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Oligomeric Structure of gp41, the Transmembrane Protein of Human Immunodeficiency Virus Type 1

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We characterized the structural forms of the human immunodeficiency virus *env*-encoded proteins with a panel of monoclonal and polyclonal antibodies. Western blot (immunoblot) assays with antibodies specific for gp41 invariably recognized a major component of 160 kilodaltons and a less intense component of 120 kilodaltons in viral lysates. We demonstrated that these species are noncovalently associated tetramers and trimers of gp41 which represent the native form of this protein in virions. These complexes were stable when boiled in the presence of low concentrations of sodium dodecyl sulfate but were dissociated to gp41 monomers at high sodium dodecyl sulfate concentrations. Moreover, two human monoclonal antibodies preferentially recognized the oligomeric complexes over monomeric gp41 in Western blots, indicating the presence of epitopes recognized by the human immune system on the gp41 multimers which are not efficiently expressed by the dissociated monomers. The demonstration of the existence of multimeric *env* complexes and the enhanced and altered antigenicity of such multimers may be relevant to the design of subunit and recombinant human immunodeficiency virus *env* vaccines.

The human immunodeficiency virus (HIV) *env* gene encodes an intracellular precursor glycoprotein, gPr160, that is normally processed in infected cells by proteolytic cleavage to form gp120, the external viral glycoprotein, and gp41, the viral transmembrane protein (1, 18, 26, 32). gp120 remains associated with virions by virtue of noncovalent interactions between several regions of this protein and gp41 (13); however, this association is weak and most gp120 is released from cells and viruses in soluble form (11, 28). As a consequence, relatively low quantities of gp120 are generally present in purified HIV preparations (23). Significant levels of gPr160 are not present in extracellular HIV (33). This is consistent with the general conclusion that *env* precursor polyproteins are primarily cell-associated products and are normally not incorporated into extracellular virions (22). Seemingly contradictory to these findings is the visualization of heavy bands corresponding to proteins of 120 and 160 kilodaltons (kDa) in Western blots (immunoblots) of purified human T-cell lymphotropic virus type III (HTLV-III) lysates stained with seropositive human sera; these bands are referred to as gp120 and gp160 and are considered to be of major diagnostic importance (7). In this report, we demonstrate that most of the components visualized in the 120- to 160-kDa region do not correspond to gp120 or its precursor but rather represent oligomers of gp41. We describe conditions for converting these oligomers to monomeric gp41, and we demonstrate that such oligomers show preferential reactivity with human monoclonal antibodies to gp41.

MATERIALS AND METHODS

Viruses. Lysates of gradient-purified preparations of the HTLV-III strain of HIV type 1 (HIV-1) were obtained from Organon Teknika or Electro-Nucleonics, Inc. These lysates were supplied in Tris buffer containing 0.5% Nonidet P-40 (NP-40) and 0.5 M NaCl. Lysates of the ARV-2 strain of HIV-1 were prepared by pelleting supernatant medium of infected H9 cell cultures and suspending the pelleted virus directly in buffers containing 0.1% sodium dodecyl sulfate (SDS).

Antibodies. A specific antiserum to gp120 was prepared by immunizing Fisher rats with purified recombinant gp120 obtained from Genentech (16). Anti-peptide antibodies were prepared by immunizing chimpanzees with a keyhole limpet hemocyanin-conjugated synthetic peptide corresponding to residues 735 to 752 of the HTLV-IIIb *env* gene, and specific antibodies were purified by immunoaffinity chromatography on a Sepharose column coupled with bovine serum albumin-peptide conjugates. Human monoclonal antibodies to gp41 and reverse transcriptase were obtained from Epstein-Barr virus-transformed B-cell cultures derived from HIV-infected persons. The cells were generally cloned by limiting dilution (12a); antibody 50-69 was from cells cloned in agar. All three monoclonal antibodies to gp41 are of the immunoglobulin G2 isotype. The rat monoclonal antibodies to viral *gag* products were obtained from hybridomas prepared by fusion of NS-1 cells with splenocytes of Fisher rats immunized with HTLV-IIIb lysates.

Radioimmunoprecipitation and Western blotting assays. For immunoprecipitation assays of HIV-infected cultures, H9 cells were infected with the ARV-2 strain of HIV-1 and after 2 days, glycoproteins in the infected cells were labeled with [^{3}H]glucosamine for an additional 24 h. The cells were then lysed and immunoprecipitated as previously described (24) and analyzed by electrophoresis on 8% polyacrylamide gels (15), followed by fluorography (4). Western blots were performed either with commercial strips obtained from Organon Teknika or with strips prepared in our laboratory. Approximately 1 μg of viral lysate was analyzed per lane. Samples were prepared for gel analysis by dilution in buffer composed of 0.01 M Tris hydrochloride (pH 7.4), 10% glycerol, 0.01% bromophenol blue, 1% dithiothreitol, and the desired concentration of SDS. Similar patterns were

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obtained when dithiothreitol was omitted. Gels were run on a Bio-Rad Mini-Protean II apparatus with a modification of the discontinuous buffer system described by Neville (20); these gels resulted in better resolution of the high-molecular-weight bands and more consistent patterns after blotting than did gels run with the Laemmli system. The upper-reservoir buffer consisted of 0.040 M boric acid, 0.041 M Tris hydrochloride, and 0.10% SDS (pH 8.4); the stacking gel buffer was 0.54 M Tris hydrochloride (pH 6.1); and the resolving gel and lower-chamber buffer was 0.40 M Tris hydrochloride (pH 9.2). In this system, SDS was present only in the upper-reservoir buffer. After separation, the gels were blotted to nitrocellulose (0.2-μm pore size) with a Bio-Rad Laboratories Trans-blot apparatus. The blotting buffer consisted of 0.192 M glycine, 0.025 M Tris, and 20% methanol (pH 8.3), and blotting was performed at 4°C with precooled buffer for 2 h at 50 V for a 0.75-mm-thick gel. The strips were treated with the first antibody diluted in phosphate-buffered saline buffer containing 0.1% Tween 20, reacted with the appropriate alkaline phosphatase-conjugated second antibody diluted in the same buffer, and developed with Nitro Blue Tetrazolium–5-bromo-4-chloro-3-indolyl phosphate as the substrate.

Two-dimensional gel analysis of oligomeric forms of gp41. ARV-2 virions were denatured in the presence of 0.1% SDS and separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE). Individual lanes were cut out, further denatured by heating at 56°C in the presence of 1% SDS for 30 min, and then electrophoresed at 90°C onto a second gel. These gels were then blotted onto nitrocellulose and probed with antisera to viral proteins.

RESULTS

The specificities of antibodies to the HIV *env*-encoded proteins used in this study were characterized by radioimmunoprecipitation of lysates of [^{3}H]glucosamine-labeled H9 cells infected with the ARV-2 strain of HIV (27) (Fig. 1). Human anti-HIV-1 serum obtained from a patient with acquired immunodeficiency syndrome recognized three bands, corresponding to gPr160, gp120, and gp41. Hyperimmune rat antiserum to recombinant gp120 reacted only with gPr160 and gp120, while chimpanzee antibodies to a synthetic peptide of gp41 (corresponding to amino acids 735 to 752 of the HIV *env*-encoded protein sequence; 5) and three human monoclonal antibodies (12a) recognized both the *env* gene precursor, gPr160, and gp41. These results confirmed that the rat serum was specific for epitopes of gp120, while the chimpanzee antibodies and the three monoclonal antibodies were directed against epitopes present on gp41.

When these antibodies were characterized by Western blotting with a commercially prepared kit, an anomalous pattern was observed. The human seropositive serum yielded an intense band corresponding to a component of 160 kDa and a less intense band corresponding to a component of 120 kDa (Fig. 2, lane 1). These two bands were also recognized by the gp41-specific anti-peptide serum (lane 3) and by the three monoclonal anti-gp41 antibodies (lanes 4 to 6). These antibodies also recognized the gp41 band to various degrees; the anti-peptide serum was the most reactive for the monomeric band, and monoclonal antibody 50-69 was the least reactive. The 120-kDa band recognized by the anti-gp41 antibodies was somewhat broader and migrated slightly faster than the bona fide gp120 band recognized by rat anti-gp120 serum (Fig. 2, lane 2). As controls, monoclonal antibodies specific for reverse transcriptase (p66 and p51; Fig. 2, lane 7) and *gag*-encoded components p24 (lane 8) and p17 (lane 9) were also tested; these gave the expected patterns and did not react with any of the *env*-specific bands. These results indicate that the 120- and 160-kDa components did not contain determinants of reverse transcriptase or

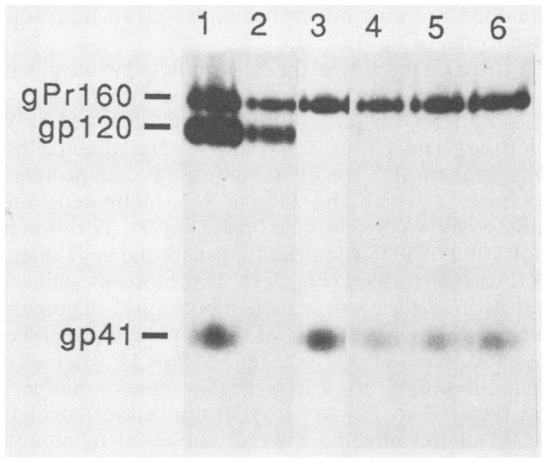

FIG. 1. Specificities of antibodies for intracellular HIV *env*-encoded proteins. SDS-PAGE analysis of cell lysates of [^{3}H]glucosamine-labeled ARV-2-infected H9 cells immunoprecipitated with the following antibodies: lane 1, human serum from a patient with acquired immunodeficiency syndrome; lane 2, rat polyclonal anti-gp120 serum; lane 3, chimpanzee antibodies specific for amino acids 735 to 752 of the HTLV-IIIb *env* gene; lane 4, human monoclonal antibody 50-69; lane 5, human monoclonal antibody 98-6; lane 6, human monoclonal antibody 98-43.

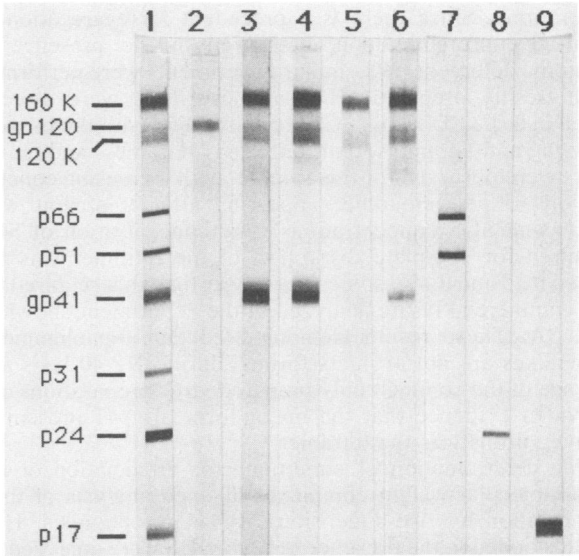

FIG. 2. Western blot analysis of HIV proteins. Commercially prepared test strips containing solubilized HTLV-IIIb lysates (Organon Teknika) were treated by standard protocols with the following antibodies: lane 1, human serum from a patient with acquired immunodeficiency syndrome; lane 2, rat polyclonal anti-gp120 serum; lane 3, chimpanzee anti-gp41 peptide antibodies; lane 4, human monoclonal antibody 98-43; lane 5, human monoclonal antibody 50-69; lane 6, human monoclonal antibody 98-6; lane 7, human monoclonal antibody 52-91, specific for reverse transcriptase; lane 8, rat monoclonal antibody Q-10, specific for p24; lane 9, rat monoclonal antibody B4-F8, specific for p17. K, Kilodaltons.

gag-encoded proteins but rather appeared to represent oligomeric forms of gp41.

To further characterize the high-molecular-weight bands recognized by the anti-gp41 antibodies, HIV lysates were solubilized under various conditions and analyzed by Western blotting. The patterns obtained were affected by the concentration of SDS used to denature the viral proteins, the temperature at which the sample was incubated, and the antibodies used to develop the blots (Fig. 3). Titration of the concentration of SDS required to disrupt the gp41 oligomers at 100°C was performed (Fig. 3A). This blot was stained with the anti-peptide serum to visualize both gp41 oligomers and monomers. In the absence of SDS, only the high-molecular-weight bands were observed (Fig. 3A, lane 1), whereas in the presence of 0.05% to 0.15% SDS, some monomer was formed (lanes 2 to 4). At an SDS concentration of 0.2%, almost all of the oligomers were converted to monomeric gp41 (lane 5), and at 1% SDS, only the monomer and a little of an 80-kDa component, consistent in size with a gp41 dimer (band X), were observed (lane 6). Further titrations were analyzed with monoclonal antibody 50-69, which reacted well with the gp41 oligomers but not with monomeric gp41 (Fig. 3B). Samples prepared in the presence of 0.5% NP-40 and 0.1% SDS retained the oligomeric gp41 bands after incubation at temperatures ranging from 37 to 100°C, while for samples prepared in 1% SDS, the oligomers were stable after incubation at 37°C but disappeared upon treatment at temperatures of 56°C and higher (Fig. 3B). The absence of a significant band at the expected position for monomeric gp41 in this blot confirms the preferential reactivity of this antibody for gp41 oligomers.

The experiments described above were performed with commercially prepared HIV lysates; these samples had been subjected to a protocol to ensure inactivation of viral infectivity, and they contained 0.5% NP-40 and high NaCl concentrations. Since there is a precedent for aggregation of viral glycoproteins upon incubation in the presence of nonionic detergent (29), similar experiments were performed with freshly prepared HIV samples which were directly lysed in 0.1% SDS (Fig. 3C). Upon staining with the broadly reacting anti-peptide serum, only gp41 tetramers and monomers were observed for this sample, with increasing concentrations of the monomer appearing after treatment with increasing SDS concentrations. The concentration of SDS required for complete disruption of the tetramer was between 0.25 and 0.4%, somewhat higher than that required for the commercial lysate analyzed in the experiment shown in Fig. 3A. These results demonstrated that the oligomeric complexes are not artifacts formed during NP-40 lysis and storage of the commercially prepared virus preparations and provided evidence that the major form of gp41 present in native virions was the tetramer.

The determination of conditions for dissociation of the high-molecular-weight complexes allowed analysis of their composition by two-dimensional gel electrophoresis. HIV lysates boiled in the presence of 0.1% SDS were analyzed by electrophoresis in the first dimension. Gel strips were cut out and treated with 1% SDS at 56°C and then electrophoresed into a second gel, which was analyzed by immunoblotting with human antiserum (Fig. 4A) and anti-gp41 peptide serum (Fig. 4B). Under these conditions, most viral proteins fell on a diagonal line, as expected, and products of dissociated complexes fell off the diagonal. gp41 was clearly resolved as the only dissociation product of the 160-kDa band, demonstrating directly that the larger components are homo-oligomeric complexes of gp41.

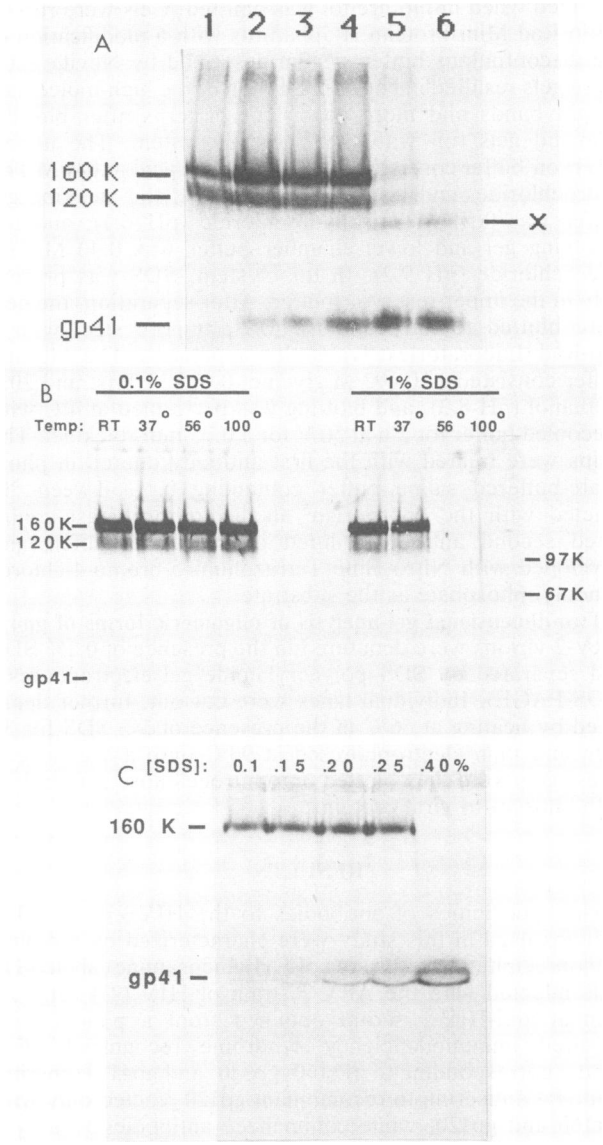

FIG. 3. (A) Effect of SDS concentration on Western blot pattern of gp41. Solubilized HTLV-IIIb lysate was boiled for 1 min with different concentrations of SDS and separated by SDS-PAGE. The gel was blotted to nitrocellulose and probed with chimpanzee anti-gp41 peptide serum. The SDS concentrations used were as follows: lane 1, 0%; lane 2, 0.05%; lane 3, 0.10%; lane 4, 0.15%; lane 5, 0.20%; lane 6, 1.0%. (B) Analysis of the stability of gp41 oligomers. HTLV-IIIb lysates were adjusted to either 0.1 or 1% SDS and incubated for 5 min either at room temperature (RT) or at the indicated temperatures. The samples were then separated by SDS-PAGE, blotted to nitrocellulose, and analyzed by immunostaining with antibody 50-69. Prestained proteins were used as standard molecular weight markers. (C) Analysis of gp41 forms present in freshly prepared HIV lysates. Virus was pelleted from supernatant medium of H9 cells infected with the ARV-2 strain of HIV and solubilized directly in 0.01 M Tris buffer containing 0.1% SDS. Samples were adjusted to the indicated SDS concentrations and, after being boiled for 1 min, analyzed by SDS-PAGE and Western blotting. The immunoblot was stained with chimpanzee anti-gp41 peptide serum. K, Kilodaltons.

As indicated in Fig. 2 and 3, different antibodies to gp41 exhibited differential reactivities to the oligomeric and monomeric forms of gp41. This effect was quantitated by densitometry scans of the strips shown in Fig. 2, which were

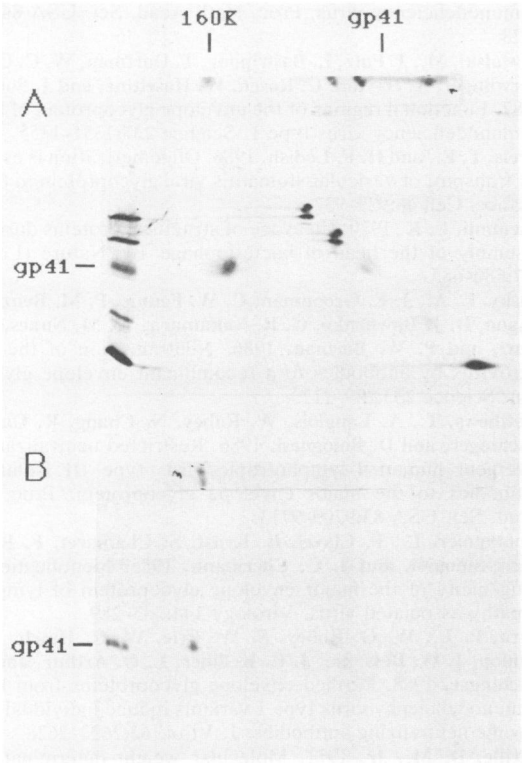

FIG. 4. Two-dimensional gel analysis of oligomeric forms of gp41. Samples of HIV lysates were treated with 0.1% SDS and separated by SDS-PAGE. Strips of the first-dimension gel were then treated with 1% SDS and analyzed on a second gel. After being blotted, the viral proteins were analyzed by immunostaining with either human anti-HIV serum (A) or chimpanzee anti-gp41 peptide serum (B). K, Kilodaltons.

stained with the different antibodies (Table 1). The anti-peptide serum and antibody 98-43 recognized the gp41 oligomers and monomers equally well, while antibody 50-69 and, to a lesser extent, antibody 98-6 reacted preferentially with the larger forms. The fact that human monoclonal antibodies isolated from patients infected with the virus exhibited this selectivity for oligomeric forms of gp41 suggests that the oligomers possess naturally immunogenic epitopes which are poorly expressed on dissociated monomeric gp41.

DISCUSSION

The data presented above provide evidence that gp41 exists in virions as homocomplexes which are extremely stable, surviving boiling in the presence of low concentrations of SDS and 1% dithiothreitol. These complexes do not react with antibodies to any of the other viral proteins tested (Fig. 2), and upon two-dimensional gel analysis of dissociated complexes, they were shown to contain only gp41 (Fig. 4). Furthermore, in radioimmunoprecipitation assays using lysates of HIV-infected cells labeled with ^{3}H-labeled glucosamine (Fig. 1) or HIV lysates labeled with ^{125}I (23), no cellular or serum proteins coprecipitated with gp41 and its precursor. The major oligomer migrated slower on gels than did gp120, while the next most abundant form migrated slightly faster (Fig. 2). On the basis of these results, it appears that the larger complex corresponds to a gp41 tetramer and the smaller complex corresponds to a trimer. Consistent with this assignment was the occasional detection of a minor band corresponding in size to a dimer (Fig. 2 and 3A). In view of the greater stability and yield of the largest component and the fact that it was the only oligomeric form observed in freshly prepared viral lysates (Fig. 3C), we propose that the basic quaternary structure of gp41, and consequently of the native HIV surface structure, is a tetramer.

TABLE 1. Comparison of relative reactivities of different anti-gp41 antibodies for monomeric and tetrameric forms of gp41[a]

Antibody	Ratio of gp41/160-kDa protein peak intensities
Chimpanzee anti-gp41 peptide	1.25
Human monoclonal antibody 98-43	0.93
Human anti-HIV serum	0.86
Human monoclonal antibody 98-6	0.30
Human monoclonal antibody 50-69	0.10

[a] The gel strips analyzed in Fig. 2 were quantitated with a Bio-Rad 620 video densitometer.

There is precedence for the existence of oligomeric forms of the surface glycoproteins of enveloped viruses. Chemical cross-linking studies demonstrated trimeric or tetrameric forms of *env* proteins of murine leukemia virus (21, 30) and murine mammary tumor virus (25), and recently it has been reported that the Rous sarcoma virus *env* proteins exist as oligomers both intracellularly and in virions (10). The influenza virus hemagglutinin protein also forms a trimeric complex which partially survives boiling in SDS (9), and trimerization is required for its transport out of the endoplasmic reticulum (8, 12). The influenza neuraminidase protein has been shown to exist as a tetramer (31), and the glycoprotein of vesicular stomatitis virus is an oligomer, and in this case too, oligomerization has been shown to be essential for transport (14). In view of the demonstrated functions of the glycoprotein oligomers in other virus systems, it is likely that oligomerization of the HIV *env* proteins plays a key role in the transport of these proteins through the cell and in their functional activities in virions.

The preferential reactivities of some human monoclonal antibodies with oligomeric forms of gp41 suggest that such native structures possess immunoreactive epitopes which are less readily expressed on the isolated monomers. Furthermore, in view of the equimolar synthesis and association of gp120 and gp41, the demonstration of a stable tetrameric structure for gp41 argues that the native forms of the HIV *env*-encoded proteins in viral and cellular membranes include complexes composed of four gp41 subunits associated with four gp120 subunits. Support for this is provided by electron microscopy, which demonstrates that the surface structures of HIV are knoblike structures with a diameter of 15 nm and a height of 9 nm (11). This compares with the reported dimensions for the influenza virus hemagglutinin trimer of 14 by 4 nm (6, 34) and the influenza virus neuraminidase tetramer of 10 by 6 nm (31). The molecular mass of the hemagglutinin trimer is 225 kDa, and that of the neuraminidase tetramer is 240 kDa. Thus, the size of the HIV structures, visualized by electron microscopy, is consistent with the hypothesis that these structures are heterocomplexes of gp41 and gp120.

HIV vaccine trials in animals performed with purified gp120 have been disappointing in that, in contrast to the natural immune response to the virus in humans, vaccinated animals produce primarily type-specific responses with lim-

ited neutralizing activity (2, 3, 17, 19). The demonstration in this paper of the oligomeric nature of HIV *env*-encoded proteins and the enhanced antigenicities of such structures suggest that the disruption of native, higher-order structure upon purification of *env* subunits may result in reduced and altered immunogenicity. This raises the possibility that vaccine formulations which retain the native oligomeric structure of *env*-encoded proteins may provide a more effective immune response.

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