Increased Incorporation of Chimeric Human Immunodeficiency Virus Type 1 gp120 Proteins into Pr55<sup>ag</sup> Virus-like Particles by an Epstein–Barr Virus gp220/350-Derived Transmembrane Domain

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Received February 24, 1997; returned to author for revision April 4, 1997; accepted June 9, 1997

Noninfectious Pr55<sup>ag</sup> virus-like particles containing high quantities of oligomeric human immunodeficiency virus type 1 (HIV-1) envelope (Env) proteins represent potential candidate immunogens for a vaccine against HIV-1 infection. Thus, chimeric env genes were constructed encoding the HIV-1 exterior glycoprotein gp120 which was covalently linked at different C-terminal positions to a transmembrane domain (TM) from the Epstein–Barr virus (EBV) major Env glycoprotein gp220/350. All chimeric Env-TM polypeptides as well as the wild-type HIV Env proteins were equally produced and incorporated at the outer surface of insect cells using the baculovirus expression system. In the presence of coexpressed HIV Pr55<sup>ag</sup> polyproteins significantly decreased amounts of wild-type Env proteins were presented at the cell surface, whereas the membrane incorporation of the Env-TM chimeras was not affected. Biochemical and immunoelectron microscopical analysis of particles that were efficiently released from these cells displayed the incorporation of both wild-type Env and chimeric Env-TM proteins on the surface of VLPs. However, the quantities of particle-associated chimeric Env-TM proteins exceeded those of incorporated wild-type Env proteins by a factor of 5–10. Chemical cross-linking and subsequent polyacrylamide gel electrophoresis of VLP-entrapped Env proteins revealed that the chimeric Env-TM proteins form homodimers and a higher-order oligomer, similar to that observed for wild-type Env proteins. Thus, the results of this study clearly demonstrate that the replacement of the gp41 transmembrane domain of HIV Env by an EBV-derived membrane anchor provides an effective strategy to incorporate high quantities of oligomeric HIV gp120 proteins on the surface of Pr55<sup>ag</sup> virus-like particles.

INTRODUCTION

Various polyvalent particulate structures have been previously demonstrated to possess intrinsic adjuvant properties (Jenkins et al., 1991; Layton et al., 1993; Schirmbeck et al., 1994). Consequently, particle-based antigen delivery systems have been established by recombinant DNA technology to stimulate the immune response to selected immunodominant epitopes of various viruses for vaccine purposes. For example, chimeric hepatitis B virus (HBV) core and surface antigen (von Brunn et al., 1991; Schlienger et al., 1992; Michel et al., 1993; Schirmbeck et al., 1994) as well as recombinant papilloma virus L1/L2 (Lin et al., 1993; Breitburd et al., 1995; Kirnbauer et al., 1996) and yeast retrotransposon Ty-p1 virus-like particles (Griffiths et al., 1991; Harris et al., 1992) have been recently shown to elicit both B-cell and T-cell responses to the particulate carrier moiety and the inserted foreign determinants. Also retroviral Gag precursor proteins have been previously shown to include all information required for the formation of particulate structures (Delchambre et al., 1989; Wills and Craven, 1991). The incorporation of foreign antigens into retroviral particles has been originally described using the Gag proteins of Rous sarcoma virus (RSV) and Moloney murine leukemia virus (MLV) as a particulate carrier (Jones et al., 1990; Weldon, Jr., et al., 1990). For HIV, expression of Pr55<sup>ag</sup> by recombinant vaccinia viruses and baculoviruses results in the formation of noninfectious virus-like particles (VLPs) that lack HI-viral RNA (Gheysen et al., 1989; Vernon et al., 1991; Royer et al., 1991). The identification of distinct domains within the HIV-1 Pr55<sup>ag</sup> precursor, which are dispensable for the formation and release of VLPs from eukaryotic cells (Wagner et al., 1991), led to a variety of Gag mutants allowing the incorporation of different epitopes derived from other HIV reading frames such as nef into chimeric VLPs (Wagner et al., 1994). When administered to BALB/c mice, these particles readily induced cytolytic T-cells specifically recognizing Env-derived sequences that were inserted into different positions of the gag reading frame (Griffiths et al., 1993; Wagner et al., 1995; Wagner et al., 1996). However, although such particles elicited a strong antibody response against the Pr55<sup>ag</sup> polyprotein, antibody titers to the presented foreign epi-

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0042-6822/97 $25.00
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topes were comparably low and showed only a weak or no neutralizing activity (Luo et al., 1992; Griffiths et al., 1993; Wagner et al., 1996).

Alternatively, the incorporation of oligomeric HIV Env proteins into Pr55$^{ag}$ VLPs may be a promising strategy in the design of a rational HIV candidate vaccine. The exterior Env protein gp120 represents the major target for neutralizing antibodies, which may play an important role in inducing protection from infection or disease progression (Steimer et al., 1991; Haigwood et al., 1992; Sattentau and Moore, 1995). The assembly of recombinant pseudovirions that contain the Pr55$^{ag}$ structural protein as well as full-length HIV Env glycoproteins was previously reported using the vaccinia virus expression system (Haffar et al., 1990; Vzorov et al., 1991) or stable transfected mammalian cells (Rovinski et al., 1992; Krausslich et al., 1993; Rovinski et al., 1995). Alternatively, the baculovirus expression system provides by far the most efficient system for the overexpression of the Pr55$^{ag}$ polyprotein and its assembly into VLPs (Wagner et al., 1992). However, the expression patterns of the HIV Env glycoproteins in the baculovirus system showed some unusual features in that the gp160 precursor seemed to be inefficiently cleaved and mainly remained cell associated (Rusche et al., 1987; Hu et al., 1987; Wells and Comans, 1990).

For HIV and SIV, determinants within the cytoplasmic domain of the transmembrane protein and the viral matrix protein have been implicated in modulating the cell surface expression of the Env proteins (Owens et al., 1991) and directing their subsequent incorporation into virions (Yu et al., 1992; Gonzalez et al., 1993; Dorfman et al., 1994; Bugelski et al., 1995; Freed et al., 1995; Freed and Martin, 1995). Alterations in the cytoplasmic domain of the SIV and HIV-2 transmembrane protein significantly increased the level of Env proteins on the surface of viruses (Mulligan et al., 1992; Zingler and Littman, 1993; LaBranche et al., 1995), whereas various deletions in the C-terminal region of the cytoplasmic region of HIV-1 gp41 were demonstrated to diminish the incorporation of Env proteins into HIV virions (Dubay et al., 1992; Yu et al., 1993). However, determinants that positively or negatively affect the incorporation of Env proteins into Pr55$^{ag}$ VLPs appear to depend largely on the cell culture system and have not been identified so far.

Previous reports by others and us indicated that herpesvirus-derived glycoproteins were efficiently incorporated at the outer surface of Pr55$^{ag}$ virus-like particles by using the baculovirus expression system (Osterrieder et al., 1995; Garnier et al., 1995). Based on this observation, we investigated the possibility of increasing the packaging of HIV Env proteins on the outer surface of Pr55$^{ag}$ VLPs by replacing gp41 of the gp160 precursor protein with a heterologous membrane anchor derived from the EBV major glycoprotein gp220/350. Our results clearly indicate a significantly enhanced incorporation of Env-TM chimeras on the outer surface of Pr55$^{ag}$ VLPs when compared to wild-type Env proteins. Moreover, results obtained with chemical cross-linking analysis suggest an oligomeric structure of particle-entrapped gp120 derivatives. Therefore the presented system is well suited for studying the protein incorporation into retroviral VLPs. In addition, these noninfectious enveloped Pr55$^{ag}$ VLPs provide an attractive approach for the development of a candidate vaccine against HIV infection.

**MATERIALS AND METHODS**

**Media, cells, and viruses**

Spodoptera frugiperda Sf9 cells were maintained in TC100 medium supplemented with 10% FCS (Gibco-BRL, Grand Island, NE). Wild-type Autographa californica nuclear polyhedrosis viruses (AcNPVs) and recombinant baculoviruses were propagated on Sf9 insect cells as described previously (Smith et al., 1985). Titers of baculovirus stocks were determined by plaque assay (O’Reily et al., 1992). For large scale preparation of recombinant antigens from serum-free cell culture supernatants (SF900; Gibco), HighFive cells derived from Trichoplusia ni egg cell homogenates (Invitrogen Inc., San Diego, CA) were used for the infection with the recombinant baculoviruses.

**HIV-Sequence**

All amino acid positions given in the text refer to the HIV-1$_{LAI}$ sequence.

**Monoclonal antibodies**

A HIV gp120-specific murine monoclonal antibody (mab NEA 9305) recognizing a central motive of the V3-loop region (RIQRPGGAVTIGK) and a gp41-specific murine monoclonal antibody (mab NEA 9303) were purchased from DuPont Canada, Inc. (Markham, Ontario). The gp120-specific murine mab 133/237 recognizing amino acids 64 to 73 and mab 133/192 recognizing a discontinuous epitope within gp120 (Niedrig et al., 1992) as well as a human CD4-IgG construct (CD4-2D-H23; Langner et al., 1993) were generously provided by Dr. Matthias Niedrig (Robert Koch Institute, Berlin). A Pr55$^{ag}$ specific monoclonal antibody (mab 16/4/2) has been previously mapped to amino acids 307–336 within the p24(CA) moiety of the Pr55$^{ag}$ polyprotein (Wolf et al., 1990). Mab 64/D7 reacts with the external portion of the EBV major glycoprotein gp220/350 (unpublished observation).

**Construction of plasmids**

A baculovirus transfer vector encoding the HIV-1 gag gene (pVLPr55) has been constructed as described previously (Wagner et al., 1992). Subcloning of the coding sequence of the HIV-1 gp160 Env protein and chimeric env genes including parts of the external glycoprotein gp120 was achieved in a pUC18 derivative named pUC-
EH. pUC-EH was created by replacing the original polylinker of pUC18 by a 5'-EcoRI–BclI–MroI–XbaI–PstI–HindIII-3' multiple cloning site. For this purpose, two synthetic oligonucleotides a (5'-AAT TCT GAT CAT CCG GAT CTA GAC TGC AGA-3') and b (5'-AGC TTC TGC AGT CTA GAT CCG GAT CAG C-3') were annealed and inserted into the EcoRI–HindIII-linearized vector fragment of pUC18. DNA-fragments encoding the complete gp160, the full-length gp120, or carboxy (C)-terminally by 5 or 20 amino acids truncated variants of gp120 were obtained from plasmid pH102 by polymerase chain reaction (PCR). Primers c (5'-ATA TTA GAA TTC ATG AGA GTG AAG GAG AAA TAT CAG C-3'), including a 5'-EcoRI restriction site (italics) as well as the ATG start codon (bold-faced), and d (5'-ATA TTA CGT CAG TTA TAG CAA AAT CCT TTC C-3'), containing a 5'-PstI site (italics), were used to amplify the gp160 open reading frame. The amplified DNA product was digested with EcoRI/PstI and inserted into the EcoRI/PstI vector fragment of pUC-EH, resulting in pUCgp160. PCR products encoding gp120 and the C-terminally truncated gp120 derivatives were obtained by using the 5'-primer c together with the 3'-primer e (5'-ATA TTA TCC GGA CAC CAC CCT CTT CTT CTA TCC), primers c and f (5'-ATA TTA TCC GGA GGA CCT TGC CTT GGT GGC TAC TCC-3'), and primers c and g (5'-ATA TTA TCC GGA TTC ATT ATA TAA TTC ACT TCT CC-3'). The primers e, f, and g include a MroI restriction site indicated in italics, respectively. The amplified DNA fragments were digested with EcoRI/MroI and inserted into the EcoRI/MroI sites of pUC-EH vector. The resulting plasmids were designed as pUCgp120, pUCgp120-5, and pUCgp120-20. In order to allow stable anchoring of gp120 and its derivatives on cellular membranes, the transmembrane region of the Epstein–Barr virus (EBV) glycoprotein gp220/350 was linked to the C-terminus of the gp120 variants. For this purpose, a gene fragment encoding the 22 amino acid spanning transmembrane domain (TD) and the short cytoplasmic region (CR) (27 amino acids) of the gp220/350 gene was amplified by PCR from the plasmid pBRBamHI-L (Skare and Strominger, 1980) using primers h (5'-ATA TTA TCC GGA AGC GGG GGA CCA GGA TTC ATT CTA GTA CTT CAA TGG GCC TCT CTG-3') containing a spacer that encodes a flexible stretch of 6 amino acids (S-S-G-S-G-A-G-; bold-faced) as well as a MroI site (italics) and primer i (5'-ATA TTA TCT CAG TTA TAC ATA CTT CTC GCC TTC-3'), containing a stop codon (bold-faced) and a PstI restriction site (italics). After digestion with MroI/PstI, the amplified PCR fragment was inserted into the chimeric gp120 constructs between the MroI and PstI sites of the plasmids pUCgp120, pUCgp120-5, and pUCgp120-20. The resulting vectors were termed pUCgp120-TM, pUCgp120-5TM, and pUCgp120-20TM, respectively. To establish recombinant baculoviruses the pUC-derived Env constructs were subcloned into the EcorI/PstI-digested pVL1393 baculo transfer vector. The resulting plasmids (pVLgp120, pVLgp120-TM, pVLgp120-5TM, and pVLgp120-20TM) encode the full-length gp160 as well as the HIV-1 exterior Env glycoprotein gp120 fused at different C-terminal positions in gp120 to a type I transmembrane region from the EBV Env protein gp220/350 (Fig. 1). All subcloned DNA-sequences including the flanking regions were verified by double-strand DNA sequencing.

Generation of recombinant baculoviruses

The Autographa californica nuclear polyhedrosis virus (AcNPV) recombinants used in this study were AcPr55 (Wagner et al., 1992), Acgp160, Acgp120-TM, Acgp120-5TM, and Acgp120-20TM. Recombinant baculoviruses were established in Sf9 insect cells by cotransfection of infectious Autographa californica nuclear polyhedrosis viral DNA and pVL baculovirus transfer vector DNA containing the foreign gene (Smith et al., 1985). Polyhedrin-negative plaques were isolated following several rounds of plaque purification, and high-titer stocks of recombinant baculoviruses were prepared as described previously (Wagner et al., 1992).

Detection and quantification of chimeric proteins

Recombinant proteins were expressed in Sf9 or High-Five insect cells after (co)infection with AcNPV recombinants at a multiplicity of infection (m.o.i.) of 10 for each virus. Synthesis of the recombinant proteins was demonstrated at Day 3 p.i. by separating the cell lysates on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels followed by conventional Western blot analysis (Sambrook et al., 1989). Recombinant proteins were specifically visualized using the indicated monoclonal antibodies. The concentration of Pr55<sup>Ag</sup> proteins was determined using a commercial p24 capture assay (Abbott Diagnostics, Wiesbaden, Germany). The quantities of particle entrapped Env proteins were calculated by comparative Western immunoblotting of normalized particle preparations and serial twofold dilutions (0.2–3.2 μg) of highly purified rgp120 protein (Genentec Inc., San Francisco, CA). Additionally, a commercial gp120 capture ELISA kit (Intracel Corp., Cambridge, MA) was used to verify the quantities of particle-associated Env proteins.

APAAP immunostaining and FACSscan analysis

Cell surface expression of the chimeric Env proteins was shown at Day 3 after (co)infection of Sf9 cells with the recombinant AcNPVs. After extensive washing in PBS (10 mM phosphate buffer, pH 8.0, containing 0.15 M NaCl), cells were fixed with 2% glutaraldehyde for 10 min. Following three more washes in PBS, the fixed cells were incubated with different dilutions of the indicated Env-specific monoclonal antibodies or a human CD4-IgG construct for 1.5 hr at 37°. Unbound antibodies were removed by three washing steps using PBS. Antibodies, which specifically bound to gp120 exposed on the cell surface, were detected by an alkaline phosphatase anti-alkaline

AID VY 8669 / 6a3e$$$$561 07-30-97 14:49:39 viral AP: VY
phosphatase (APAAP) staining procedure as described in the manufacturers protocol (Dako Diagnostika, Hamburg, Germany). Surface expression of Env glycoproteins on infected cells was further determined by flow cytometry using a FACScan analyzer (Becton–Dickinson, San Jose, CA). For this purpose, 2 × 10⁵ Sf9 cells were harvested at Day 2 after (co)infection with the indicated AcNPV recombinants and resuspended in 100 μl of a 1:500 dilution of the anti-gp120 mouse monoclonal antibodies 133/237 or 133/192 dissolved in PBS supplemented with 10% FCS and 10 mM Na3N. After 30 min incubation at 37°C, cells were washed in PBS and incubated for another 30 min in a solution of 100 μl fluorescein isothiocyanate (FITC)-conjugated rabbit-anti-mouse F(ab’2)-fragment (20 μg/ml; Dako). After three more washes in PBS, cells were fixed for 1 hr in 70% methanol, sedimented (500 g), and resuspended in PBS. The cellular RNA was degraded by incubation with RNase A (1 mg/ml; Boehringer Mannheim, Mannheim, Germany) for 30 min at 37°C. Propidium iodide was added to a final concentration of 1 μg/ml to determine the DNA content. Cells were analyzed with a FACScan flow cytometer (Becton–Dickinson) using Cell-fit software (Becton–Dickinson) in a dual parameter fluorescence analysis of antibody and DNA. A pulse processor used on the red fluorescence signal allowed to exclude cell debris as well as dead and aggregated cells from the analysis.

Purification and biochemical characterization of VLPs

Serum-free supernatants of HighFive cells expressing Pr55gal in addition to either gp160 or one of the Env-TM proteins were collected 3–5 days p.i. and clarified by centrifugation at 2,000 g for 10 min at 4°C. Concentration of virus-like particles (VLPs) from precleared culture supernatant was achieved by Centrisate I tubes (Satorius to suspend overnight in PBS, pH 8.0, at 4°C. Fractions at 4°C, and resuspended in PBS to a final concentration of 0.6 ml were collected and divided each into two aliquots. Aliquot 1 was diluted with PBS to a total volume of 1 ml and VLPs were pelleted at 16,000 rpm for 40 min at 4°C in an Eppendorf centrifuge. Antigen preparations were resuspended in sample buffer (Laemmli, 1970) and separated by electrophoresis on a SDS–10% polyacrylamide gel, followed by Western immunoblot as described previously (Wagner et al., 1994).

Immunoprecipitation of VLPs from p24(CA) peak fractions (fractions 12–14) obtained after sucrose sedimentation analysis was performed with 2 to 10 μl of a CD4-IgG chimeric antibody according to a protocol described by Sambrook et al. (1989), except that no detergent was used in the buffers. Immunoprecipitates were diluted in sample reducing buffer, separated by electrophoresis on SDS–10% polyacrylamide gels, and analyzed by immunoblotting using monoclonal antibodies directed either to the V3 domain within gp120 (mab NEA 9305) or the p24(CA) moiety within Pr55gal (mab 16/4/2).

Electron microscopy

Sf9 cells (10⁶) were harvested 3 days after (co)infection with the indicated AcNPV recombinants (m.o.i. = 10 for each virus). Specimens for ultrathin sectioning were prepared as described previously (Wagner et al., 1994). Sections of 25- to 75-nm thickness were cut with a diamond knife and mounted on uncoated copper grids. The sections were poststained with 100 mmol lead (II) citrate, pH 12.6. All pictures were taken with Siemens Elmiskop 101 electron microscope. The magnification was calibrated with a cross line grating replica. Immunelectron microscopy of VLPs was performed essentially as described by Osterrieder et al. (1995). Purified VLPs were adsorbed to grids after fixation with 2% glutaraldehyde. Grids were washed in TBS, blocked with 3% gelatin in TBS for 1 hr at RT, and incubated with the V3-specific mab NEA 9305 in TBS for 1 hr at RT. After three washes in TBS, grids were floated on an anti-mouse IgG immunogold conjugate (particle size: 5 nm; Sigma, Deisenhofen, Germany) for 1 hr at RT. After three washes in TBS grids were contrasted with phosphoric tungstic acid and examined in an electron microscope (Zeiss EM 10C/CR).

Chemical cross-linking

 Supernatants of HighFive cells coinfect with Pr55gal and Env-TM recombinant baculoviruses were clarified by centrifugation at 2,000 g for 10 min at 4°C and then centrifuged through a 30% sucrose cushion at 100,000 g for 2.5 hr at 4°C. Pelleted VLP preparations were allowed to suspend overnight in PBS, pH 8.0, at 4°C. Aliquots (2 ml) of the concentrated material were layered onto a 10-ml 10–60% sucrose gradient in PBS and centrifuged at 100,000 g for 16 hr at 4°C. Antigens sedimenting at a sucrose density of 1.15–1.19 g/cm³ were collected, diluted in 3 vol of PBS, repelleted at 100,000 g for 2.5 hr at 4°C, and resuspended in PBS to a final concentration of 1–5 mg protein per milliliter. Highly purified recombinant gp160 produced in Chinese hamster ovary cells by Immuno (Vienna, Austria) was suspended in PBS and served as a control antigen. Probes were treated with 0.3 or 1.5 mM bis(sulfosuccinimidyl)suberate (BS²) (Pierce Chemical Co., Rockford, IL), incubated at 37°C for 30 min, and quenched for 15 min with 50 mM Tris, pH 7.5. Non BS³-treated antigens served as controls. The probes were solubilized by the addition of a solution containing 4% SDS, 100 mM Tris, pH 6.8, 10% glycerin, 10% 2-mercaptoethanol, 0.01% bromphenol blue and boiling for 10 min. Each sample was then subjected to electrophoresis on SDS–5% polyacrylamide gels under reducing buffer conditions. Proteins were transferred to nitrocellulose, immunoblotted with antibodies directed against gp120.
FIG. 1. Schematic representation of chimeric Env-TM glycoprotein mutants. The wild-type HIV-1 glycoprotein precursor gp160 is diagrammed at the top of the figure. The gp41 transmembrane domain of gp160 was replaced by a heterologous Epstein–Barr virus (EBV)-derived type I transmembrane region (TM), consisting of a 22 amino acid spanning transmembrane domain (TD) and a short cytoplasmic region (CR) (27 amino acids), which was covalently linked to the C-terminus of gp120 by a flexible-S-G-S-A-G-hinge region (gp120-TM). Additionally derivatives of the gp120-TM construct were generated lacking 5 (gp120-5TM) or 20 C-terminal amino acids (gp120-20TM) of the gp120 moiety. The gp120-TM protein as well as the gp120-5TM and gp120-20TM polypeptides were designated as Env-TM. Abbreviations of recombinant baculoviruses expressing the different chimeric genes are given on the right, the number of amino acids encoded by the chimeric genes are indicated on the left side of the figure. A diagram of the particle forming Pr55<sup>agg</sup> polyprotein is shown below.

FIG. 2. Analysis of Sf9 cell lysates 3 days after infection with different recombinant baculoviruses at a m.o.i. of 10. Cells were harvested 3 days postinfection (p.i.) and analyzed by immunoblotting. Env proteins were visualized by using the gp120/V3-specific monoclonal antibody NEA 9305. Predominant protein bands with apparent molecular weights consistent with those of the wild-type Env proteins gp160 and gp120 as well as the chimeric Env-TM polyproteins (137 kDa for gp120-TM, 135 kDa for gp120-5TM, and 132 kDa for gp120-20TM) were visualized in lysates of insect cells infected with the corresponding Env recombinant AcNPVs (Fig. 2A). The de...

RESULTS

Expression of chimeric Env-TM glycoproteins in insect cells

A set of recombinant baculoviruses was constructed to study the incorporation of genetically engineered Env-TM glycoproteins into HIV Pr55<sup>agg</sup> virus-like particles (VLPs). The chimeric Env glycoprotein gp120-TM was established by replacing the gp41 transmembrane protein of the HIV gp160 Env-precursor with a heterologous type I transmembrane region (TM) of the Epstein–Barr virus (EBV) major glycoprotein gp220/350 (Fig. 1). A flexible hinge region consisting of six amino acids (-S-G-S-G-A-G-) was introduced between gp120 and the heterologous membrane anchor to support correct and independent folding of both domains. Derivatives of gp120-TM have 5 (gp120-5TM) or 20 amino acids (gp120-20TM) deleted from the C-terminus of the gp120 moiety to avoid unspecific processing at cryptic cleavage sites located within the C-terminus of the exterior HIV Env-protein (Morikawa et al., 1990, 1993). To investigate and compare the synthesis of both HIV wild-type Env polypeptides and chimeric Env-TM proteins, Sf9 insect cells were infected with various Env recombinant baculoviruses at a m.o.i. of 10. Cells were harvested 3 days postinfection (p.i.) and analyzed by immunoblotting. Env proteins were visualized by using the gp120/V3-specific monoclonal antibody NEA 9305. Predominant protein bands with apparent molecular weights consistent with those of the wild-type Env proteins gp160 and gp120 as well as the chimeric Env-TM polyproteins (137 kDa for gp120-TM, 135 kDa for gp120-5TM, and 132 kDa for gp120-20TM) were visualized in lysates of insect cells infected with the corresponding Env recombinant AcNPVs (Fig. 2A). The de...
tected quantities of chimeric Env-TM polypeptides were comparable to that of the wild-type HIV Env-polypeptides. Proteins migrating at a lower molecular weight are due to unspecified degradation of the recombinant proteins by cellular proteases. Identical results were obtained when the Env-specific mab 133/237 binding amino acids 64–73 and mab 133/192, recognizing a discontinuous epitope within gp120, were used for immunological detection of the Env glycoproteins (data not shown). Thus, C-terminal truncations of gp120 by either 5 or 20 amino acids and fusion of the gp120 derivatives to a heterologous EBV-derived membrane anchor neither resulted in a reduced expression nor in a decreased stability of these polypeptides when compared to wild-type HIV Env proteins. Lysates of uninfected insect cells or cells which were infected by wild-type AcNPVs (AcWt) or Pr55gag recombinant baculoviruses (AcPr55) (Wagner et al., 1992) were negative for Env-TM or wild-type HIV Env proteins. A monoclonal antibody recognizing the transmembrane protein gp41 specifically detected both, and lentiviral Env proteins (data not shown). In contrast uninfected cells or cells which were infected by wild-type AcNPVs expressing the Pr55gag polyprotein and one of the Env protein recombinant AcNPV. For control, cells remained uninfected or were coinfected with AcWt and AcPr55. Simultaneous expression of Pr55gag and gp160 or one of the chimeric Env-TM polypeptides in infected insect cells was confirmed by immunofluorescence using monoclonal antibodies to the p24(CA) moiety of the Pr55gag precursor (mab 16/4/2) and gp120 (mab NEA 9305) (data not shown). When analyzed by immunoblotting, lysates of Sf9 cells harvested 3 days after coinfection with the indicated AcNPV recombinants exhibited no differences in the overall amount of the Pr55gag precursor (Fig. 4A). More importantly, similar amounts of Env glycoproteins were visualized in all tested cell lysates by Western immunoblotting irrespective of whether gp160 or one of the chimeric Env-TM proteins was coexpressed with Pr55gag (Fig. 4B). As assessed by a commercial p24 sandwich assay, coexpression of Pr55gag with one of the Env polypeptides led to a 40–60% reduction in the Pr55gag synthesis when compared to cells infected with AcPr55, only. Similarly, the overall quantities of synthesized Env-TM chimeras and wild-type HIV Env proteins were reduced by a factor of 1.5 to 2. This indicates that the capacity of the coinjected cells to produce recombinant proteins from the polyhedrin promoter is limited and almost equally distributed to each of the expression units. However, analyzing the influence of coexpressed Pr55gag on the cell surface presentation of the Env proteins, flow cytometric analysis demonstrated the amounts of presented wild-type Env to be significantly reduced by a factor of two to three when compared to the presented modified Env-TM proteins (Fig. 5). This observation indicates that the incorporation of wild-type Env proteins into the membrane of baculovirus-infected insect cells may be limited by a specific interaction of the transmembrane protein gp41 and domains within the Pr55gag polypeptide. In contrary, apart from the fact that coexpression of Pr55gag and Env proteins reduces the amounts of cell-associated Env-TM by a factor of 1.5 to 2, the incorporation of the chimeric

Influence of coexpressed Pr55gag on the synthesis and surface expression of chimeric Env-TM polypeptides

To determine whether the incorporation of either wild-type Env proteins or the Env-TM chimeras into the cell membrane is affected by the coexpression the particle forming Pr55gag precursor, Sf9 cells were simultaneously infected with AcNPVs expressing the Pr55gag polypeptide and one of the Env protein recombinant AcNPV. For control, cells remained uninfected or were coinfected with AcWt and AcPr55. Simultaneous expression of Pr55gag and gp160 or one of the chimeric Env-TM polypeptides in infected insect cells was confirmed by immunofluorescence using monoclonal antibodies to the p24(CA) moiety of the Pr55gag precursor (mab 16/4/2) and gp120 (mab NEA 9305) (data not shown). When analyzed by immunoblotting, lysates of Sf9 cells harvested 3 days after coinfection with the indicated AcNPV recombinants exhibited no differences in the overall amount of the Pr55gag precursor (Fig. 4A). More importantly, similar amounts of Env glycoproteins were visualized in all tested cell lysates by Western immunoblotting irrespective of whether gp160 or one of the chimeric Env-TM proteins was coexpressed with Pr55gag (Fig. 4B). As assessed by a commercial p24 sandwich assay, coexpression of Pr55gag with one of the Env polypeptides led to a 40–60% reduction in the Pr55gag synthesis when compared to cells infected with AcPr55, only. Similarly, the overall quantities of synthesized Env-TM chimeras and wild-type HIV Env proteins were reduced by a factor of 1.5 to 2. This indicates that the capacity of the coinjected cells to produce recombinant proteins from the polyhedrin promoter is limited and almost equally distributed to each of the expression units. However, analyzing the influence of coexpressed Pr55gag on the cell surface presentation of the Env proteins, flow cytometric analysis demonstrated the amounts of presented wild-type Env to be significantly reduced by a factor of two to three when compared to the presented modified Env-TM proteins (Fig. 5). This observation indicates that the incorporation of wild-type Env proteins into the membrane of baculovirus-infected insect cells may be limited by a specific interaction of the transmembrane protein gp41 and domains within the Pr55gag polypeptide. In contrary, apart from the fact that coexpression of Pr55gag and Env proteins reduces the amounts of cell-associated Env-TM by a factor of 1.5 to 2, the incorporation of the chimeric

Cell surface expression of chimeric Env-TM polypeptides

The cell surface expression of wild-type HIV Env proteins and the Env-TM chimeras was assessed by APAAP immunostaining and flow cytometric analysis. For that purpose Sf9 insect cells were either nontreated, infected with wild-type baculoviruses or with AcNPVs expressing gp160 or the gp120-TM chimeras, and then harvested early at Day 2 p.i. to avoid virus-mediated cytopathic effects. APAAP immunostaining was performed with the gp120-specific monoclonal antibodies (mabs) 133/192, 133/237, NEA 9305, as well as a chimeric human CD4-IgG construct. In all cases, a clear gp160- and gp120-specific staining of the cell membrane was noticed (data not shown). To compare the cell surface expression of wild-type Env and the chimeric Env-TM glycoproteins under more quantitative aspects, FACS analysis was performed with infected Sf9 cells by using the gp120-specific mab 133/237. All variants of the chimeric gp120-TM glycoprotein as well as wild-type Env polypeptides were detected at the cell surface to a comparable extent (Fig. 3). Similar results were obtained with the gp120-specific mab 133/192 (binding to a discontinuous epitope within gp120) for immunological detection of the various cell surface-exposed Env glycoproteins (data not shown). In contrast uninfected cells and cells that were infected with wild-type baculoviruses or with AcNPVs expressing the Pr55gag polypeptide were not recognized by the Env-specific antibodies. These data suggest that the targeting of the external glycoprotein gp120 to the surface of Sf9 insect cells was not affected by the substitution of the autologous transmembrane protein gp41 with the heterologous, EBV gp220/350-derived transmembrane domain.
FIG. 3. One-dimensional histograms representing flow cytometric measurements of different Env proteins expressed on the surface of Sf9 insect cells. Fluorescence intensities are indicated in log scale. Sf9 cells infected with baculoviruses expressing (C) Pr55 (D, E) gp160, or (F – H) the indicated hybrid Env-TM proteins at a m.o.i. of 5 per cell for 48 hr were stained for FACScan analysis with (A – C, E – H) a gp120-specific monoclonal antibody (mab 133/237) and then labeled with FITC-conjugated anti-mouse IgG antibody. (A) Noninfected Sf9 insect cells or (B) cells infected with AcWt were used as negative controls. (D) The background of fluorescence was determined by treating Acgp160-infected Sf9 cells with an irrelevant monoclonal antibody against the EBV-glycoprotein gp220/350 (mab 64D7) and consecutive incubation of the cells with a FITC-conjugated anti-mouse IgG antibody. (I) Comparative overlapping of histograms of noninfected Sf9 insect cells (black histogram) and cells infected with Acgp120-TM (red histogram) or Acgp160 (blue histogram). The data represent the mean of three experiments, with standard deviations being equivalent to 5 to 10% of the mean.

Incorporation of chimeric Env-TM glycoproteins into budding virus-like particles

In order to assess the incorporation of wild-type Env proteins and chimeric Env-TM polypeptides into budding virus-like particles, HighFive insect cells were either infected with AcPr55 alone or coinfectected with recombinant baculoviruses expressing the Pr55 polyprotein (AcPr55) and AcWt or one of the AcNPV recombinants Acgp160, Acgp120-TM, Acgp120-5TM, or Acgp120-20TM, respectively. Seventy-two hours postinfection cell culture supernatants and infected cells were separated and analyzed either by sucrose sedimentation analysis or ultrathin sectioning electron microscopy. Morphological analysis of coinfectected cells revealed efficient budding of re-
HIV Pr55\textsuperscript{gag} VLPs PRESENTING CHIMERIC ENVELOPE PROTEINS

**FIG. 5.** One-dimensional histograms representing flow cytometric measurements of Env proteins expressed on the outer surface Sf9 insect cells. Fluorescence intensities are indicated in log scale. Sf9 cells coinfected with baculoviruses expressing the Pr55\textsuperscript{gag} polyprotein and (B) gp160 or (C-E) the indicated chimeric Env-TM proteins (m.o.i. = 5 for AcPr55 and m.o.i. = 10 for each Env-recombinant AcNPV) for 48 hr were stained for flow cytometric analysis with (A-F) the gp120-specific monoclonal antibody 133/237 and then labeled with FITC-conjugated anti-mouse IgG antibody (DAKO). (A) Sf9 insect cells coinfected with AcWt and AcPr55 were used as negative controls. Cell populations analyzed were >95% infected with AcPr55, as determined by Pr55 gag expression on permeabilized cells by using fluorescence microscopy. (F) Comparative overlapping of histograms of Sf9 insect cells coinfected with AcPr55 and AcWt (black histogram), Acgp120-TM (red histogram), or Acgp160 (blue histogram). The data represent the mean of three experiments, with standard deviations being equivalent to 5 to 10% of the mean.

**FIG. 6.** Influence of the Pr55\textsuperscript{gag} polyprotein on the surface expression of gp160 and chimeric Env-TM polypeptides on Sf9 insect cells. Comparative overlapping of histograms of Sf9 insect cells infected with (A) AcWt, (B) Acgp160, and (C) Acgp120-TM (black histograms), or coinfected with AcPr55 and (A) AcWt, (B) Acgp160, and (C) Acgp120-TM (red histograms). Cells were stained with the gp120-specific mab 133/237 and labeled with FITC-conjugated anti-mouse IgG antibody. The data represent the mean of three experiments, with standard deviations being equivalent to 5 to 10% of the mean.

combinant VLPs, which resembled immature HIV-virions and Pr55\textsuperscript{gag} VLPs (Gheysen et al., 1989; Wagner et al., 1994). The sedimentation of cell culture supernatant-derived particulate antigens was followed in 600-µl fractions of 10 to 60% sucrose gradients using a commercial sandwich assay (Abbott). Pr55\textsuperscript{gag} was identified at a density of 1.13–1.19 g/cm\textsuperscript{3}, with a peak at 1.16 g/cm\textsuperscript{3} for all coinfections tested (Fig. 7A), which is consistent with the density reported for immature HIV-virions and recombinant Pr55\textsuperscript{gag} based VLPs. In all anti-p24(CA)-reactive fractions, the presence of VLPs was demonstrated by electron microscopical analysis after negative staining.
However, only fractions 12–14 were free of contaminating baculoviruses. Analysis of the collected fractions by immunoblotting with both an anti-p24(CA) mab (16/4/2) and a gp120-V3-specific monoclonal antibody (NEA 9305) revealed coincidence of the Pr55*Gag precursor and the Env proteins in sucrose fractions 10–14.

The amounts of particle-entrapped Env proteins were calculated by Western immunoblotting. For that purpose, VLPs were pelleted from pooled p24(CA) peak fractions 12–14 (1.15–1.19 g/cm³). Aliquots of resuspended VLP preparations were separated by SDS-PAGE, immunoblotted, and visualized with anti-Pr55*Gag mab 16/4/2 and a gp120/V3-specific monoclonal antibody (Figs. 7B and C). Antibody 16/4/2 detected comparable amounts of the unprocessed Pr55*Gag protein in all antigen preparations irrespectively from whether AcWt, Acgp120-TM, Acgp120-5TM, Acgp120-20TM, or Acgp160 was used in the coinfection experiment (Fig. 7B). However, an approximately 50% decreased particle release was consistently observed from all infected insect cells, when compared to cells infected with AcPr55 alone (Fig. 7A). An examination of these VLP preparations with a gp120/V3-specific monoclonal antibody (NEA 9305) revealed almost identical quantities of the chimeric glycoproteins gp120-TM, gp120-5TM, and gp120-20TM, whereas only small amounts of the wild-type Env proteins were associated with these combined p24(CA)-positive sucrose fractions (Fig. 7C, lane 7). Supernatants harvested from uninfected cells or obtained from cells that were coinfected with AcWt and Acgp120-TM did not contain any of the HIV antigens.

To further demonstrate that wild-type Env proteins and the derivatives of gp120 were not only copurified with the VLPs, communoprecipitates of VLPs from sucrose gradient peak fractions 12–14 were analyzed by SDS-PAGE and immunoblotting (Figs. 7D and 7E). The CD4-
IgG chimeric antibody precipitated the VLPs associated with gp120-TM, gp120-5TM, gp120-20TM, and gp160 as shown by the positive reaction of the anti-p24(CA) mab (16/4/2) and the gp120/V3-specific antibody in the immunoblot analysis. Even small quantities of VLP-entrapped wild-type Env proteins were sufficient for the precipitation of hybrid Pr55/gp160 VLPs, as evidenced from the differences in the signal intensity obtained for Pr55\textsuperscript{gag} with mab 16/4/2 and for HIV-Env achieved with a gp120/V3-specific monoclonal antibody (Figs. 7D and 7E, lanes 7). The CD4-IgG chimera did not precipitate Pr55\textsuperscript{gag} particles without coexpressed HIV-1 Env sequences (Figs. 7D and 7E, lanes 3).

The presence of HIV-Env and chimeric Env-TM polypeptides on the surface of VLPs was further confirmed by immunoelectron microscopy. The CD4-IgG chimeric antibody specifically bound to sucrose gradient-purified Pr55\textsuperscript{gag}/gp160 (Fig. 8B) as well as Pr55\textsuperscript{gag}/gp120-TM VLPs (Figs. 8C and 8D) as detected by an anti-mouse IgG gold conjugate. Negative controls included Pr55\textsuperscript{gag}/gp120-TM VLP preparations incubated with an irrelevant antibody recognizing the external portion of the EBV major glycoprotein gp220/350 (mab 64D7) as well as VLPs purified from the supernatants of HighFive cell cultures infected with AcPr55 and incubated with the CD4-IgG construct. In these preparations, no gold-labeling of VLPs was observed (Fig. 8A).

To further estimate the amounts of wild-type Env proteins or gp120 derivatives incorporated into the membrane of recombinant VLPs, antigens harvested from sucrose gradient fractions 12–14 were quantified using a commercial p24(CA) sandwich assay (Abbott). Identical amounts of VLPs (20 μg) were separated by SDS–PAGE together with twofold serial dilutions of purified rgp120 (0.2 to 3.2 μg) as a calibration curve. The recombinant proteins were readily detected by an anti-gp120/V3 monoclonal antibody. Comparison of the signal intensities detected in the VLP preparations with those of the calibration curve suggested an estimated proportion of about 0.2 μg wild-type Env proteins incorporated into 20 μg of Pr55\textsuperscript{gag}/gp160 VLPs. However, as compared to wild-type HIV Env polypeptides significantly increased concentrations of hybrid Env-TM proteins, ranging from 0.8 to 1.2 μg were quantified in 20 μg of Pr55\textsuperscript{gag}/gp120-TM preparations. Similar results were obtained for Pr55\textsuperscript{gag}/gp120-5TM and Pr55\textsuperscript{gag}/gp120-20TM VLPs (data not shown).

Oligomeric organization of particle-entrapped Env-TM glycoproteins

Previous studies have indicated that the HIV gp160 precursor as well as the gp41 transmembrane protein are capable of forming stable oligomers, whereas the isolated gp120 subunit readily dissociates into monomers (Schawaller et al., 1989; Pinter et al., 1989; Earl et al., 1990). To investigate the oligomeric structure of particle-entrapped chimeric Env-TM proteins, sucrose gradient sedimented VLP preparations were treated with the noncleavable, membrane-impermeable cross-linking agent bis(sulfosuccinimidyl)suberate (BS\textsubscript{3}). After quenching the cross-linking reaction, the VLPs were solubilized in presence of reducing agents, separated by low percentage SDS–PAGE, and detected by immunoblotting with a gp120/V3-specific monoclonal antibody. Non-cross-linked anti-gp120 reactive bands of approximately 137, 135, and 132 kDa represent the monomeric forms of the chimeric gp120-TM, gp120-5TM, and gp120-20TM Env proteins (Figs. 9A, lanes 1–3). The molecular weights of the anti-gp120 reactive bands have been estimated with reference to particle-entrapped gp160, gp120 (Fig. 9A, lane 4), and purified rgp160 Env protein species (lane 5) and were consistent with other commercial high molecular weight standards (Sigma, Deisenhofen, Germany). By using low concentrations of BS\textsubscript{3} (0.3 mM) an additional dominant anti-gp120 reactive protein band appeared in samples of Pr55\textsuperscript{gag}/Env VLPs presenting the different Env-TM derivatives, apparently migrating as 240- to 250-kDa proteins (Fig. 9B, lanes 1–3). In contrast only minute quantities of cross-linked products were detected in Pr55\textsuperscript{gag}/gp160 VLP probes (Fig. 9B, lane 4). Thus, these results indicate a close and, in comparison to the wild-type Env proteins, enhanced association of particle-entrapped Env-TM polypeptides.

DISCUSSION

We and others have previously shown, that the expression of the HIV Pr55\textsuperscript{gag} precursor protein by recombinant...
FIG. 8. Immunoelectron microscopy of concentrated particles. Sucrose gradient sedimented (A) Pr55\textsuperscript{gag}, (B) Pr55\textsuperscript{gag}/gp160 and (C) Pr55\textsuperscript{gag}/gp120-TM VLP preparations (fractions 11–14, Fig. 7) were adsorbed to grids and incubated with anti-gp120 mab 133/237. Bound mab 133/237 was detected with an anti-mouse IgG gold conjugate and analyzed by electron microscopy. Env proteins were specifically detected on VLPs secreted by Sf9 insect cells coinfected with Pr55\textsuperscript{gag}, and Env-recombinant baculoviruses. Clusters of immunogold-labeled Env proteins are indicated by black arrows. (D) A representative immunogold-labeled Pr55\textsuperscript{gag}/gp120-TM VLP with distinct regions of clustered gp120-TM proteins. No specific labeling of particles was seen in negative controls.
baculoviruses results in the formation of large quantities of noninfectious particulate structures that retain many of the physical properties of immature HIV virions (Gheysen et al., 1989; Overton et al., 1989; Royer et al., 1991; Wagner et al., 1992). These virus-like particles (VLP) can be easily produced in a substantially pure form using conventional purification techniques and have been demonstrated to be very potent stimuli of the immune system. Accordingly we and others have previously demonstrated the capability of presenting selected HIV-derived immunogenic determinants by inserting these epitopes at various positions within the particle forming Pr55gag polyprotein (Luo et al., 1992; Griffiths et al., 1993; Wagner et al., 1994; Brand et al., 1995).

Aiming at the development of a more complex VLP-based candidate vaccine we constructed antigenetically extended Pr55gag virus-like particles presenting high quantities of oligomeric HIV-1 gp120 proteins at the outer particle surface. Therefore, various chimeric HIV-1 Env proteins were established in which the complete transmembrane protein gp41 was replaced by a heterologous transmembrane and cytoplasmic moiety of the EBV glycoprotein gp120/20TM. As compared to the wild-type HIV gp160 protein the particle-associated chimeric gp120-TM derivatives possess some intrinsic advantages for vaccine purposes: (i) an exclusion of immunosuppressive effects and adverse immune reactions suggested to be induced by the HIV-1 transmembrane protein gp41 (Young, 1988; Ruegg et al., 1989; Robinson, Jr. et al., 1990; Denner et al., 1994); (ii) a significantly, by a factor of 5 – 10, increased incorporation of oligomeric gp120 chimeras on the surface of immunostimulatory Pr55gag lipoprotein particles; and (iii) the absence of the diagnostically relevant gp41, which will allow a serological discrimination of vaccinees and HIV-infected people in future vaccination studies.

Biochemical and FACScan analysis revealed that all variants of the chimeric Env-TM protein were synthesized, transported to the cell membrane, and incorporated on the outer surface of insect cells at levels comparable to that of wild-type HIV Env-proteins. These results extend previous observations that the transmembrane and cytoplasmic domain of gp41 is dispensable for an efficient trafficking and glycoprotein incorporation into the membranes of various mammalian cell-lines (Gabuzda et al., 1992; Wilk et al., 1992, 1996; Salzwedel et al., 1993). However, the simultaneous expression of the Pr55gag polyprotein caused a significantly reduced cell surface exposure of wild-type Env proteins, when compared to that of the chimeric Env-TM proteins. Moreover the exchange of gp41 by the EBV gp220/350-derived transmembrane domain even resulted in a 5- to 10-fold increased amount of particle-associated glycoproteins compared to wild-type Env proteins.

The mechanisms and signals involved in the selective incorporation of cellular and heterologous proteins into HIV-virions and Pr55gag VLPs are not completely understood so far and appear to be cell-type-specific. For the HIV Env proteins, several data demonstrated the requirement of an interaction between distinct regions within the extremely long cytoplasmic domain of the HIV transmembrane protein gp41 and the p17 matrix domain of the Pr55gag precursor (Yu et al., 1992, 1993; Dorfman et al., 1994; Freed et al., 1995; Freed and Martin, 1995), resulting in a specific incorporation of Env proteins into HIV virions that is limited to approximately 72 Env projections (Gelderblom et al., 1989). Alterations in the cytoplasmic moiety of the SIV and HIV-2 transmembrane domain significantly increased the level of Env proteins on virions (Mulligan et al., 1992; Zingler and Littman, 1993; LaBranche et al., 1995) and VLPs (Yamshchikov et al., 1995), whereas various deletions in the C-terminal region of the cytoplasmic region of the HIV-1 gp41 were shown to reduce the incorporation of Env proteins into virus particles (Dubay et al., 1992; Yu et al., 1993). Regarding the increased incorporation of Env-TM into VLPs one could assume that these chimeric proteins, lacking a long cytoplasmic domain such as that of wild-type gp41, are capable of being incorporated into VLPs without the need of a specific interaction with the Pr55gag polyprotein. This suggestion is supported by a very recent report of Wilk and coworkers, demonstrating that recombinant gp160 harboring the membrane spanning region and long cytoplasmic domain from the cellular glycoprotein CD22 were
excluded from particle incorporation, probably because of steric hindrances. However, the deletion of the spacious foreign cytoplasmic domain restored the particle incorporation of the chimeric Env protein, approximately to wild-type level (Wilk et al., 1996). Glycoprotein incorporation in Pr55\(^{\text{gag}}\)/Env particles and subsequent immunoprecipitation of sedimented pseudovirions with gp120-specific antibodies. The chimeric Env-TM proteins, as well as wild-type gp160, have been detected mainly in those sucrose fractions that also contained VLPs. Furthermore, Pr55\(^{\text{gag}}\) VLPs presenting chimeric or wild-type Env proteins but not Env negative Pr55\(^{\text{gag}}\) VLPs were specifically coprecipitated by Env-specific antibodies, indicating a close association of the Env proteins with the particulate carrier component. Particle-associated Env protein consisted of almost equal quantities of unprocessed and processed forms of the gp160 precursor protein or uncleaved Env-TM chimeras. These observations support previous reports indicating that the targeting of HIV-Env proteins to the surface of VLPs does not essentially require endoproteolytic cleavage (Rovinski et al., 1995; Tobin et al., 1996). Furthermore, immunoelectron microscopic analysis was performed to determine the distribution of the chimeric Env-TM proteins on the surface of the Pr55\(^{\text{gag}}\) VLPs, as it currently provides the most convincing means to demonstrate the specific association and location of Env proteins on recombinant Pr55\(^{\text{gag}}\) particles (Gelderblom et al., 1987; Gonda, 1994). The VLP surface labeling unambiguously demonstrated the presence of chimeric Env-TM proteins on the exterior of sucrose gradient-purified particles, confirming a close association of the Env chimeras with the VLPs.

A common feature for the Env glycoproteins of HIV and other enveloped viruses is their oligomeric structure. Our cross-linking analysis indicate that the chimeric Env-TM proteins are organized as dimers and higher order oligomers on the particle surface. Due to the large size of this complex, it is difficult to determine its degree of oligomerization with absolute certainty, as well-characterized molecular weight markers are not available. However, several considerations led us to conclude that the higher order form is tetrameric. First, consistent recovery of a predominant dimer band suggests that these form the “building block” of any higher order structure. Thus, the supposed tetramers would consist of two noncovalently associated dimers. Second, the estimated molecular mass is closer to that which would be expected for a tetrameric rather than a trimeric molecule. Third, the chimeric Env-TM and wild-type HIV-Env proteins form higher-order oligomers with a similar molecular mass, and gp160, gp120, as well as gp41 are suggested to have tetrameric structures (Pinter et al., 1989; Schawaller et al., 1989; Earl et al., 1990; Owens and Compans, 1990; Weiss et al., 1990), as do the glycoprotein precursors of HIV-2 and SIV (Rey et al., 1989; Chakrabarti et al., 1990; Rey et al., 1990). However, as compared to the wild-type Env proteins, the dimeric form of the Env-TM polyproteins appears to be more stable. Thus, given all of the considerations, we suggest that the Env-TM proteins form dimers that assemble into tetramers composed of two dimers. For wild-type HIV Env proteins, the relatively conserved gp41 ectodomain is responsible for oligomerization of gp160, whereas the cytoplasmic and transmembrane domains are not essential (Earl et al., 1990; Earl and Moss, 1993; Hallenberger et al., 1993; Poumbourios et al., 1995). Regarding that chimeric Env-TM polyproteins lacking the complete gp41 protein have been shown to readily oligomerize, our results extend previous studies, suggesting that HIV-1 Env protein oligomerization is mediated by multiple domains within gp41 and gp120 (Poumbourios et al., 1995). Further studies are needed to elucidate whether domains within the EBV-transmembrane or cytoplasmic region are involved in the aggregation of the chimeric Env-TM proteins. However, it is likely that the overall conformation of the extracellular gp120 domain of the Env-TM proteins may not be significantly affected by the addition of the heterologous EBV-derived transmembrane region, although the oligomeric state of the Env proteins might be analyzed more precisely in future studies by using antibodies specifically recognizing Env oligomers.

Aiming at the development of more complex HIV candidate vaccines we have suggested a novel strategy to incorporate high quantities of chimeric gp120 oligomers at the outer surface of Pr55\(^{\text{gag}}\) virus-like particles that have been previously demonstrated to be highly immunogenic in absence of adjuvant (Wagner et al., 1996). The HIV Pr55\(^{\text{gag}}\) polyprotein and oligomeric envelope proteins represent major targets for the cell-mediated (Gag, Env) (Nixon et al., 1988; Koup et al., 1991; Buseyne et al., 1993; Harrer et al., 1996) and cross-neutralizing humoral immune response (oligomeric Env-proteins) (Steimer et al., 1991; Sattentau and Moore, 1995, Fouts et al., 1997). Work in progress demonstrates that these nonreplicating Pr55\(^{\text{gag}}\)/Env pseudovirions are capable of inducing both humoral and CD8+ CTL responses in rodents and monkeys (Dempl et al., 1997; Teewuken et al., manuscript in preparation). Therefore, nonreplicating Pr55\(^{\text{gag}}\)/Env pseudovirions provide a promising candidate immunogen for the development of a safe and efficacious particle-based AIDS vaccine.

ACKNOWLEDGMENTS

The excellent technical assistance of Elke Perthen is appreciated. We are grateful to Matthias Niedrig, Robert-Koch Institute, Berlin, Ger-
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