8.0). An aliquot (2 μL) was spotted onto a poly(ethylenimine)−cellulose thin-layer plate. The reaction products [ATP and inorganic phosphate (P_i)] were separated by chromatography in a solution of 1 M formic acid and 0.5 M LiCl.

Surface Plasmon Resonance Analysis. The interaction between proteins or the interaction between protein and peptide was quantitatively analyzed on a BIAcore X apparatus (BIAcore, Uppsala, Sweden) at 25 °C using surface plasmon resonance (SPR) measurements. DP1, DP1(1−200), and DP2(792−1163) were amine-coupled on the surface of

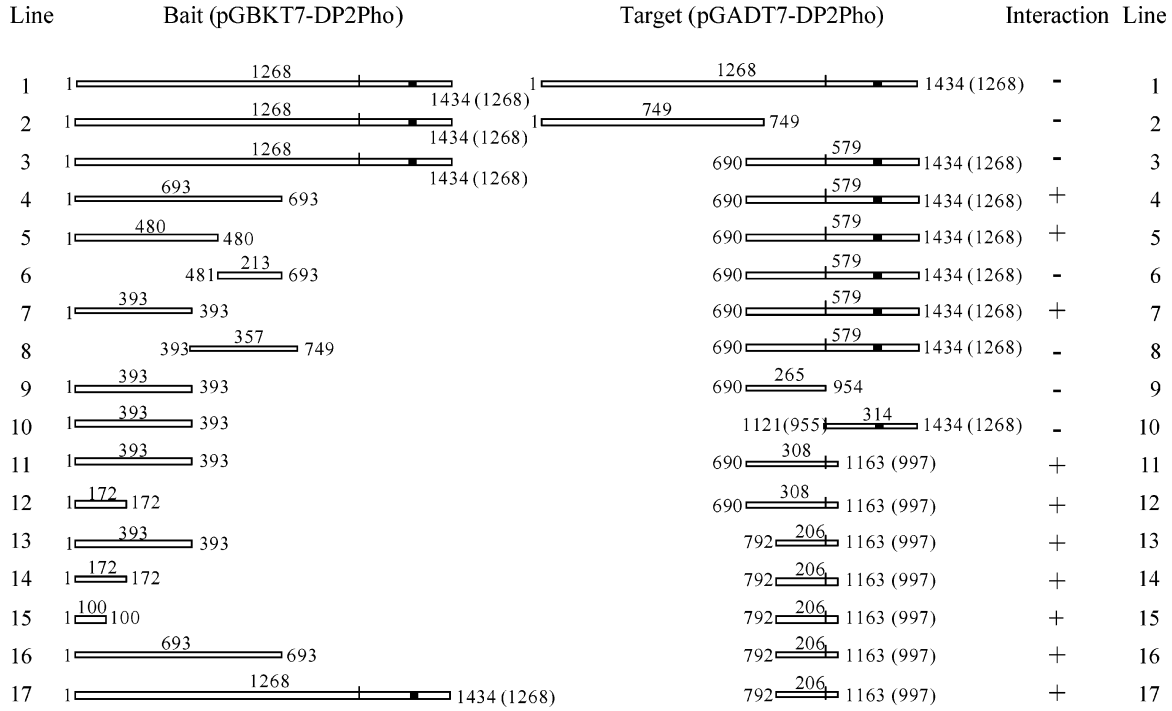

FIGURE 1: Mapping of the interactions between the domains of DP2 in vivo by yeast two-hybrid analysis. The yeast two-hybrid constructs are described under Materials and Methods. Interactions of two proteins in yeast cells were qualified by colony-lift filter assay of β-galactosidase using X-gal as substrate. The numbers in parentheses are the number of peptides without the 166 amino acid intein (955−1120). A short bar shows the catalytic sites for DNA polymerization. A black block indicates the region of the putative zinc-finger motif. (+) indicates the colonies that turned blue within 8 h incubation; (−) shows that the colonies remained white even after 8 h incubation. pGBKT7-, GAL4 DNA-binding domain fusion; pGADT7-, GAL4 activation domain fusion.

CM5 sensor chips (BIAcore) in 10 mM sodium acetate buffer (pH 4.0), respectively. A flow rate of 20 μL/min was used for kinetic analysis. Five different concentrations were injected. The obtained sensorgrams were analyzed using Bioevaluation software version 3 (BIAcore). The dissociation constant (K_D) was calculated on the basis of a simple 1:1 binding model.

RESULTS

The N-Terminus of DP2 Interacted with the C-Terminus. We have reported that the amino-terminal fragment (amino acids 1−300) of DP2 is highly stable and could be purified in high purity and quantity (*10*). Gel filtration chromatography showed that DP2(1−300) formed a trimer or a dimer, which suggested that one DP2(1−300) domain interacts with another in the PhoPolD complex.

In this study, we used a yeast two-hybrid assay to identify the intrasubunit domain interaction besides that of DP2(1−300). The full length or fragments of the DP2 gene were fused to the GAL4 DNA-binding domain or to the GAL4 activation domain as described under Materials and Methods. After cotransformation of *S. cerevisiae* strain Y187 with a pair of plasmids, we utilized the colony-lift filter method using X-gal as substrate for the β-galactosidase assay. The results are shown in Figure 1. No interaction was detected between two full-length DP2 (Figure 1, line 1), between the full-length DP2 and N-terminal half (1−749) (Figure 1, line 2), or between the full-length DP2 and C-terminal half (690−1434) (Figure 1, line 3). As a 166 amino acid mini-intein is coded from site 955 to 1120, region 690−1434 is actually 579 amino acids in length. Since the full-length DP2 did not interact with either the N-terminal half or C-terminal half, we checked the interaction between the N-terminal half and the C-terminal half of DP2. We found the N-terminal domains such as DP2(1−693), DP2(1−480), and DP2(1−393) all interacted with DP2(690−1434) (Figure 1, lines 4, 5, and 7), while interaction between the rest of the N-terminus and DP2(690−1434) was not detected (Figure 1, lines 6 and 8). To determine which part of DP2(690−1434) was related to the interaction, we separated DP2(690−1434) into two fragments, DP2(690−954) and DP2(1121−1434). Considering the 166 amino acid mini-intein, region 1121−1434 is actually equal to 955−1268 in parentheses as shown in Figure 1, line 10. The interaction between DP2-(1−393) and DP2(690−954), and between DP2(1−393) and DP2(1121−1434), was investigated. To our surprise, no interaction was detected (Figure 1, lines 9 and 10). Since the active residues for DNA polymerization of PhoPolD were Asp1122 and Asp1124 (*8*), which were the second and fourth amino acids at the N-terminus of DP2(1121−1434), we speculated that the region including the active residues might be involved in the interaction. Therefore, we lengthened DP2(690−954) to obtain DP2(690−1163), which contained the two active residues for DNA polymerization at the C-terminus. Interaction between DP2(1−393) and DP2(690−1163), and between DP2(1−172) and DP2(690−1163), was detected (Figure 1, lines 11 and 12). The N-terminal regions related to the interaction could even be shortened to DP2(1−100), while the C-terminal region was shortened to DP2(792−1163), which was a 206 amino acid fragment (Figure 1, lines 13−15). Interaction between DP2-(1−693) and DP2(792−1163) and that between full-length DP2 and DP2(792−1163) was also detected (Figure 1, lines 16 and 17).

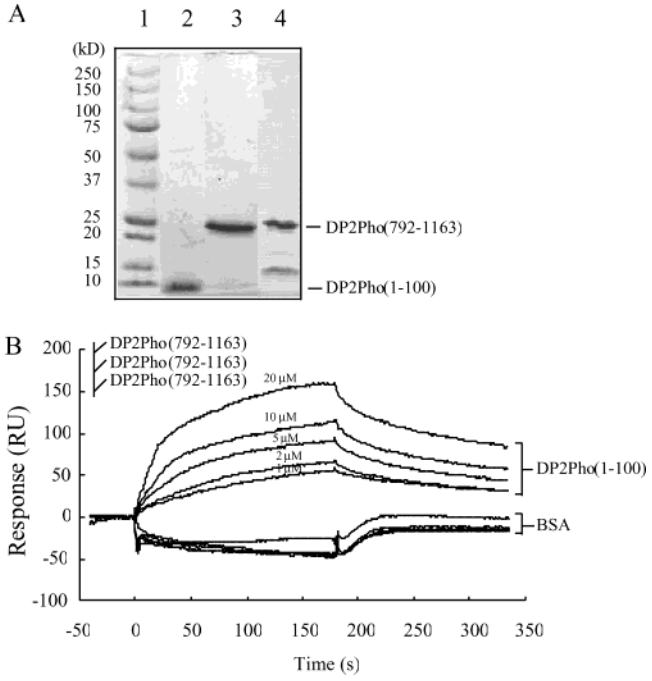

FIGURE 2: Interaction between DP2(1−100) and DP2(792−1163). (A) SDS−PAGE of purified DP2 fragments: lane 1, molecular mass marker; lane 2, purified DP2(1−100) after heat treatment and anion-exchange chromatography; lane 3, denatured DP2(792−1163) eluate of the Ni column chromatograph (6 M urea); lane 4, refolded DP2(792−1163) after dialysis. (B) Surface plasmon resonance analysis of the interaction of DP2(792−1163) and DP2(1−100). 4700 RU of DP2(792−1163) was immobilized on a CM5 sensor chip. Samples containing different concentrations of DP2(1−100) in 50 mM Tris-HCl, 150 mM NaCl, 1% glycerol, and 0.005% Tween 20 were injected over the immobilized DP2(792−1163) for 3 min at 20 μL/min. The background resulting from the injection of buffer alone was subtracted from the data before plotting. K_D was determined between 0 and 300 s. A control injection of BSA (top to bottom: 20, 10, 5, 2, 1 μM) over the immobilized DP2(792−1163) CM5 sensor chip was also performed, resulting in negligible results.

The β-galactosidase activity of several cotransformants was measured using a liquid culture assay. The β-galactosidase activity of the pGKBT7-DP2 and pGADT7-DP2(792−1163) cotransformant was 2.3 units, while that of the pGKBT7-DP2(1−693) and pGADT7-DP2(792−1163) cotransformant was 11 units. Since the colony-lift filter method (substrate: X-gal) is about 10^6-fold more sensitive than the liquid culture assay (substrate: ONPG), most of the interactions could not be calculated using the liquid culture assay.

Because of the limitations of the liquid culture assay, we used the SPR assay to confirm the direct interaction between DP2(1−100) and DP2(792−1163). DP2(1−100) and DP2(792−1163) were overexpressed in *E. coli*. DP2(1−100) was purified to homogeneity after heat-treatment and anion-exchange chromatography. It was an 11.2 kDa protein on SDS−PAGE as predicted (Figure 2A, lane 2). DP2(792−1163) was overexpressed in *E. coli* with a His tag on its N-terminus. The predicted molecular mass of DP2(792−1163) was 23.2 kDa. DP2(792−1163) precipitated in the form of inclusion bodies and was dissolved in 6 M urea. The denatured protein was purified through Ni column chromatography with 6 M urea (Figure 2A, lane 3). Refolding of DP2(792−1163) was conducted by stepwise dialysis. Refolded DP2(792−1163) (Figure 2A, lane 4) was immobilized on the surface of a CM5 sensor chip via amine coupling. Different concentrations of DP2(1−100) were injected over the chip's surface, and a sensorgram was recorded. As shown in Figure 2B, the sensorgram clearly indicated the DP2(1−100)−DP2(792−1163) interaction. The dissociation constant K_D was 3.16 μM. BSA was used as a control under the same conditions. There was no interaction between DP2(792−1163) and BSA.

DP2(1290−1310) Interacted with DP1(1−200). We had reported that the C-terminus (1255−1332) of DP2, which contained a conserved putative zinc-finger motif (two cysteine pairs) from Cys1289 to Cys1308, was involved in the subunit interaction because all truncated mutants lacking 1255−1332 of DP2 were incapable of forming a PhoPolD complex (*10*).

We used the yeast two-hybrid assay to confirm the domains interacting between DP1 and DP2. First, the interaction between DP1 and DP2 was confirmed (Figure 3A, line 1). Interaction between DP1 and the C-terminus of DP2, DP2(690−1434) or DP2(1290−1434), was also detected (Figure 3A, lines 2 and 4), while no interaction between DP1 and DP2(1375−1434) was detected (Figure 3A, line 5). The results are consistent with those obtained using truncated mutants of PhoPolD (*10*). Furthermore, small fragments of the C-terminus of DP2, such as DP2(1290−1332), DP2(1290−1310), DP2(1290−1304), and DP2(1293−1304), were fused to the GAL4 activation domain. Interaction between DP1 and all of these small fragments of DP2 was detected (Figure 3A, lines 6−9). The smallest fragment, NTKFRRPPLDGK, which corresponds to DP2(1293−1304), was the internal region of the two cysteine pairs (putative zinc-finger motif) with only 12 amino acids. The results proved direct interaction between DP1 and the internal region of the putative zinc-finger region.

Furthermore, using the SPR assay, we measured the dissociation constant of DP1 and a synthetic 21 amino acid peptide, VKCNTKFRRPPLDGKCPICGG, which corresponds to region 1290−1310 of DP2. DP1 was purified as described (*8*). Purified DP1 was immobilized on the surface of a CM5 sensor chip via amine coupling. Different concentrations of the 21 amino acid peptide were injected over the chip's surface, and a sensorgram was recorded. The sensorgram detected interaction between DP1 and the 21 amino acid peptide (Figure 3B). The dissociation constant K_D was 4.81 nM. A random 21 amino acid synthetic peptide, MKEGIPSVARFCLGKTPDRFG, which has the same p*I* of 9.8 as the 21 amino acid peptide derived from DP2, was injected over the chip surface as a control. There was no interaction between DP1 and the random 21 amino acid peptide.

To clarify which part of DP1 interacts with DP2(1290−1310), DP1(1−200) and DP1(201−622) were fused with the DNA-binding domain in pGBKT7, respectively. Because the independent transformant of DNA-BD fused DP1(1−200) had β-galactosidase activity, DP1(1−200) could not be used as bait in the yeast two-hybrid assay. On the other hand, DP1(201−622) was found not to interact with either of the fragments using the yeast two-hybrid assay (Figure 3A, lines 11−15). Therefore, the interaction between DP1 and the 21 amino acid peptide corresponding to DP2(1290−1310) most probably involved DP1(1−200). Because the overexpressed DP1(1−200) was stable and easily purified (*10*), we used

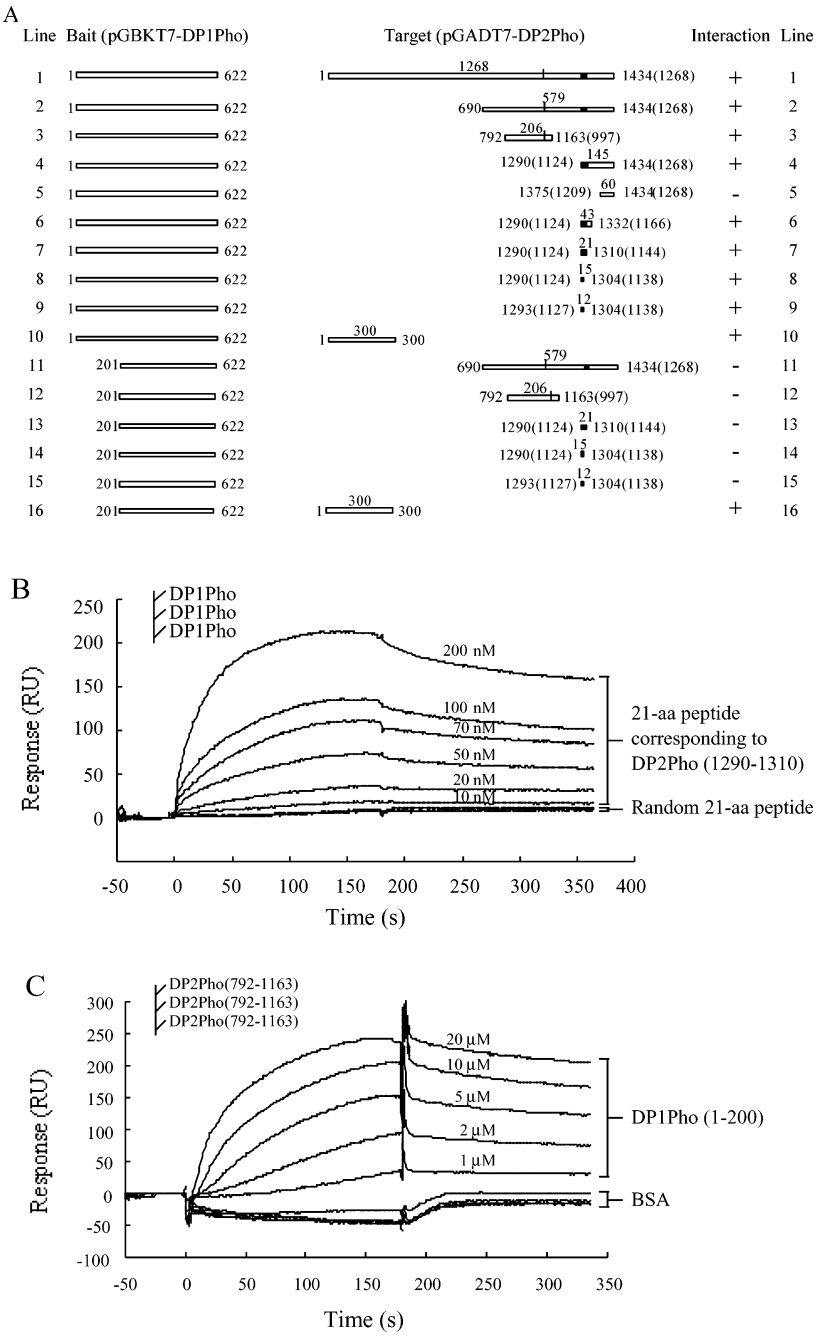

FIGURE 3: Interactions between DP1 and the domains of DP2. (A) Yeast two-hybrid analysis. The marks are described in Figure 1. Because the independent transformant of DNA-BD fused DP1(1−200) had β-galactosidase activity, DP1(1−200) could not be used as bait in the yeast two-hybrid assay. (B) Surface plasmon resonance analysis of the interaction between DP1 and the 21 amino acid peptide corresponding to DP2(1290−1310). 5400 RU of the DP1 was immobilized on a CM5 sensor chip. Samples containing different concentrations of the 21 amino acid peptide in 50 mM Tris-HCl, 100 mM NaCl, 1% glycerol, and 0.005% Tween 20 were injected over the immobilized DP1 for 3 min at 20 μL/min. The background resulting from the injection of buffer alone was subtracted from the data before plotting. K_D was determined between 0 and 350 s. A control injection of the random 21 amino acid peptide (top to bottom: 1000, 500, 100 nM) passed over the immobilized DP1 sensor chip was also performed, resulting in a negligible result. (C) Surface plasmon resonance analysis of the interaction of DP2(792−1163) and DP1(1−200). 4700 RU of DP2(792−1163) was immobilized on a CM5 sensor chip. Samples containing different concentrations of DP1(1−200) in 50 mM Tris-HCl, 150 mM NaCl, 1% glycerol, and 0.005% Tween 20 were injected over the immobilized DP2(792−1163) for 3 min at 20 μL/min. The background resulting from the injection of buffer alone was subtracted from the data before plotting. K_D was determined between 0 and 300 s.

purified DP1(1−200) and the synthetic 21 amino acid peptide to measure the interaction with the SPR assay. DP1(1−200) was immobilized on the surface of a CM5 sensor chip via amine coupling. DP1(1−200) interacted with the 21 amino acid peptide corresponding to DP2(1290−1310), with a dissociation constant K_D of 4.3 nM. There was no detectable interaction between DP1(1−200) and the random 21 amino acid peptide.

DP2(1−300) Interacted with DP1(201−622). The second DP2 domain involved in the interaction with DP1 was also found by yeast two-hybrid assay. The N-terminal domain of DP2, DP2(1−300), was found to interact with DP1 (Figure

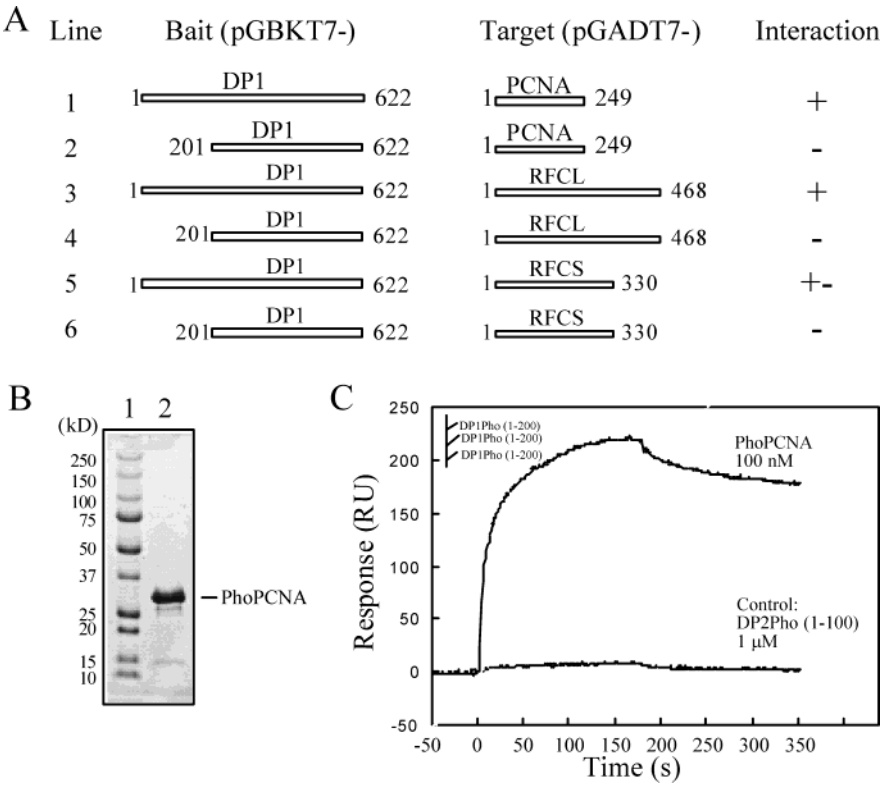

FIGURE 4: Interaction between DP1 and its associated proteins. (A) Interaction between DP1Pho and PhoPCNA and between DP1Pho and PhoRFC detected with the Y2H assay. (B) SDS−PAGE of purified PhoPCNA: lane 1, molecular mass marker; lane 2, purified PhoPCNA after heat treatment and anion-exchange chromatography. (C) Surface plasmon resonance analysis of the interaction of DP1Pho(1−200) and PhoPCNA. 866 RU of DP1(1−200) was immobilized on a CM5 sensor chip. Sample containing 100 nM PhoPCNA in 50 mM Tris-HCl, 150 mM NaCl, 1% glycerol, and 0.005% Tween 20 was injected over the immobilized DP1(1−200) for 3 min at 20 μL/min. The background resulting from the injection of buffer alone was subtracted from the data before plotting. A control injection of 1 μM DP2Pho-(1−100) over the immobilized DP1(1−200) CM5 sensor chip was also performed, resulting in negligible results.

3A, line 10). Furthermore, interaction between DP2(1−300) and DP1(201−622) was detected (Figure 3A, line 16). DP1-(201−622) could be overexpressed in *E. coli*, but it was unstable and precipitated in the form of inclusion bodies. Therefore, direct interaction between protein DP1(201−622) and DP2(1−300) was not measured with the SPR assay.

DP2(792−1163) Interacted with DP1(1−200). The interaction between DP1 and DP2(792−1163) was also detected using the yeast two-hybrid assay (Figure 3A, line 3). Because no interaction was detected between DP1(201−622) and DP2(792−1163) (Figure 3A, line 12), we conclude that DP1-(1−200) and DP2(792−1163) must be involved. The direct interaction between purified proteins DP1(1−200) and DP2-(792−1163) was measured using the SPR assay. DP2(792−1163) was immobilized on the surface of a CM5 sensor chip via amine coupling. DP1(1−200) was injected over the chip's surface, and the sensorgram was recorded. DP1(1−200) interacted with DP2(792−1163), with a dissociation constant K_D of 0.79 μM (Figure 3C). BSA was used as a negative control under the same conditions.

DP1(1−200) Interacted with PhoPCNA. Besides the interaction between domains DP1 and DP2, we also checked the relation between PhoPolD and its associated protein, proliferating cell nuclear antigen (PCNA).

PH0665 from the *P. horikoshii* genome has homology with PCNA. The PH0665 gene product consisted of 249 amino acids with a predicted molecular mass of 28 kDa. The PhoPCNA gene was inserted into pGADT7 or pGBKT7 as bait or target protein in the yeast two-hybrid assay. Interaction between DP1 and PhoPCNA was detected when PhoPCNA was used as the target, but no interaction between DP1(201−622) and PhoPCNA was detected with the Y2H assay (Figure 4A, lines 1 and 2).

To examine the direct interaction between DP1(1−200) and PhoPCNA in vitro, PhoPCNA was overexpressed in *E. coli* and purified to homogeneity by heat treatment to denature the majority of *E. coli* proteins, poly(ethylenimine) treatment to remove DNA, ammonium sulfate precipitation, and anion-exchange chromatography (Figure 4B).

The direct interaction between PhoPCNA and DP1, and that between PhoPCNA and DP1(1−200), was measured by SPR. DP1 and DP1(1−200) were immobilized on CM5 sensor chips, respectively. The sensorgram showed interaction between PhoPCNA and DP1, and between PhoPCNA and DP1(1−200) (Figure 4C).

The interaction between DP2 and PhoPCNA was also checked with the yeast two-hybrid assay. We could not detect interaction between PhoPCNA and full-length DP2PolD, or between PhoPCNA and any fragment of DP2PolD, neither as target protein nor as bait protein.

DP1 Interacted with PhoRFCS. In the genome of *P. horikoshii* OT3, two genes, PH0112 and PH0113, which have homologous sequences to the eukaryotic RFC, are arranged in tandem, and these two genes are adjacent to PhoPolD (PH0121 and PH0123). In PH0112, a region encoding an intein was found. The intein was removed, and the two exteins were fused by the splicing overlap extension strategy. The intein-removed gene product, which corresponded to the

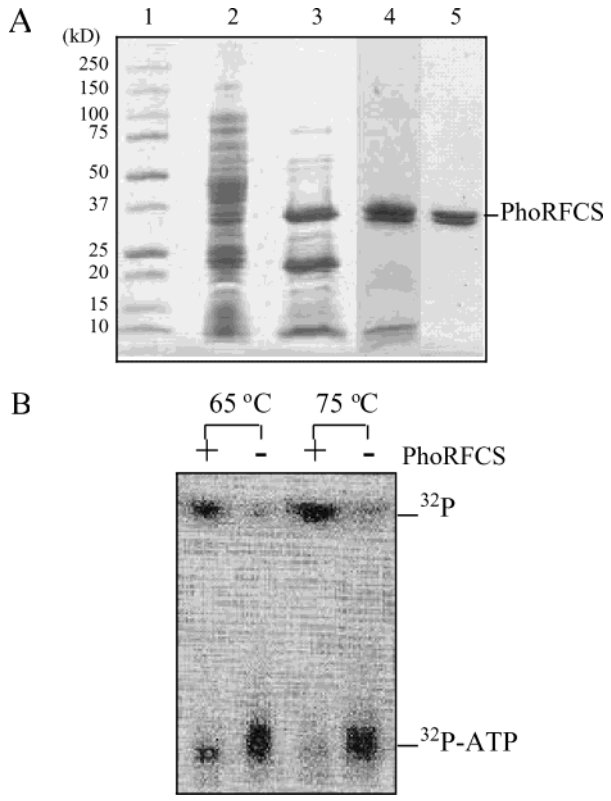

FIGURE 5: Purification and ATPase activity of PhoRFCS. (A) Expression and purification of recombinant PhoRFCS: lane 1, molecular mass marker; lane 2, cells of recombinant *E. coli* harboring PhoRFCS after induction; lane 3, cell-free extract; lane 4, soluble fraction after heat treatment; lane 5, purified PhoRFCS after hydroxyapatide (Econo-Pac CHT-II) and gel filtration chromatography. (B) ATPase activity of PhoRFCS. The measurement of activity is described under Materials and Methods. (+) indicates the presence of 0.6 μg of PhoRFCS; (−) indicates the absence of PhoRFCS.

small subunit of PhoRFC (PhoRFCS), consisted of 330 amino acids, and its molecular mass was calculated to be 37.87 kDa. The PH0113 gene product corresponded to the large subunit of PhoRFC (PhoRFCL), which consisted of 468 amino acids and had a calculated molecular mass of 53.91 kDa.

The interaction between PhoPolD and PhoRFC was investigated by Y2H assay with the full length or fragments of PhoPolD as bait and with the full-length PhoRFCS or PhoRFCL as a target. Interestingly, interaction between DP1 and the large subunit of PhoRFC (PhoRFCL) was detected. The interaction between DP1 and the small subunit of PhoRFC (PhoRFCS) was unclear (Figure 4A, lines 3−6). No interaction between DP2 and the subunit of PhoRFC was detectable using the full length or fragments of DP2. Because until now there has been no report about the direct interaction between DNA polymerase and RFC, we used the SPR assay to confirm the results of the Y2H assay.

First, we expressed PhoRFCL and PhoRFCS in *E. coli*. Though PhoRFCL could be overexpressed in *E. coli*, it was almost insoluble. Even a small amount of PhoRFCL degraded gradually during purification. Therefore, we could not obtain enough soluble PhoRFCL protein to make SPR measurements. Meanwhile, PhoRFCS was overexpressed in *E. coli* as a soluble product. It was purified to near homogeneity, and the ATPase activity of PhoRFCS at 65 and 75 °C was confirmed (Figure 5).

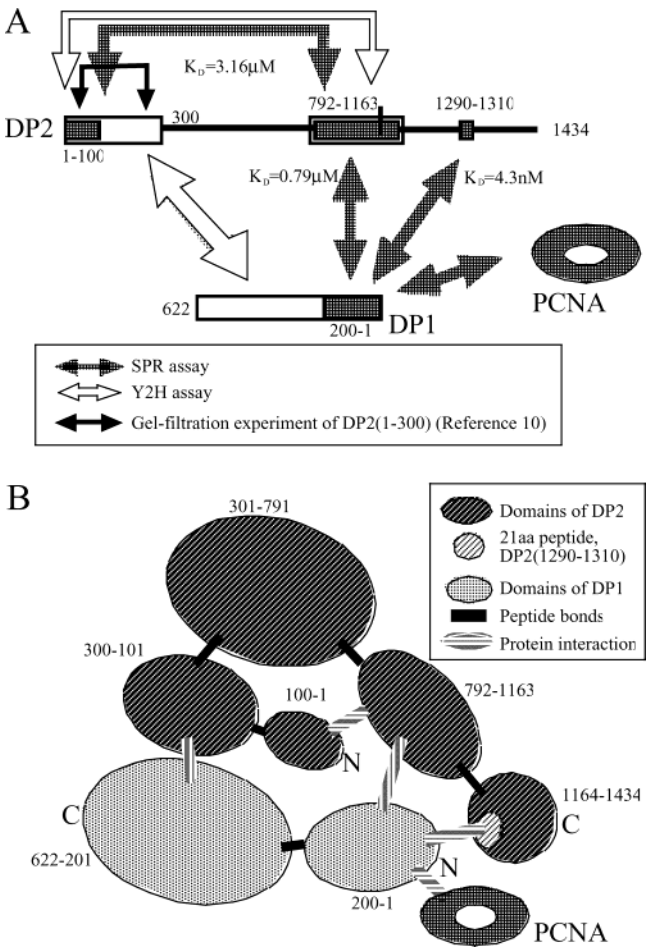

FIGURE 6: Model of interactions in PhoPolD. (A) Arrows indicate the interaction detected with the Y2H and SPR assays. The short bar inside DP2 shows the catalytic sites of DNA polymerization. (B) The possible folding and interactions of the subunits of PhoPolD.

We examined the direct interaction between DP1 and purified PhoRFCS using SPR measurements. The binding of PhoRFCS to DP1 and DP1(1−200) decreased dramatically when the NaCl concentration increased. Therefore, the interaction between DP1 and PhoRFCS detected by Y2H seems to be a result of electrostatic interaction.

DISCUSSION

A Model of Interaction of the PhoPolD Complex. In this report, the yeast two-hybrid assay and surface plasmon resonance assay were utilized to identify interactions between the domains of large and small subunits of the PhoPolD complex. A model of the interaction is depicted in Figure 6 to summarize the knowledge of PhoPolD so far, including results from this study.

PCNA Interacted with the PhoPolD Complex in the Region DP1(1−200). We found that DP1 and DP1(1−200) interacted with PhoPCNA using the Y2H and SPR assays as shown in Figure 4. Usually, in the protein that interacts with PCNA, there is a PCNA-interacting motif. Several kinds of motifs have been found recently, such as a PCNA-interacting protein box (PIP box) (*14*), a replication factory targeting sequence (RFTS) (*15*), and a KA box (*16*). But there is no candidate sequence for a PIP box or other kinds of motifs in DP1(1−200) or in DP1. In *P. furiosus*, DP2 was found to

interact with PfuPCNA, while DP1 only interacted very weakly with PfuPCNA (*4*). In the extreme C-terminal region of DP2 from *P. furiosus*, two motifs of the PIP box-like sequence were found, which corresponded to region 1264−1271 (ETILNSHL) and region 1424−1431 (VISLDDFF) of DP2 from *P. horikoshii*, respectively. However, no interaction was detected between PhoPCNA and full-length DP2, or between PhoPCNA and the fragments of DP2 that include the two regions, in our Y2H assay.

The N-Terminal and C-Terminal Regions of DP2 Could Interact with Each Other. We found that the N-terminal region of DP2 interacted with the C-terminal region as shown in Figure 1 and Figure 2C. The β-galactosidase activity of the pGKBT7-DP2(1−693) and pGADT7-DP2(792−1163) cotransformant was 11 units, while that of the pGKBT7-DP2 and pGADT7-DP2(792−1163) cotransformant was 2.3 units, in a liquid culture assay. The results suggest that the interaction between domain DP2(1−693) and domain DP2-(792−1163) would be intrasubunit, not intersubunit. The PhoPolD complex was a tetramer with two small subunits and two large subunits (L_2S_2). We had reported that the N-terminal 1−300 amino acids of DP2, DP2(1−300), must play an important role in the oligomerization of DP2 (*10*). Full-length DP2 was unstable when it was expressed in *E. coli* independently. All results suggest that the interaction between the N-terminal region and C-terminal region of DP2 and interaction between two DP2(1−300) domains are not enough to make the independent DP2 stable. Some other interaction is needed for the proper folding and stability of DP2.

A 21 Amino Acid Peptide, DP2(1290−1310), Is Probably Involved in the Complex L_2S_2 Formation and the Regulation of Exonuclease Activity through Interaction with DP1(1−200). The Y2H assay indicated that DP1 interacted with the C-terminus of DP2, DP2(1290−1434), but not with the far C-terminus of DP2, DP2(1375−1434), as shown in Figure 3A. Therefore, DP2(1290−1374) is one of the regions in DP2 related to the interaction with DP1. We have demonstrated that a region of DP2, DP2(1255−1332), was very important for the formation of the PhoPolD complex through truncated experiments (*10*). The region of DP2(1255−1332) contained a conserved putative zinc-finger motif (Cys1289-Cys1308) with two pairs of cysteines. The results of the Y2H assay support those of the truncated experiments. The region related to the interaction with DP1 could be shortened to 12 amino acid DP2(1293−1304), which was the internal region of the putative zinc-finger motif (Figure 3A, line 9).

Recently, we found that the 3′−5′ exonuclease activity-related sites were on the small subunit of PhoPolD and that the 3′−5′ exonuclease activity was Mn^{2+}-dependent (*11*). DP1, mutant SL(DEL 1289−1298), and SL(DEL 1299−1308) all had 3′−5′ exonuclease activity when Mn^{2+} was used as a metal cofactor. Interestingly, a 21 amino acid peptide corresponding to DP2(1290−1310), which contained a defective putative zinc-finger motif with only three cysteines, increased the 3′−5′ exonuclease activity of DP1, mutant SL(DEL 1289−1298), and mutant SL(DEL 1299−1308) when Mn^{2+} was used as the metal cofactor (*11*). We also found that the 21 amino acid peptide corresponding to DP2(1290−1310) not only interacted with the full-length DP1 with a dissociation constant of 4.81 nM as shown in Figure 3B but also interacted with DP1(1−200) with a dissociation constant of 4.3 nM. We reported that the truncated mutant S(201−622)L had enhanced exonuclease activity compared with the wild-type PhoPolD and protein S(1−200) inhibited the exonuclease activity of S(201−622)L. Thus, the region 1290−1310 of DP2 is probably involved in PhoPolD complex formation and regulating the exonuclease activity through its interaction with DP1(1−200).

DP2(792−1163) Might Be One of the Key Domains Involved in DNA Polymerization and Stability in the PhoPolD Complex, in Which Multiple Sites of DP2 Interact with DP1. We found that DP1 and DP1(1−200) also interacted with DP2(792−1163) using the Y2H and SPR assays (Figure 3A,C), and DP1 and DP1(201−622) interacted with DP2(1−300) using the Y2H assay (Figure 3A). No interaction was detected between DP1(201−622) and DP2-(792−1163) with the Y2H assay (Figure 3A, line 12). The dissociation constant for DP2(792−1163) and DP1(1−200) was 0.79 μM, while that for DP2(792−1163) and DP2(1−100) was 3.16 μM, suggesting that the intersubunit interaction between DP2(792−1163) and DP1(1−200) is 3-fold stronger than the intrasubunit interaction between DP2(792−1163) and DP2(1−100). Because the truncated mutant S(201−622)L was stable and had both polymerase and exonuclease activities, DP1(1−200) alone might play limited roles involved in the stability and activity of the PhoPolD complex whereas DP1(1−200) is involved in the regulation of the exonuclease activity through interaction with DP2(1290−1310). Therefore, several interactions including the one between DP2(792−1163) and DP1(1−200), probably the one between DP1(201−622) and DP2(1−300), the one between DP2(1−100) and DP2(792−1163), and other interactions unidentified were contributed to form the L_2S_2 PhoPolD complex (Figure 6B).

The interaction between DP2(792−1163) and DP2(1−100) was confirmed using the Y2H and SPR assays (Figure 1, line 15, and Figure 2C); however, the interaction was lost by the deletion in the vicinity of the catalytic residues, Asp1122 and Asp1124 (Figure 1, lines 9 and 10), suggesting the functional importance of the boundary sequence of the catalytic residues located at the C-terminus. Using a hydrophobicity analysis, we found three hydrophobic regions in the C-terminus of DP2(792−1163) (data not shown). The polypeptide DP2(792−1163) was expressed as an insoluble form in *E. coli* probably due to its hydrophobicity. Fortunately, we succeeded in solubilization of the polypeptide by refolding processes. The solubilized DP2(792−1163) could interact with both DP2(1−100) and DP1(1−200) as shown in Figures 2B and 3C. Since DP2(792−1163) contains the catalytic residues for DNA polymerization, Asp1122 and Asp1124, at the beginning of the third hydrophobic region in its C-terminus and interacts with DP2(1−100) and DP1-(1−200) as depicted in Figure 6, the polypeptide DP2(792−1163) probably forms the most important domain deeply involved in both the catalysis of DNA polymerization and stabilization of the PhoPolD complex through multiple protein−protein interactions including hydrophobic interactions with other domains.

Our study on the interaction of domains from the family D DNA polymerase of *P. horikoshii* provides initial domain-interaction information on archaeal PolD. Further study on the molecular structure of PolD will clarify the mechanisms

of subunit folding, subunit communication, and interaction between PolD and other proteins.

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