Preparations of Recombinant HIV-1 p66 Antigen to Improve the Specificity of Immune Complex Transfer Enzyme Immunoassay of Antibody IgG to HIV-1 Reverse Transcriptase

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Recombinant HIV-1 p66 (rp66, a subunit of reverse transcriptase (RT), a heterodimer of p66 and p51) was produced in *Escherichia coli* in three different ways. First, rp66 was produced as a part of the fusion protein of *lacZ* protein and HIV-1 *pol* protein consisting of three components: protease (p10), RT (p51/p66), and integrase (p31), and was released from the fusion protein by the protease (*pol*-rp66). Second, rp66 with Ser-Ser at the *N*-terminus was produced as a fusion protein with maltose-binding protein containing a factor Xa site between the two proteins (MBP-Ser-Ser-rp66) and was released from the fusion protein by factor Xa (Ser-Ser-rp66). Third, rp66 with Met-Gly at the *N*-terminus was produced in transformed cells (Met-Gly-rp66). The recombinant proteins were purified from sonic extracts of transformed cells by ammonium sulfate fractionation and various column chromatographies. MBP-Ser-Ser-rp66 and Met-Gly-rp66

were readily purified in sufficient amounts for labeling with 2,4-dinitrophenyl groups and β -D-galactosidase from *E. coli*, but *pol*-rp66 and Ser-Ser-rp66 were not for enzyme-labeling. Ser-Ser-rp66 was not only polymerized but also degraded to considerable extents. The purified preparations were labeled with 2,4-dinitrophenyl groups and β -D-galactosidase and were tested in immune complex transfer enzyme immunoassay of antibody IgG to HIV-1 RT using serum samples from 600 HIV-1 seronegative and 30 HIV-1 seropositive subjects. Among various combined uses of the two labeled preparations, the uses of 2,4-dinitrophenylated MBP-Ser-Ser-rp66 and *pol*-rp66 with β -D-galactosidase-labeled Met-Gly-rp66 showed the highest (99.8%) and the second highest (99.5%) specificities, which were higher than that with the labeled preparations used in the previous study (98.0%). J. Clin. Lab. Anal. 14:169–179, 2000. © 2000 Wiley-Liss, Inc.

Key words: reverse transcriptase; recombinant protein; enzyme immunoassay; β -D-galactosidase; specificity; immunoglobulin G; HIV-1 infection; AIDS

INTRODUCTION

HIV-1 reverse transcriptase (RT) (heterodimer of p66 and p51) or p66 has been reported to be highly reactive with serum samples from HIV-1 seropositive subjects. By the conventional ELISA (1) and Western blotting (2), the seropositive rate with recombinant HIV-1 RT (rRT) as antigen was higher than those with *gag* proteins (p17 and p24) and regulatory proteins (*nef*, *vif*, etc.) as antigens and was as high as that with *env* gp41 as antigen. By a two-site enzyme immunoassay using rRT-coated microplates and rRT-alkaline phosphatase conjugate, seroconversion was detected as early as by the conventional ELISA using five recombinant proteins (gp120, gp41, p24, p17, and p15) as antigens and the gelatin particle-agglutination test using a lysate of HIV-1 as antigen (3). In addition, the specificity of immunoassays using rRT

as antigen appears to be fairly high. In a two-site enzyme immunoassay using rRT-coated microplates and rRT-alkaline phosphatase conjugate for anti-HIV-1 antibodies, no reaction has been observed with sera from subjects infected with either HIV-2 or hepatitis B virus (3). HIV RT has been described to be antigenically distinct from those of human T-lymphotropic virus type I (HTLV-I) and II (4).

An ultrasensitive enzyme immunoassay (immune complex transfer enzyme immunoassay) has been developed for antibody IgG to HIV-1 using rRT as antigen and β -D-galactosidase from *E. coli* as label (5). Antibody IgG to RT was allowed

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to react simultaneously with 2,4-dinitrophenyl-bovine serum albumin-rRT conjugate and rRT- β -D-galactosidase conjugate. The immune complex formed of the three components was trapped on polystyrene beads coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG and, after washing, was transferred to polystyrene beads coated with affinity-purified (antihuman IgG γ -chain) IgG in the presence of excess ϵ N-2,4-dinitrophenyl-L-lysine. Using this enzyme immunoassay, diagnoses of HIV-1 infection using urine and whole saliva have become easier than by conventional methods. With 100 μ L urine samples, the lowest signals for asymptomatic carriers and patients with AIDS-related complex (ARC) and AIDS were 20-, 11-, and 7.2-fold, respectively, higher than the highest signal for HIV-1 seronegative subjects (5). With only one μ L whole-saliva samples, the lowest signals for asymptomatic carriers and patients with ARC and AIDS were 38-, 78-, and 3-fold, respectively, higher than the highest signal for HIV-1 seronegative subjects (6). In addition, diagnosis of HIV-1 infection was possible with whole-saliva samples dried on filter papers (7). With 10- μ L serum samples, the lowest signals for asymptomatic carriers and patients with ARC and AIDS were 56,000-, 66,000-, and 5,200-fold, respectively, higher than the highest signal for HIV-1 seronegative subjects, and this enzyme immunoassay was 300- to 1,000-fold more sensitive than Western blotting for HIV-1 p66 band (8). Finally, no significant reaction has been observed with sera from HTLV-I-infected subjects (5). However, low but not unequivocally negative signals have been observed in some serum samples from HIV-1 seronegative subjects.

This report describes the preparations of HIV-1 rp66 to improve the specificity of immune complex transfer enzyme immunoassay of antibody IgG to HIV-1 RT in serum.

MATERIALS AND METHODS

Plasmid Constructions

Recombinant *Taq* DNA polymerase (TaKaRa *Taq*) and ligase included in a ligation kit (Ver. 1) were obtained from Takara Shuzo Co., Kyoto, Japan. Restriction enzymes used were obtained from New England Biolabs, Inc., Beverly, MA. Plasmids for the production of recombinant HIV-1 p66 (rp66) were produced using pNLΔBg lacking a part of *env* DNA (9), which derived from pNL4-3 (10) constructed from NY5 (GenBank accession number HIVNL43 [M19921]) and LAV (11). The sequence of p66 derived from NY5. Oligodeoxynucleotides used were obtained from BEX, Tokyo, Japan. The sequences corresponding to p66 in the plasmids constructed were confirmed to be correct by Takara Shuzo Co.

pSEGALPOL11

The plasmid for the production of rp66 with the native amino-acid sequence (*pol*-rp66) was constructed by insertion of HIV-1 *pol* DNA and *E. coli lacZ* DNA into pSE420

(Invitrogen, CH Groningen, Netherlands) using three restriction enzyme sites located in the order of *Nco* I, *Bgl* II, and *Xho* I from 5'- to 3'-end in the polylinker. First, HIV-1 *pol* DNA containing 3,000 base pairs (bps) (2,097–5,096) corresponding to protease, p66 and integrase was prepared from pNLΔBg by treatment with *Bgl* II and *Sal* I, and was inserted into pSE420 after treatment with *Bgl* II and *Xho* I (pSEPOL21). Second, *E. coli lacZ* DNA containing 2,997 bps corresponding to β-D-galactosidase subunit lacking *N*-terminal 9 amino acids and *C*-terminal 16 amino acids of pMC1871 (Amersham Pharmacia Biotech, Buckinghamshire, England) was inserted into LITMUS 28 (New England Biolabs) by treatment with *Bam*H I and *Eco*R I (pLITGALBE2). Third, *E. coli lacZ* DNA of pLITGALBE2 was inserted into pSEPOL21 by treatment with *Nco* I and *Bgl* II (pSEGALPOL11).

pMALMBPSP2SP1Ser-Ser-p662121

The plasmid for producing a fusion protein of rp66 having two serine residues at the *N*-terminus of rp66 with maltose binding protein (MBP) (MBP-Ser-Ser-rp66) was constructed so as to link the two proteins with a spacer of 29 amino acids (Asn-Ser-Ser-Ser-Asn-Ser-Asn-Thr-Ser-Ser-Asn-Asn-Asn-Pro-Ser-Ser-Ser-Asn-Asn-Asn-Pro-Ser-Ser-Ser-Ser-Ile-Glu-Gly-Arg) containing a factor Xa site to release Ser-Ser-rp66 from the fusion protein (Figs. 1 and 2). First, a DNA fragment (first spacer DNA, SP1) containing *Sac* I- and *Hind* III-splitted sequences at 5'- and 3'-ends, respectively, four restriction enzyme sites in the order of *Eco*R I, *Nhe* I, *Sal* I, and *Xba* I from 5'- to 3'-end and the factor Xa site between *Nhe* I and *Sal* I sites was produced by annealing two oligodeoxynucleotides (5'-CGAATTCGAACGCTAGCTC-GAGCATCGAAGGTCGACTGCAGTCTAGA and 5'-AGCTTCTAGACTGCAGTCGACCTTCGATGCTCGAG-CTAGCGTTTCGAATTCGAGCT) and, was inserted into pMAL-c2 (New England Biolabs) after digestion with *Sac* I and *Hind* III (pMALMBPSP1102). Second, HIV-1 p66 DNA was produced by PCR using pNLΔBg as template and two primers (5'-GCACCCGTCGACCCATTAGTCCTATTGAG-A and 5'-GGTCTAGATCATAGTACTTTCCTGATTCCA) so as to have *Sal* I and *Xba* I sites at the 5'- and 3'-ends, respectively, besides 1,680 bps (2,550-4,229) corresponding to p66, and was inserted into pMALMBPSP1102 by treatment with *Sal* I and *Xba* I (pMALMBPSP1p662001). Third, a DNA fragment (second spacer DNA, SP2) containing *Eco*R I- and *Xba* I-splitted sequences at its 5'- and 3'- ends, respectively, was produced by annealing two oligodeoxynucleotides (5'-AATTCGAACACTAGTTCCAACAACAATCCGTCTT-CCAGCAACAACAACCCGT and 5'-CTAGACGGGTTG-TTGTGCTGGAAGACGGATTGTTGTTGGAAGTCTG-TTCG) and was inserted into pMALMBPSP1p662001 after digestion with *Eco*R I and *Nhe* I (pMALMBPSP2SP1-p662101). Fourth, HIV-1 p66 DNA was prepared from pMBPSP2SP1p662101 by treatment with *Sal* I and *Hind* III,

Fig. 1. Construction of plasmids for the production of MBP-Ser-Ser-rp66.

pLITp663 **pLITp663 digested with *Bgl* II and *BsmB* I** **Fragment Ser-Ser** **pLITSer-Ser-p667** **pMALMBPSP2SP1p662101 digested with *Sal* I and *Hind* III** ***BsmB* I-*Hind* III fragment of pLITSer-Ser-p667** **pMALMBPSP2SP1-Ser-Ser-p662121**

Fig. 2. Construction of pMALMBPSP2SP1SerSerp662121 from pMALMBPSP2SP1p662101.

and was inserted into LITMUS 28 after treatment with *Xho* I and *Hind* III (pLITp663). LITMUS 28 had a *Bsm*B I site overlapping with the *Xho* I site (Fig. 2). Fifth, a DNA fragment for addition of Ser-Ser at the *N*-terminus of rp66 containing a *Bam*H I-splitted sequence at the 5'-end, another *Bsm*B I site, and the complementary sequence at the 3'-end to the *Bsm*B I-splitted sequence of pLITp663 was produced by annealing of two oligodeoxynucleotides (5'-GATCCGTCTCATCGATC-CTC and 5'-GGGTGAGGATCGATGAG-ACG) and was inserted into pLITp663 after digestion with *Bgl* II and *Bsm*B I (pLITSer-Ser-p667). Sixth, Ser-Ser-p66 DNA was prepared from pLITSer-Ser-p667 by treatment with *Bsm*B I and *Hind* III, and was inserted into pMALMBPSP2SP1p662101 after treatment with *Sal* I and *Hind* III (pMALMBPSP2SP1Ser-Ser-p662121).

pSEMet-Gly-p662

The plasmid for the production of rp66 with Met-Gly at the *N*-terminus (Met-Gly-rp66) was constructed by insertion of HIV-1 p66 DNA into pSE420. HIV-1 p66 DNA was produced by PCR using pNLΔBg as template and two primers (5'-CGACAGCCATGGGTCCCATTAGTCCTATTGAGA and 5'-GGTCTAGATCATAGTACTTTCCTGATTCCA) so as to have *Nco* I and *Xba* I sites at its 5'- and 3'-ends, respectively, besides 6 bps corresponding to Met-Gly and 1,680 bps (2,550–4,229) corresponding to p66, and was inserted into pSE420 by treatment with *Nco* I and *Xba* I (pSEMet-Gly-p662).

Escherichia coli

Competent cells of *E. coli* K12 strain DH5α [*F*⁺Φ80-*dlacZ*Δ*M15*Δ(*lacZYA-argF*)*U169 endA1 recA1 hsdR17* (*r*_K⁺)*deoR thi-1 supE44 λ⁻ gyrA96 relA1*] for the productions of *pol*-rp66 and MBP-Ser-Ser-rp66 and *E. coli* β strain BL21 [*F*⁺ *ompT hsdS_B*(*r*_B⁻ *m*_B⁻) *gal dcm*] for the production of Met-Gly-rp66 were obtained from Life Technologies, Rockville, MD, and Novagen Inc., Madison, WI, respectively.

Production of Recombinant HIV-1 p66 (rp66)

Production of *pol*-rp66

DH5α cells transformed with pSEGALPOL11 were grown in Terrific broth medium (12) without glycerol containing 20 mmol/L glucose and 100 μg/mL penicillin G potassium salt at 37°C to an absorbance of 0.8 at 600 nm. Expression of *lacZ* protein-*pol*-rp66 fusion protein was induced at 25°C for 22 hr by addition of an equal volume of ice-chilled Terrific broth medium without glycerol containing 2 mmol/L isopropyl-β-D-thiogalactopyranoside and 100 μg/mL penicillin G potassium salt.

Productions of MBP-Ser-Ser-rp66 and Met-Gly-rp66

DH5α and BL21 cells transformed with pMALMBPSP2SP1Ser-Ser-p662121 and pSEMet-Gly-p662, respectively, were grown in Luria-Bertani medium (12) containing 80 μg/mL penicillin G potassium salt at 25 and 37°C, respectively, to an absorbance of 0.6 at 600 nm. Expressions of MBP-Ser-Ser-rp66 and Met-Gly-rp66 were induced at 25°C for 12 hr and at 37°C for 4 hr, respectively, by addition of isopropyl-β-D-thiogalactopyranoside at a final concentration of 0.3 mmol/L.

The cultured cells harvested by centrifugation at 4°C were washed with 300 mL 10 mmol/L Tris-HCl, pH 8.0, containing 0.1 mol/L NaCl and 1 mmol/L EDTA, and were stored at -20°C until use.

Buffers

The frequently used buffers were 20 mmol/L Tris-HCl, pH 7.4, containing 1 mmol/L EDTA, 10 mmol/L β-mercaptoethanol, 1 mmol/L phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO), 10 μg/mL pepstatin A (Peptide Institute Inc., Osaka, Japan), 10 μg/mL E-64 (Peptide Institute Inc.) (buffer A), 50 mmol/L sodium phosphate buffer, pH 7.0, containing 1 mmol/L EDTA, and 1 mmol/L β-mercaptoethanol (buffer B), buffer B containing 0.1% Triton X-100 (reduced) (Sigma) (buffer B-Tr), 20 mmol/L Tris-HCl, pH 7.4, containing 1 mmol/L EDTA (buffer C), buffer C containing 1 mmol/L β-mercaptoethanol (buffer C-SH), 20 mmol/L Tris-HCl, pH 8.0, containing 1 mmol/L EDTA (buffer D), 15 mmol/L sodium phosphate buffer, pH 6.8, containing 0.1% Triton X-100 (buffer E), 10 mmol/L sodium phosphate buffer, pH 7.5, containing 50 mmol/L NaCl and 0.1% Triton X-100 (buffer F), 20 mmol/L sodium phosphate buffer, pH 7.0, containing 0.1% Triton X-100 (buffer G), and 10 mmol/L sodium phosphate buffer, pH 7.0, containing 0.1 g/L bovine serum albumin (fraction V, Intergen Co., Purchase, NY), 1.0 mmol/L MgCl₂ and 1.0 g/L NaN₃ (buffer H).

Purification of HIV-1 rp66

The purifications were carried out at 4°C throughout except the column chromatographies with Ultrogel AcA 44 or 34 (Biosepra, Villeneuve la Garenne, France) at room temperature. Protein concentrations were determined with a BCA (bicinchoninic acid) protein assay kit (Pierce, Rockford, IL).

Purification of *pol*-rp66

The cells from 18 L culture medium suspended in 500 mL buffer A containing 0.1% Triton X-100 (reduced) were sonicated, and the sonic supernatant fluid (520 mL) was fractionated with solid (NH₄)₂SO₄. The precipitate between 35 and 50% (NH₄)₂SO₄ saturations dissolved in 50 mL buffer A was dialyzed three times against 2 L buffer B-Tr overnight and,

after centrifugation, was applied to a column (5.0 × 5.1 cm) of DEAE Sepharose FF (Amersham Pharmacia Biotech) in buffer B-Tr. The flow through fractions (288 mL, 604 mg protein) were applied to a column (2.5 × 4.0 cm) of Heparin Sepharose CL-6B (Amersham Pharmacia Biotech) in buffer B-Tr, and *pol*-rp66 was eluted with a linear gradient of 50 to 250 mmol/L phosphate in 200 mL buffer B-Tr. The fractions between 220 and 250 mmol/L phosphate were dialyzed three times against 1 L buffer B-Tr overnight (148 mL, 37 mg protein) and were applied to a column (2.5 × 4.2 cm) of Macro-Prep ceramic hydroxyapatite (Type II, 20 μm, Bio-Rad Laboratories, Hercules, CA) in buffer B-Tr. *pol*-rp66 was eluted with a linear gradient of 50 to 200 mmol/L phosphate in 100 mL buffer B-Tr. The fractions between 90 and 100 mmol/L phosphate (20 mL, 6.4 mg protein) were concentrated to 0.39 mL and were applied to a column (1.5 × 45 cm) of Ultrogel AcA 44 in 0.1 mol/L sodium phosphate buffer, pH 7.0, containing 0.01% Triton X-100 (reduced). The fractions, Nos. 34 to 40 (3.1 mL, 4.5 mg protein), were used for labeling with 2,4-dinitrophenyl groups and β-D-galactosidase.

Purification of MBP-Ser-Ser-rp66

The cells from 18 L culture medium suspended in 900 mL buffer A were sonicated, and the sonic supernatant fluid (920 mL) was fractionated with solid (NH₄)₂SO₄. The precipitate between 35 and 60% (NH₄)₂SO₄ saturations was dissolved in 150 mL buffer C-SH and, after dialyzing three times against 2 L buffer C-SH overnight, was applied to a column (2.5 × 20 cm) of Amylose Resin (New England Biolabs) in buffer C-SH. MBP-Ser-Ser-rp66 was eluted with buffer C-SH containing 10 mmol/L maltose and 0.2 mol/L NaCl and dialyzed against 2 L buffer B overnight. The dialysate (48 mL, 91 mg protein) was applied to a column (2.5 × 4.1 cm) of Affi-Gel heparin (Bio-Rad Laboratories) in buffer B and, MBP-Ser-Ser-rp66 was eluted with a linear gradient of 0 to 1 mmol/L NaCl in 200 mL buffer B. The fractions between 0.16 and 0.4 mol/L NaCl were dialyzed three times against 2 L buffer B overnight (50 mL, 41 mg protein) and were applied to a column (2.5 × 2.0 cm) of Macro-Prep ceramic hydroxyapatite (Type II, 20 μm) in buffer B. MBP-Ser-Ser-rp66 was eluted with a linear gradient of 50 to 300 mmol/L phosphate in 100 mL buffer B. The fractions between 160 and 220 mmol/L phosphate (27 mL, 25 mg protein) were concentrated to 1.2 mL and were applied to a column (1.5 × 57 cm) of Ultrogel AcA 34 in 0.1 mol/L sodium phosphate buffer, pH 7.0. Fraction Nos. 54 to 62 (31 mL, 11 mg protein) were used for labeling with 2,4-dinitrophenyl groups and β-D-galactosidase.

Purification of Ser-Ser-rp66

The precipitate between 35 and 60% (NH₄)₂SO₄ saturations obtained as described above for MBP-Ser-Ser-rp66 was dissolved in 150 mL buffer D and, after dialyzing twice against 2 L buffer D overnight, was applied to a column (5.0 × 12.2

cm) of DEAE Sepharose FF in buffer D. MBP-Ser-Ser-rp66 was eluted with a linear gradient of 0 to 0.3 mol/L NaCl in 250 mL buffer D, and the fractions between 0.11 and 0.16 mol/L NaCl (295 mL) were brought to 60% (NH₄)₂SO₄ saturation. The precipitate was dissolved in 17 mL buffer C and, after dialyzing three times against 2 L buffer C overnight, was applied to a column (2.5 × 12 cm) of Amylose Resin in buffer C. MBP-Ser-Ser-rp66 was eluted with buffer C containing 10 mmol/L maltose and 0.2 mol/L NaCl and dialyzed against 2 L 50 mmol/L Tris-HCl, pH 8.0, containing 0.1 mol/L NaCl overnight. The dialysate (16.8 mL, 38 mg protein) was incubated with 168 μL 10% Triton X-100 in water, 16.8 μL 1 mol/L CaCl₂ and 282 μL 1 mg/mL factor Xa solution (New England Biolabs) at 30°C for 5 hr. The reaction mixture, after overnight dialysis against 2 L buffer E, was applied to a column (2.5 × 4.1 cm) of SP Sepharose FF (Amersham Pharmacia Biotech) in buffer E, and Ser-Ser-rp66 was eluted with a linear gradient of 0 to 0.5 mol/L NaCl in 200 mL buffer E. The fractions between 0.08 and 0.4 mol/L NaCl (140 mL, 14 mg protein), after dialyzing twice against 2 L buffer F overnight, were applied to a column (1.5 × 2.8 cm) of rabbit (anti-MBP) IgG-Sepharose 4B in buffer F, which had been prepared by coupling 10 mg IgG from rabbit anti-MBP serum (Shibayagi Co., Gumma, Japan) to 1 g CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech). The flow through fractions (151 mL, 15 mg protein) were applied to a column (2.5 × 2.0 cm) of Affi-Gel heparin in buffer F, and Ser-Ser-rp66 was eluted with a linear gradient of 0.05 to 1 mol/L NaCl in 100 mL buffer F. The fractions between 0.1 and 0.6 mol/L NaCl (54 mL, 7.6 mg protein), after dialyzing twice against 2 L buffer G overnight, were applied to a column (1.5 × 2.8 cm) of Macro-Prep ceramic hydroxyapatite (Type II, 20 μm) in buffer G, and Ser-Ser-rp66 was eluted with a linear gradient of 0.02 to 0.3 mol/L phosphate in 50 mL buffer G. The fractions between 130 and 300 mmol/L phosphate (52 mL, 2.4 mg protein) were concentrated to 1.2 mL and were applied to a column (1.5 × 58 cm) of Ultrogel AcA 44 in 0.1 mol/L sodium phosphate buffer, pH 7.0, containing 0.01% Triton X-100. The fractions Nos. 37 to 47 (7.3 mL, 0.6 mg protein, Ser-Ser-rp66-I), Nos. 48 to 59 (7.8 mL, 1.0 mg protein, Ser-Ser-rp66-II) and Nos. 60 to 70 (7.3 mL, 0.4 mg protein, Ser-Ser-rp66-III) were used for labeling with 2,4-dinitrophenyl groups and β-D-galactosidase.

Purification of Met-Gly-rp66

The cells from 18 L culture medium suspended in 900 mL buffer A containing 0.1% Triton X-100 (reduced) were sonicated, and the sonic supernatant (895 mL) was fractionated with solid (NH₄)₂SO₄. The precipitate between 35 and 50% (NH₄)₂SO₄ saturations dissolved in 180 mL buffer B-Tr was dialyzed twice against 2 L buffer B-Tr overnight and was applied to a column (5.0 × 9.0 cm) of DEAE Sepharose FF in buffer B-Tr. The flow through fractions (501 mL, 351 mg

protein) were applied to a column (2.5×6.1 cm) of Affi-Gel heparin in buffer B-Tr, and Met-Gly-rRT was eluted with a linear gradient of 0.05 to 1 mol/L NaCl in 300 mL buffer B-Tr. The fractions between 0.16 and 0.38 mol/L NaCl (72 mL) were dialyzed twice against 2 L buffer B-Tr overnight (90 mL, 114 mg protein) and were applied to a column (1.5×8.4 cm) of Macro-Prep ceramic hydroxyapatite (Type II, 20 μ m) in buffer B-Tr. Met-Gly-rp66 was eluted with a linear gradient of 0.05 to 0.5 mol/L phosphate in 150 mL buffer B-Tr. The fractions between 90 and 160 mmol/L phosphate (53 mL, 59 mg protein) were concentrated to 5.7 mL. The concentrated fractions were divided into three portions, and each portion was applied to a column (1.5×58 cm) of Ultrogel AcA 44 in 0.1 mol/L sodium phosphate buffer, pH 7.0, containing 0.01% Triton X-100 (reduced). Fraction Nos. 45 to 57 of three eluate fractions were combined (19 mL, 37 mg protein) and were used for labeling with 2,4-dinitrophenyl groups and β -D-galactosidase.

Labeling of rp66 With 2,4-Dinitrophenyl Groups and β -D-Galactosidase

Thiol groups introduced into rp66 preparations were allowed to react with 6-maleimidohexanoyl- ϵ -N-2,4-dinitrophenyl-L-lysine (13) and maleimide groups introduced into β -D-galactosidase from *E. coli* using *N*-succinimidyl-6-maleimidohexanoate (14).

Immune Complex Transfer Enzyme Immunoassay of Antibody IgG to HIV-1 RT

The immunoassay was performed as described previously (15). An aliquot (10 μ L) serum samples was incubated with 130 or 140 μ L buffer H containing 0.4 mol/L NaCl, 50 μ g inactive β -D-galactosidase, 100 to 300 fmol 2,4-dinitrophenylated preparations and 30 to 100 fmol β -D-galactosidase-labeled preparations in the presence or absence of 10 μ L nonspecific rabbit serum at room temperature for 2 hr. The reaction mixture was incubated for 1 hr with 2 colored polystyrene beads of 3.2 mm diameter coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG (0.1 g/L) (16). After washing the colored polystyrene beads were incubated for 1 hr with 2 white polystyrene beads of 3.2 mm diameter coated with affinity-purified (antihuman IgG γ -chain) IgG (0.1 g/L) (16) in 150 μ L buffer H containing 0.1 mol/L NaCl and 1.0 mmol/L ϵ -N-2,4-dinitrophenyl-L-lysine. β -D-Galactosidase activity bound to the white polystyrene beads was assayed at 30°C for 1 hr or 20 hr by fluorometry using 4-methylumbelliferyl- β -D-galactoside as substrate (17). The fluorescence intensity was measured relative to 1×10^{-8} mol/L 4-methylumbelliferone.

Serum Samples

Serum samples used were collected from 610 HIV-1 seronegative subjects (372 males aged 22–77 yr and 238 fe-

males aged 20–73 yr) and 30 HIV-1 seropositive subjects (18 male asymptomatic carriers aged 17–46 yr, 2 female asymptomatic carriers aged 29–39 yr, 4 male patients aged 10–52 yr with ARC, 1 female patient aged 42 yr with ARC, 5 male patients aged 14–46 yr with AIDS) and were stored at -20°C until use. Seropositivity was confirmed by the gelatin particle agglutination test with a commercial kit (SERODIA-HIV; Fujirebio Inc., Tokyo, Japan) (18). A positive serum included in a commercial HIV Western blot kit (Ortho Diagnostic Systems Inc., Raritan, NJ) was used after 4,000-fold dilution with buffer H containing 0.4 mol/L NaCl.

RESULTS AND DISCUSSION

Production of Recombinant HIV-1 p66 (rp66)

HIV-1 rp66 was produced in *E. coli* in three different ways. First, rp66 with the native amino-acid sequence was produced as a part of the fusion protein of *lacZ* protein and HIV-1 *pol* protein consisting of three components: protease (p10), RT (p51/p66), and integrase (p31) and was released from the fusion protein by the protease (*pol*-rp66). Second, rp66 with Ser-Ser at the *N*-terminus was produced as a fusion protein with maltose-binding protein containing a factor Xa site between the two proteins (MBP-Ser-Ser-rp66) and was released from the fusion protein with factor Xa (Ser-Ser-rp66). Third, rp66 with Met-Gly at the *N*-terminus was produced in transformed cells (Met-Gly-rp66).

Purification of HIV-1 rp66

HIV-1 rp66 was purified from sonic extracts of transformed *E. coli* cells. HIV-1 *pol*-rp66 was purified by ammonium sulfate fractionation and successive column chromatographies with DEAE Sepharose, Heparin Sepharose, hydroxyapatite, and Ultrogel AcA 44. MBP-Ser-Ser-rp66 was purified as a fusion protein by ammonium sulfate fractionation and successive column chromatographies with Amylose Resin, Affi-Gel heparin, hydroxyapatite, and Ultrogel AcA 34. To obtain Ser-Ser-rp66, MBP-Ser-Ser-rp66 was purified by ammonium sulfate fractionation and column chromatographies with DEAE Sepharose and Amylose Resin and was treated with factor Xa. Ser-Ser-rp66 was purified by column chromatographies with SP Sepharose, (anti-MBP) IgG-Sepharose, Affi-Gel heparin, hydroxyapatite, and Ultrogel AcA 44. Met-Gly-rp66 was purified by ammonium sulfate fractionation and successive column chromatographies with DEAE Sepharose, Affi-Gel heparin, hydroxyapatite, and Ultrogel AcA 44.

The amounts of *pol*-rp66, MBP-Ser-Ser-rp66, Ser-Ser-rp66 and Met-Gly-rp66 purified from a culture volume of 18 L were 4.5, 11, 2.0, and 37 mg, respectively, when the fractions of Nos. 34 to 40, 54 to 62, 37 to 70, and 45 to 57, respectively, by Ultrogel AcA 44 or 34 chromatography were pooled for labeling with 2,4-dinitrophenyl groups and β -D-

galactosidase. Thus, *pol*-rp66 and Ser-Ser-rp66 were not easily prepared in sufficient amounts for labeling with β -D-galactosidase. Gel filtrations of the purified preparations indicated that *pol*-rp66 was not significantly polymerized and that Ser-Ser-rp66 was not only much more polymerized but also much more degraded than other preparations (Fig. 3).

Positive and Negative (Nonspecific) Signals in Immune Complex Transfer Enzyme Immunoassay of Antibody IgG to HIV-1 RT With Various Labeled Preparations

The four preparations in the fractions by Ultrogel AcA 44 or 34 chromatography described above (*pol*-rp66, MBP-Ser-Ser-rp66, Ser-Ser-rp66, and Met-Gly-rp66) were labeled with 2,4-dinitrophenyl groups and β -D-galactosidase and were tested in immune complex transfer enzyme immunoassay of antibody IgG to RT in the presence of 10 μ L HIV-1-infected serum or 10 μ L serum samples from ten HIV-1 seronegative subjects. The results are shown in Table 1.

The uses of 2,4-dinitrophenylated *pol*-rp66 and Met-Gly-rp66 with β -D-galactosidase-labeled Ser-Ser-rp66 and *pol*-rp66, respectively, showed higher negative (nonspecific) signals (fluorescence intensities of bound β -D-galactosidase activity) than those of other preparations. And the uses of 2,4-dinitrophenylated *pol*-rp66, Ser-Ser-rp66, and MBP-Ser-Ser-rp66 with β -D-galactosidase-labeled Ser-Ser-rp66, *pol*-rp66, and Met-Gly-rp66, respectively, showed lower positive signals. The uses of β -D-galactosidase-labeled MBP-Ser-Ser-rp66 with other 2,4-dinitrophenylated preparations showed much higher negative signals, although the data are not shown. Higher ratios of positive to negative signals were obtained by the uses of 2,4-dinitrophenylated *pol*-rp66 and MBP-Ser-Ser-rp66 with β -D-galactosidase-labeled *pol*-rp66 or Met-Gly-rp66 and the uses of 2,4-dinitrophenylated Met-Gly-rp66 with β -D-galactosidase-labeled *pol*-rp66 and Met-Gly-rp66.

From these results, 2,4-dinitrophenylated Ser-Ser-rp66 and β -D-galactosidase-labeled Ser-Ser-rp66 and MBP-Ser-Ser-rp66 were not used in further experiments. And 2,4-dinitrophenylated *pol*-rp66, MBP-Ser-Ser-rp66, and Met-Gly-rp66 (in combination with β -D-galactosidase-labeled Met-Gly-rp66) were tested in an immune complex transfer enzyme immunoassay of antibody IgG to HIV-1 RT in the presence of 10 μ L nonspecific rabbit serum and 10- μ L serum samples from 600 HIV-1 seronegative or 30 HIV-1 seropositive subjects, because Met-Gly-rp66 was easily prepared in sufficient amounts for labeling with β -D-galactosidase. The positive serum samples were diluted 2,000- to 150,000-fold with serum from an HIV-1 seronegative subject.

The average negative signals with the labeled preparations used in the present study were similar and approximately 2-fold lower than those with the labeled preparations used in

Fig. 3. Elution profiles of various recombinant HIV-1 p66 preparations from a column of Ultrogel AcA 44 or 34. A, *pol*-rp66; B, MBP-Ser-Ser-rp66; C, Ser-Ser-rp66; D, Met-Gly-rp66. The flow rate was 0.5 ml/min, and the fraction volume was 1.0 mL. See Materials and Methods for other conditions. Bars indicate the fractions pooled for labeling with 2,4-dinitrophenyl groups and β -D-galactosidase.

the previous study (Table 2). The differences between the negative signals with the labeled preparations used in the present and those of previous studies were statistically sig-

TABLE 1. Positive and negative (nonspecific) signals in the immune complex transfer enzyme immunoassay of antibody IgG to HIV-1 RT with various labeled preparations^a

Preparations of rp66 labeled with		Signals (fluorescence intensities of bound β -D-galactosidase activity)		
2,4-dinitrophenyl groups	β -D-galactosidase	Negative (N)	Positive (P)	Ratios of P/N
Ser-Ser-rp66-II	<i>pol</i> -rp66	3.1 $\pm$ 0.6	442	143
<i>pol</i> -rp66	Ser-Ser-rp66-I	5.1 $\pm$ 9.8	153	30
<i>pol</i> -rp66	Ser-Ser-rp66-II	16.4 $\pm$ 32.7	913	56
<i>pol</i> -rp66	Ser-Ser-rp66-III	6.2 $\pm$ 2.9	960	155
<i>pol</i> -rp66	<i>pol</i> -rp66	2.3 $\pm$ 0.4	1,624	706
<i>pol</i> -rp66	Met-Gly-rp66	2.6 $\pm$ 0.7	1,473	567
MBP-Ser-Ser-rp66	<i>pol</i> -rp66	1.7 $\pm$ 0.5	1,262	742
MBP-Ser-Ser-rp66	Met-Gly-rp66	1.0 $\pm$ 0.2	994	994
Met-Gly-rp66	<i>pol</i> -rp66	6.2 $\pm$ 1.6	1,585	256
Met-Gly-rp66	Met-Gly-rp66	2.9 $\pm$ 0.8	1,437	496

^aSer-Ser-rp66-I, II and III were obtained from the pooled fractions of Nos. 37 to 47, 48 to 59, and 60 to 70, respectively, by Ultrogel AcA 44 chromatography (Fig. 1). 2,4-Dinitrophenylated and β -D-galactosidase-labeled preparations were tested in immune complex transfer enzyme immunoassay of antibody IgG to HIV-1 RT in the presence of 10 μ L of a positive sample, included in a Western blot kit and diluted 4,000-fold, or 10 μ L serum samples from ten HIV-1 seronegative subjects. Signals with the positive and ten negative samples were obtained by 1-hr and 20-hr assays, respectively, of bound β -D-galactosidase activity. MBP, maltose binding protein.

TABLE 2. Negative (nonspecific) signals and specificities in immune complex transfer enzyme immunoassay of antibody IgG to HIV-1 RT using various labeled preparations

2,4-Dinitrophenylated (DNP) and β -D-galactosidase (Gal)-labeled preparations	Sample Nos. of sera from HIV-1 seronegatives	Numbers of sera	Negative (nonspecific) signals	Percentages of sera that showed signals below indicate ones	Mean $\pm$ SD of negative (nonspecific) signals
DNP- <i>pol</i> -rp66	1-563	563	0.8-2.9	93.8	2.1 $\pm$ 1.3
Met-Gly-rp66-Gal	564-592	29	3.0-5.5	98.7	
	593-594	2	6.4-7.9	99.0	
	595-597	3	8.4-9.3	99.5	
	598-599	2	12-13	99.8	
	600	1	24	100	1.9 $\pm$ 1.2
DNP-MBP-Ser-Ser-rp66	1-560	560	0.9-2.9	93.3	
Met-Gly-rp66-Gal	561-596	36	3.0-5.8	99.3	
	597-598	2	7.4-7.8	99.7	
	599	1	8.1	99.8	
	600	1	25	100	2.0 $\pm$ 3.3
DNP-Met-Gly-rp66	1-542	542	0.4-2.9	90.3	
Met-Gly-rp66-Gal	543-582	40	3.0-5.9	97.0	
	583-587	5	6.1-7.8	97.8	
	588-592	5	8.9-9.5	98.7	
	593-597	5	13-17	99.5	4.4 $\pm$ 2.8
	598-599	2	27-37	99.8	
	600	1	55	100	
DNP- <i>pol</i> -rp66	1-44	44	1.5-2.9	7.3	
<i>pol</i> -rp66-Gal used in the previous study	45-565	521	3.0-5.9	94.2	
	566-583	18	6.0-7.8	97.2	4.4 $\pm$ 2.8
	584-588	5	8.1-9.1	98.0	
	589-595	7	10-17	99.2	
	596-599	4	20-32	99.8	4.4 $\pm$ 2.8
	600	1	47	100	

2,4-Dinitrophenylated and β -D-galactosidase-labeled preparations were tested in immune complex transfer enzyme immunoassay of antibody IgG to HIV-1 RT in the presence of 10 μ L nonspecific rabbit serum and 10 μ L serum samples from 600 HIV-1 seronegative subjects. Signals were obtained by 20-hr assay of bound β -D-galactosidase activity. Underlines indicate a tentative cutoff value of 10. MBP, maltose binding protein.

Fig. 4. Correlations between the positive signals with the labeled preparations used in the present and previous studies. Serum samples (10 μ L) from HIV-1 seropositive subjects were diluted 2,000- to 150,000-fold with serum from an HIV-1 seronegative subject, and bound β -D-galactosidase activity was assayed for 1 hr. The signals (fluorescence intensities of bound β -D-galactosidase activity) are shown as those obtained with 10- μ L serum without dilution by 1-hr assay calculated from those obtained with 10- μ L diluted serum samples by 1-hr assay. Circles, triangles, and squares indicate values for 20 HIV-1 asymptomatic carriers, 5 patients with ARC, and 5 patients with AIDS, respectively. See Results for the regression equations and correlation coefficients. DNP, 2,4-dinitrophenylated.

nificant by *t*-test ($P < 0.001$, $n = 600$). The positive signals with the labeled preparations used in the present study were at least 700,000-fold higher than a tentative cut-off value set at 10 and at least 178,000-fold higher than the highest negative signal, and such signals were well correlated with those with the labeled preparations used in the previous study (Fig. 4). The regression equations were $\log Y = 0.97 \log X + 1.09$ ($r = 0.96$) with 2,4-dinitrophenylated *pol*-rp66, $\log Y = 0.99 \log X + 0.77$ ($r = 0.95$) with 2,4-dinitrophenylated MBP-Ser-Ser-rp66 and $\log Y = 0.98 \log X + 0.87$ ($r = 0.95$) with 2,4-dinitrophenylated Met-Gly-rp66, where Y and X are signals with the labeled preparations used in the present and previous studies, respectively. The specificities with 2,4-dinitrophenylated *pol*-rp66, MBP-Ser-Ser-rp66, and Met-Gly-rp66 were 99.5, 99.8, and 98.7%, respectively, which were higher than those with the labeled preparations used in the previous study (98%) (5,8), in which a negative signal of 10 was taken as a tentative cut-off value (Table 2).

In summary, Met-Gly-rp66 and MBP-Ser-Ser-rp66 were suitable for labeling with β -D-galactosidase and 2,4-dinitrophenyl groups, respectively, since Met-Gly-rp66 and MBP-Ser-Ser-rp66 were purified in the highest and the second-highest yields, respectively, and the use of 2,4-dinitrophenylated MBP-Ser-Ser-rp66 with β -D-galactosidase-labeled Met-Gly-rp66 showed the highest specificity (Table 2). However, *pol*-rp66 may be also useful, if purified in higher yields, since *pol*-rp66 was not significantly polymerized and the use of 2,4-dinitrophenylated *pol*-rp66 and β -D-galactosidase-labeled Met-Gly-rp66 showed a similar specificity to the highest one.

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