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Oligomeric Organization of gp120 on Infectious Human Immunodeficiency Virus Type 1 Particles

CAROL D. WEISS,[1] JAY A. LEVY,[1] AND JUDITH M. WHITE[2]*

Cancer Research Institute and Department of Medicine,[1] and Departments of Pharmacology and Biochemistry and Biophysics,[2] School of Medicine, University of California, San Francisco, California 94143-0450

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The oligomeric structure of the human immunodeficiency virus type 1 envelope glycoprotein (gp120) was examined by treating infectious virions with chemical cross-linking agents and subjecting the proteins to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and velocity centrifugation. Immunoblots of cross-linked samples revealed three gp120 bands and an approximately threefold shift in gp120 sedimentation. Our finding of cross-linking solely between gp120 suggests that the gp120 subunits are closely associated in the native envelope structure.

Enveloped viruses have surface glycoproteins that mediate virus attachment and entry into cells. In all cases examined in detail, these glycoproteins form oligomers. The hemagglutinin of influenza virus, the G protein of vesicular stomatitis virus, and the spike glycoprotein of Semliki Forest virus, for example, form trimers (13, 15, 17), while the F and HN proteins of Sendai virus form tetramers (7, 12). For influenza virus, fusion and infectivity require a conformational change in hemagglutinin involving partial dissociation of oligomeric contacts (15–17).

Knowledge of the oligomeric structure of the envelope glycoprotein (gp120-gp41 and precursor gp160) of the human immunodeficiency virus (HIV) would contribute to our understanding of how this glycoprotein attaches and fuses the virus to target cells. This information could lead to new therapeutic strategies against HIV. Moreover, knowledge of the oligomeric structure is needed to evaluate the quaternary structure of recombinant forms of the envelope glycoprotein used in functional studies or immunization trials.

Virus derived from a molecular clone of the highly cytopathic HIV type 1_{SF33} (HIV-1_{SF33}) strain was used for this study (14). Intact virions from viral pellets were treated with the cleavable, membrane-impermeable cross-linking agent, 3,3-dithiobis(sulfosuccinimidylpropionate) (DTSSP). After quenching the cross-linking reaction, the proteins were solubilized, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with polyclonal anti-gp120 antiserum. By using a 100-fold concentration range of DTSSP (0.03 to 3.0 mM), a ladder of three anti-gp120-reactive bands was observed (Fig. 1A, lanes 2 to 5, arrows). Independent analyses using only fetal calf serum have shown that the minor lower-molecular-weight bands observed on the immunoblots are due to residual fetal calf serum in the virus samples (data not shown). At high concentrations of DTSSP (3.0 mM), only the third band was evident (Fig. 1A, lane 5); no further anti-gp120-reactive bands were seen with a fivefold higher concentration of DTSSP (data not shown). Sucrose gradient-purified HIV-1_{SF33} treated with 0.3 mM DTSSP gave the same three anti-gp120-reactive bands (Fig. 1A, lane 6). Similar results were obtained with the cross-linking agent dimethyl adipimidate (data not shown).

The cross-linked products were analyzed in low-percentage-acrylamide phosphate or Laemmli gels (6), and molecular weights of the anti-gp120-reactive bands were estimated with reference to the cross-linked phosphorylase *b* pentamer (Sigma). Molecular weights of the five cross-linked phosphorylase *b* species (Fig. 1A and B, lanes 1) generated a linear standard curve and were consistent with other commercial high-molecular-weight standards (Bio-Rad) and with the four bands generated by cross-linking beta-galactosidase (a tetramer) with DTSSP (data not shown). Because the phosphate gels (specified by Sigma) gave tighter bands of both the viral proteins and phosphorylase *b* standards, this system was used for most of the experiments.

In eight independent experiments, the three anti-gp120-reactive bands migrated as 212 ± 14-, 296 ± 14-, and 354 ± 18-kilodalton (kDa) proteins in 3.5% acrylamide phosphate gels (Fig. 1A, lanes 2 to 6). In comparison, the three anti-gp120-reactive bands seen in a 3.5% acrylamide Laemmli gel migrated as 130-, 204-, and 280-kDa proteins (Fig. 1B, lanes 2 to 5). Here again, the minor bands seen are due to residual fetal calf serum in the samples. In four separate experiments using 5% acrylamide Laemmli gels, the three anti-gp120 bands migrated as 118 ± 6-, 226 ± 17-, and 383 ± 19-kDa proteins (Fig. 2B, lane 1).

To determine whether the high-molecular-weight anti-gp120 reactive bands represent pure oligomers of gp120, we probed parallel blots with anti-gp41 and anti-p25 antibodies (Fig. 2A, lanes 2 and 4, respectively). The three anti-gp120-reactive bands (Fig. 2A, lanes 1 and 3) did not react with anti-gp41 or anti-p25 antibodies (Fig. 2A, lanes 2 and 4, respectively), indicating that the anti-gp120-reactive bands did not contain the viral transmembrane or gag proteins cross-linked to gp120. In addition, when duplicate samples of solubilized proteins from DTSSP-treated virions were run on higher-percentage Laemmli gels, no cross-linked gp41 or p25 products were seen (Fig. 2C, lanes 2 and 4).

Further evidence that the cross-linked anti-gp120-reactive bands represent gp120 homo-oligomers was obtained by analyzing recombinant gp120 (rgp120, Chiron) produced in mammalian cells. In both phosphate (Fig. 1A, lane 7) and Laemmli (Fig. 2B, lane 2) gels, noncross-linked rgp120 migrated identically to noncross-linked viral gp120, confirming our assignment of the first band seen in all gel systems as the gp120 monomer. Unexpectedly, with DTSSP we also detected cross-linked products of the rgp120, albeit

* Corresponding author.

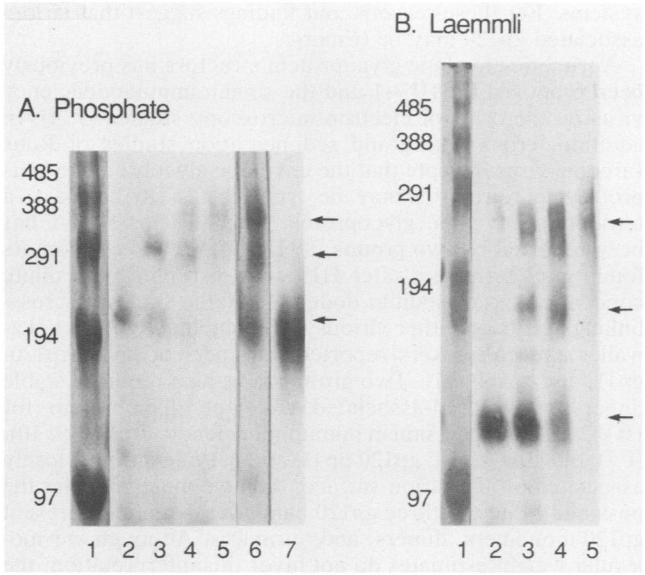

FIG. 1. Cross-linking of HIV-1_{SF33} with DTSSP yields three anti-gp120-reactive bands. Supernatants of HUT-78 cultures infected with the HIV-1_{SF33} strain were filtered through a .45-μm-pore-size filter and centrifuged in a T19 rotor at 17,000 rpm for 2 h. Viral pellets (A and B, lanes 2 to 5) were allowed to suspend overnight in phosphate-buffered saline (PBS) at 4°C to a final concentration of 2 to 5 mg of protein per ml and were stored at −70°C until use or were further purified on a 10 to 60% linear sucrose-PBS gradient in a SW41 rotor run at 40,000 rpm for 16 h (panel A, lane 6). Recombinant gp120 produced in mammalian cells by Chiron was suspended in PBS. Cross-linked or control proteins were solubilized with sodium dodecyl sulfate, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 3.5% acrylamide phosphate (A) or Laemmli (B) gels, and immunoblotted with anti-gp120 rabbit polyclonal antiserum (PB32). Primary antibodies were detected by alkaline phosphatase-conjugated secondary antibodies (Promega). Cross-linked phosphorylase *b* standards were prepared and loaded as described by Sigma and visualized after transfer by staining with Ponceau red (lanes 1). Arrows point to the three gp120 bands. The minor lower-molecular-weight bands are residual fetal calf serum in the samples. (A) (3.5% acrylamide phosphate gel) virus (40 to 50 μg) was mixed with PBS (lanes 2) or treated with 0.03, 0.3, or 3.0 mM DTSSP in PBS (lanes 3 to 5, respectively), incubated at 37°C for 45 min, and quenched with 0.1 M lysine. Samples were then solubilized with 2% sodium dodecyl sulfate, diluted with nonreducing sample buffer, and boiled for 2 min prior to loading. Sucrose gradient-purified virus (lane 6) was treated with 0.3 mM DTSSP. Recombinant gp120 was treated with PBS (lane 7). (B) (3.5% acrylamide Laemmli gel) Control and cross-linked viral pellets treated with PBS (lane 2) or 0.03, 0.3, or 3.0 mM DTSSP in PBS (lanes 3 to 5, respectively), as described above.

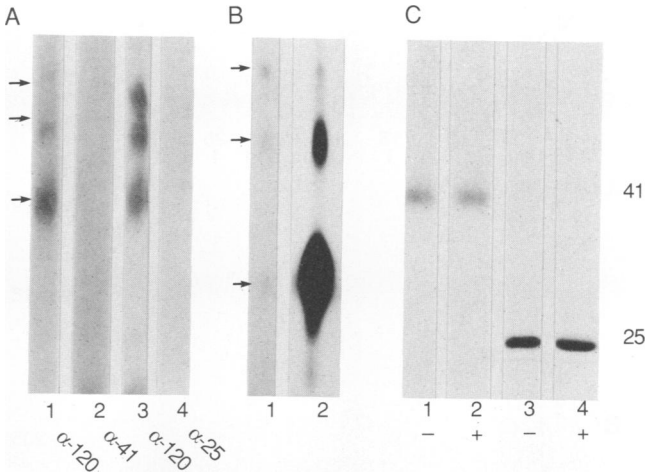

FIG. 2. Cross-linked anti-gp120-reactive bands do not contain other HIV-1 proteins. Virus was cross-linked with 0.3 mM DTSSP (+) (panel A, lanes 1 to 4 and panel C, lanes 2 and 4) or treated with PBS (−) (panel C, lanes 1 and 3). The anti-gp41 mouse monoclonal antiserum (NEA9303, Du Pont) was used against the HIV-1_{IIIB} strain (provided by M. Jennings) because neither NEA9303 nor any other available anti-gp41 antibody recognized the HIV-1_{SF33} strain. The anti-p25 human monoclonal antisera (mixture of 91-5 and 71-13, provided by S. Zolla-Pazner) was used against the HIV-1_{SF33} strain. Viral pellets and recombinant gp120 were cross-linked with 0.3 mM DTSSP, as described in Fig. 1. Transferred proteins were detected by using either alkaline phosphatase-conjugated secondary antibody (A and C) or ^{125}I-labeled protein A secondary antibody (Du Pont) and autoradiography (B). (A) Phosphate gels (3.5% acrylamide). Samples (25 μg [lane 1] and 100 μg [lane 2]) of sucrose-purified HIV-1_{IIIB} cross-linked with 0.3 mM DTSSP were probed with anti-gp120 and anti-gp41 antisera, respectively. Fourfold more virus was used for the anti-gp41 immunoblots to ensure that gp41 would be detected if present. HIV-1_{SF33} (40 to 50 μg) cross-linked with 0.3 mM DTSSP was probed with anti-gp120 (lane 3) and anti-p25 (lane 4) antisera. (B) Laemmli gel, (5% acrylamide). Viral pellet (lane 1) or recombinant gp120 (lane 2) treated with 0.3 mM DTSSP. (C) Laemmli gels. Lanes 1 and 2, 10% acrylamide; lanes 3 and 4, 7% acrylamide. HIV-1_{IIIB} (100 μg; lanes 1 and 2) was probed with anti-gp41 antiserum. HIV-1_{SF33} (40 to 50 μg; lanes 3 and 4) was probed with anti-p25 antiserum. Lanes 1 and 3, control (PBS-treated) virions; lanes 2 and 4, virions treated with 0.3 mM DTSSP.

to a much lesser extent. The two additional bands observed after cross-linking rgp120 comigrated with the second and third gp120 bands observed after cross-linking viral particles (Fig. 2B, lanes 1 and 2). Therefore, in our system, interpretation of the oligomeric state of virion-associated gp120 is simplified by the fact that the DTSSP-cross-linked anti-gp120-reactive bands represent pure gp120 oligomers.

An independent analysis of the oligomeric state of gp120 was performed by using sucrose velocity gradients (Fig. 3). HIV-1_{SF33} virions, either untreated (Fig. 3A) or treated with 3.0 mM DTSSP (Fig. 3B), were disrupted with 40 mM octyl glucoside and run on velocity sedimentation gradients containing 40 mM octyl glucoside. Proteins from odd-numbered fractions were precipitated, reduced, separated on a 5% Laemmli gel, and immunoblotted with anti-gp120 antiserum. Noncross-linked gp120 was detected in fractions 7 to 11, with maximal detection in fraction 9. After cross-linking with a high concentration of DTSSP (3.0 mM), gp120 was detected in fractions 19 to 29, with maximal detection in fraction 21. In contrast, the reverse transcriptase and p25 proteins did not shift their sedimentation positions after cross-linking, consistent with DTSSP being membrane impermeable (Table 1).

S values of the noncross-linked and cross-linked gp120 species were estimated from the positions of four internal S-value markers (arrows in Fig. 3). Averaging the data from three separate experiments, the S value for noncross-linked gp120 was 5.3 ± 0.8 and that of the peak fraction of gp120 cross-linked with a high concentration of DTSSP (3.0 mM) was 15.1 ± 1.2 (Table 1). When a small amount of the sample that was applied to the gradient in Fig. 3B was analyzed on a 3.5% phosphate gel and immunoblotted with the anti-gp120 antiserum, the dominant band seen was the third gp120 (360-kDa) band, although some of the second gp120 (257-kDa) band was also detected (data not shown).

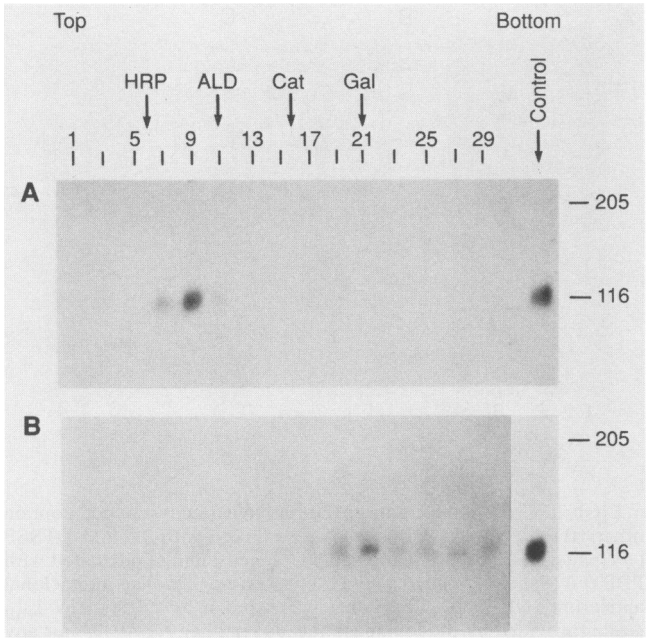

FIG. 3. Sucrose density gradient centrifugation of control and cross-linked HIV-1_{SF33} proteins. Control virus (A) or virus treated with 3.0 mM DTSSP and solubilized with 40 mM octyl glucoside (B) was loaded onto a 10 to 30% linear sucrose-PBS gradient containing 40 mM octyl glucoside and spun in a SW41 rotor at 40,000 rpm for 16 h at 4°C. Thirty fractions were collected, and proteins from odd fractions were precipitated, reduced, separated on 5% acrylamide Laemmli gels, and immunoblotted with anti-gp120 antiserum. Numbering began at the top of the gradient. Primary antibodies were detected by ^{125}I-labeled protein A (Du Pont) and autoradiography. Lane 1, Lightest fraction; lane 29, heaviest fraction; lane 30, control sample of virus. Internal S-value standards (indicated by arrows) were beta-galactosidase (Gal), catalase (Cat), alcohol dehydrogenase (ALD), and horseradish peroxidase (HRP).

Our combined cross-linking and velocity sedimentation analyses clearly show that gp120 is organized as oligomers on the virion surface. After cross-linking gp120 with DTSSP, we consistently saw three strong gp120 bands; no further gp120 bands were detected even in the presence of high concentrations of DTSSP. Molecular size estimates of the highest oligomeric form are less than 400 kDa in all gel systems. For these reasons, our findings suggest that virion-associated gp120 may be trimeric.

TABLE 1. Estimates of S values of HIV-1 proteins before and after cross-linking[a]

Protein	S value	
	Noncross-linked	Cross-linked
gp120	5.3 ± 0.8	15.1 ± 1.2[a]
RT	7.2 ± 0.9	6.7 ± 0.6
p25	3.2 ± 0.2	2.0 ± 1.3

[a] Gradient fractions from Fig. 3 were analyzed for gp120 as described in the text, for reverse transcriptase (RT) by enzymatic assay (5), and for p25 by enzyme-linked immunosorbent assay (Du Pont). S values were determined by reference to internal standards: beta-galactosidase (15.9 S), catalase (11.3 S), alcohol dehydrogenase (7.6 S), and horseradish peroxidase (3.8 S). Standards were detected by enzymatic assay according to instructions of the manufacturer (Sigma), except alcohol dehydrogenase which was visualized with Ponceau red after transfer to nitrocellulose. Linear standard curves were generated in all experiments. Values are means ± standard deviations from three separate experiments.

[b] S value of peak fraction of cross-linked gp120.

A trimeric envelope glycoprotein structure has previously been proposed for HIV-1 and the simian immunodeficiency virus on the basis of electron microscopy studies (3, 4). In addition, cross-linking and sedimentation studies of Rous sarcoma virus indicate that the envelope glycoprotein of this prototypic retrovirus may be trimeric (2). In contrast, a tetrameric envelope glycoprotein structure for HIV-1 has been reported by two groups (8, 11). Pinter and co-workers found gp41 tetramers after HIV was disrupted with dilute concentrations of sodium dodecyl sulfate (8). Using cross-linking agents on either virions or recombinant gp160, Schawaller and co-workers reported tetramers of gp41, gp120-gp41, and gp160 (11). Two groups have also reported stable dimers of the cell-associated envelope glycoprotein for HIV-1, HIV-2, and simian immunodeficiency virus (1, 9, 10). If stable dimers of gp120-gp41 are prevalent and closely associated on the virion surface, then we must consider the possibility that the three gp120 bands we detected represent gp120 monomers, dimers, and tetramers. Although our molecular weight estimates do not favor this interpretation, the lack of suitable high-molecular-weight markers for heavily glycosylated proteins preclude precise molecular weight assignments for the gp120 cross-linked products. Possible factors contributing to the differences between our findings and those of others are the source of envelope glycoproteins (viral, cell associated, or recombinant), the choice of cross-linking agent, and the methods for solubilizing the proteins. We analyzed glycoproteins on intact virions from fresh stocks that underwent few purification procedures. Conceivably, the quaternary structure of virion-associated envelope glycoproteins differs from that of recombinant or cell-associated envelope glycoproteins. Future biophysical analysis and high-resolution structural information are required to definitively resolve whether the HIV-1 envelope glycoprotein is a trimer or tetramer.

Importantly, this is the first report of chemical cross-linking solely between gp120 subunits. Our consistent finding of gp120 homo-oligomers by chemical cross-linking indicates that gp120 subunits are closely associated in the native envelope structure.

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LITERATURE CITED

1. **Earl, P. L., R. W. Doms, and B. Moss.** 1990. Oligomeric structure of the human immunodeficiency virus type 1 envelope glycoprotein. Proc. Natl. Acad. Sci. USA **87**:648–652.
2. **Einfeld, D., and E. Hunter.** 1988. Oligomeric structure of a prototype retrovirus glycoprotein. Proc. Natl. Acad. Sci. USA **85**:8688–8692.
3. **Gelderblom, H. R., M. Ozel, E. H. S. Hausmann, T. Winkel, G. Pauli, and M. A. Koch.** 1988. Fine structure of human immunodeficiency virus (HIV), immunolocalization of structural proteins and virus-cell relation. Micron Microsc. Acta **19**:41–60.
4. **Grief, C., D. J. Hockley, C. E. Fromholc, and P. A. Kitchin.**

1989. The morphology of simian immunodeficiency virus as shown by negative staining electron microscopy. J. Gen. Virol. **70**:2215–2219.
5. **Hoffman, A. D., B. Banapour, and J. A. Levy.** 1985. Characterization of the AIDS-associated retrovirus reverse transcriptase and optimal conditions for its detection in virions. Virology **147**:326–335.
6. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
7. **Morrison, T. G.** 1988. Structure, function, and intracellular processing of paramyxovirus membrane proteins. Virus Res. **10**:113–136.
8. **Pinter, A., W. J. Honnen, S. A. Tilley, C. Bona, H. Zaghouani, M. K. Gorny, and S. Zolla-Pazner.** 1989. Oligomeric structure of gp41, the transmembrane protein of human immunodeficiency virus type 1. J. Virol. **63**:267–279.
9. **Rey, M.-A., B. Krust, A. G. Laurent, L. Montagnier, and A. G. Hovanessian.** 1989. Characterization of human immunodeficiency virus type 2 envelope glycoproteins: dimerization of the glycoprotein precursor during processing. J. Virol. **63**:647–658.
10. **Rey, M.-A., A. G. Laurent, J. McClure, B. Krust, L. Montagnier, and A. G. Hovanessian.** 1990. Transmembrane envelope glycoprotein of human immunodeficiency virus type 2 and simian immunodeficiency virus SIV-mac exist as homodimers. J. Virol. **64**:922–926.
11. **Schawaller, M., G. E. Smith, J. J. Skehel, and D. C. Wiley.** 1989. Studies with crosslinking reagents on the oligomeric structure of the env glycoprotein of HIV. Virology **172**:367–369.
12. **Sechoy, O., J. R. Philippot, and A. Bienvenue.** 1987. F protein-F protein interaction within the Sendai virus identified by native bonding or chemical cross-linking. J. Biol. Chem. **262**:11519–11523.
13. **Stegmann, T., R. W. Doms, and A. Helenius.** 1989. Protein-mediated membrane fusion. Annu. Rev. Biophys. Biophys. Chem. **18**:187–211.
14. **Tateno, M., and J. A. Levy.** 1988. MT-4 plaque formation can distinguish cytopathic subtypes of the human immunodeficiency virus (HIV). Virology **167**:299–301.
15. **White, J. M.** 1990. Viral and cellular membrane fusion proteins. Annu. Rev. Physiol. **52**:675–697.
16. **White, J. M., and I. A. Wilson.** 1987. Anti-peptide antibodies detect steps in a protein conformational change: low pH activation of the influenza virus hemagglutinin. J. Cell Biol. **105**:2887–2896.
17. **Wiley, D. C., and J. J. Skehel.** 1987. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. Annu. Rev. Biochem. **56**:365–394.