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Expression and Characterization of Genetically Engineered Human Immunodeficiency Virus-Like Particles Containing Modified Envelope Glycoproteins: Implications for Development of a Cross-Protective AIDS Vaccine

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Noninfectious human immunodeficiency virus type 1 (HIV-1) viruslike particles containing chimeric envelope glycoproteins were expressed in mammalian cells by using inducible promoters. We engineered four expression vectors in which a synthetic oligomer encoding gp120 residues 306 to 328 (amino acids YNKRKRIHIGP GRAFYTTKNIIG) from the V3 loop of the MN viral isolate was inserted at various positions within the endogenous HIV-1_{LAI} *env* gene. Expression studies revealed that insertion of the heterologous V3(MN) loop segment at two different locations within the conserved region 2 (C2) of gp120, either 173 or 242 residues away from the N terminus of the mature subunit, resulted in the secretion of fully assembled HIV-like particles containing chimeric LAI/MN envelope glycoproteins. Both V3 loop epitopes were recognized by loop-specific neutralizing antibodies. However, insertion of the V3(MN) loop segment into other regions of gp120 led to the production of envelope-deficient viruslike particles. Immunization with HIV-like particles containing chimeric envelope proteins induced specific antibody responses against both the autologous and heterologous V3 loop epitopes, including cross-neutralizing antibodies against the HIV-1_{LAI} and HIV-1_{MN} isolates. This study, therefore, demonstrates the feasibility of genetically engineering optimized HIV-like particles capable of eliciting cross-neutralizing antibodies.

The human immunodeficiency virus type 1 (HIV-1) is the causative agent of AIDS and related disorders (5, 17). The envelope (*env*) glycoprotein is the major surface antigen expressed by the virus and HIV-1-infected cells. It is synthesized as a glycosylated precursor protein (gp160) that subsequently undergoes endoproteolytic cleavage to yield the large external glycoprotein, gp120, and a smaller transmembrane protein, gp41 (2, 12, 55), which contains the gp160 fusion domain. These two subunits form a noncovalent glycoprotein complex which is anchored to the virion capsid and infected cell membranes through gp41 (2, 13). The *env* glycoprotein complex is directly implicated in the infectivity, cellular tropism, cytopathology, and pathogenicity of HIV-1 (9, 10, 35, 36, 40, 43, 44, 52).

The current vaccine strategies designed to develop a safe and efficacious AIDS vaccine include whole, inactivated viruses (11, 18, 20, 21, 45, 46, 61), recombinant antigens and viruses (3, 4, 6, 16, 21, 27, 31, 48, 53, 54, 65, 68, 69), genetically engineered viruslike particles (19, 25, 26, 28, 33, 58, 60, 66), synthetic peptides (7, 8, 21, 24, 28, 30, 50, 51, 57), and anti-idiotypic antibodies (34). Vaccines composed of whole, inactivated simian immunodeficiency virus (SIV) were shown either to prevent the establishment of virus infection (11, 45) or to delay the appearance of disease (61) in macaques challenged with infectious virus. These encouraging results suggest that perhaps a protective immune response against HIV-1 can effectively be obtained by incorporating most of the viral antigens into a candidate vaccine. We (28) and others (19, 25, 33, 58, 60, 66) have previously reported that noninfectious HIV-like particles can be released from mammalian and insect cells transfected with a variety of expression vectors. Since these particles contain either most or all of the HIV-1 structural antigens, they are potential candidate immunogens for the development of improved cross-protective AIDS vaccines.

Several studies have shown that the principal neutralizing determinant of HIV-1 lies within the tip of the loop forming the third variable region (V3) of gp120 (23, 40, 42, 50, 56). Since neutralizing antibodies essentially recognize the hypervariable epitope(s) of the loop, it is conceivable to design cross-protective chimeric vaccines by inserting the V3 loop epitopes of the most predominant and divergent viral isolates into a single envelope.

In the present study, we investigated the possibility of producing HIV-like particles with chimeric envelopes. To this end, we engineered a set of expression vectors in which the *env* gene from the HIV-1_{LAI} strain was modified to produce viruslike particles containing a V3(MN) neutralization epitope inserted in different regions of gp120(LAI). Immunogenicity studies have shown that these optimized particles induce antibody responses against both autologous and heterologous V3 loop epitopes and elicit cross-neutralizing antibodies against the HIV-1_{LAI} and HIV-1_{MN} isolates.

MATERIALS AND METHODS

Cell lines and cell culture. Monkey COS-7 and Vero cells were grown and passaged biweekly in Dulbecco's modified Eagle's medium (Flow Laboratories, McLean, Va.) supplemented with 10% heat-inactivated fetal bovine serum (Bock-

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neck), glutamine (2 mM), penicillin (50 IU/ml), and streptomycin (50 μg/ml). A CD4$^+$ HeLa cell line (41) was obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, National Institute of Allergy and Infectious Diseases; HeLa T4$^+$) from Richard Axel. This cell line was maintained in the presence of 0.5 mg of Geneticin (GIBCO Laboratories, Long Island, N.Y.) per ml.

Antibodies. The following mouse monoclonal antibodies were obtained from Dupont Canada, Inc., Markham, Ontario: anti-HIV-1 reverse transcriptase (RT), NEA-9304; neutralizing anti-HIV-1 gp120 monoclonal antibody 5023 (14) raised against a synthetic peptide containing 15 amino acids present within the V3 loop of HIV-1(III_B), NEA-9305; and anti-HIV-1 gp41, NEA-9303. The anti-CD4 monoclonal antibody OKT4 was obtained from Ortho Diagnostic Systems, Inc., Raritan, N.J. The neutralizing anti-gp120 human monoclonal antibody 268-11D (22) recognizes a core epitope consisting of the HIGPGR sequence in the third variable domain of the *env* glycoprotein of the viral MN strain.

Construction of recombinant expression plasmid vectors. The nucleotide and amino acid numbering used throughout for HIV was designated by Myers et al. (47). The expression plasmid vector pMTHIVd25 (Fig. 1A) was constructed from pMTHIV (28) by deleting a 25-bp DNA fragment (nucleotides 753 to 777; LAI sequence) containing viral RNA packaging sequences (1, 39). In this vector, transcription of the HIV-1 coding sequences is regulated by the inducible human metallothionein II_a (MT) promoter and a simian virus 40 polyadenylation sequence. A collection of pMTHIVd25-based expression vectors containing modifications in the *env* gene coding sequence was generated (Fig. 1B). Vectors pMTHIVST, pMTHIVBG, and pMTHIVKP were constructed by inserting synthetic oligonucleotide cassettes encoding amino acid residues 306 to 328 from the V3(MN) loop sequence (47) into the indicated *Stu*I, *Bgl*II, and *Kpn*I restriction sites, respectively (Fig. 1B). To construct plasmid pMTHIVSB, the *Stu*I-*Bgl*II DNA fragment depicted in Fig. 1B was replaced by a synthetic oligonucleotide encoding the same V3 loop residues. For all constructs, the synthetic DNA cassettes were designed to encode additional amino acid residues to maintain the reading frame and create unique restriction sites flanking the heterologous V3(MN) loop DNA segment. The nucleotide sequences of all constructs were confirmed by DNA sequencing.

DNA-mediated cell transfections. COS-7, Vero, and HeLa T4$^+$ cells were grown to 80% confluence and transfected with 20 μg of plasmid DNA either by Lipofectin (Bethesda Research Laboratories [BRL], Gaithersburg, Md.) or by the Transfinity (BRL) calcium phosphate procedure. Cells transfected with plasmids containing the human MT promoter were induced 24 to 36 h after transfection with 5 μM $CdCl_2$ for 12 to 16 h. Unless otherwise indicated, most cells and culture supernatants were analyzed for protein expression at 48 h posttransfection.

Isolation of HIV-1 viruslike particles from cell culture supernatants. Culture media (10 ml) from cells transfected with individual expression constructs were collected and clarified by centrifugation at 2,000 × *g* (Sorvall RT 6000B; Dupont Company, Wilmington, Del.) for 15 min at 4°C. Viruslike particles were isolated by ultracentrifugation as previously described (28).

Sucrose gradient fractionation and RT assay. To purify HIV-like particles for immunogenicity studies, pelleted particles obtained by ultracentrifugation of cell culture supernatants were resuspended in 200 μl of TNE buffer (10 mM Tris-HCl [pH 8.0], 100 mM NaCl, and 1 mM EDTA),

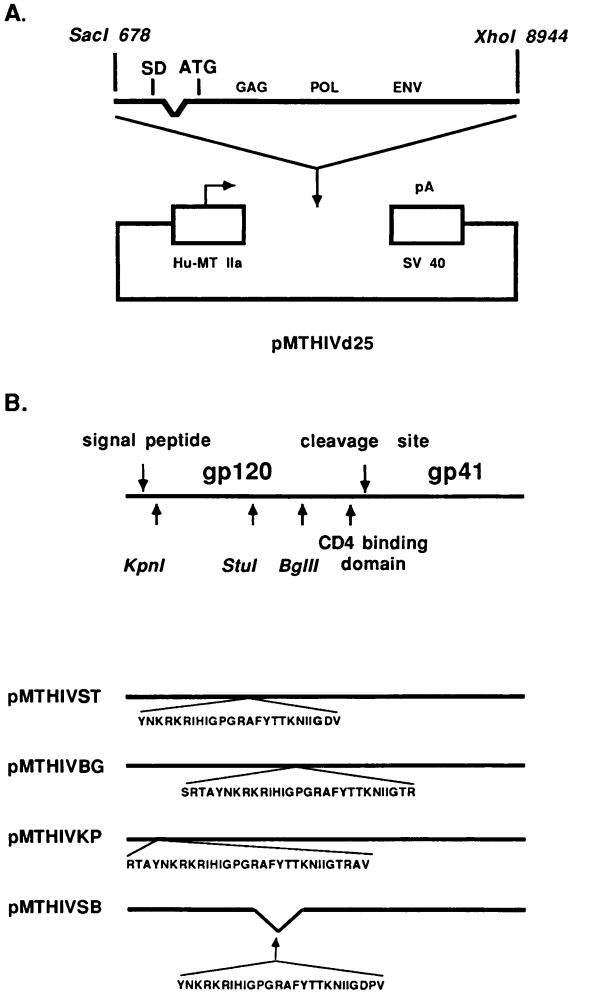

FIG. 1. Diagrammatic representation of constructs and vectors used to express HIV-like particles with normal and modified envelope proteins. (A) A 25-bp DNA fragment (nucleotides 753 to 777 from HIV-1_{LAI}) which contains known viral RNA packaging sequences (1, 39) was deleted from plasmid pMTHIV (28) to generate the expression vector pMTHIVd25. In this vector, transcription is driven by the human MT II_a promoter. (B) Vectors pMTHIVST, pMTHIVBG, and pMTHIVKP were constructed by inserting synthetic oligonucleotide cassettes encoding the 23 gp120 amino acids YNKRKRIHIGPGRAFYTTKNIIG (residues 306 to 328) from the V3 loop of the MN isolate (47) into the depicted *Stu*I, *Bgl*II, and *Kpn*I restriction sites, respectively. To construct plasmid pMTHIVSB, the *Stu*I-*Bgl*II DNA fragment was simply replaced by a synthetic DNA cassette encoding the same V3(MN) loop residues. The predicted amino acid sequence of inserted epitopes is indicated for each individual construct. SD, splice donor; pA, simian virus 40 polyadenylation site; MT, MT promoter.

overlaid onto a continuous sucrose gradient (20 to 60% wt/vol), and sedimented at 100,000 × *g* in a Beckman SW40 rotor for 1.5 h at 4°C. The gradient fractions were collected from the bottom in 500-μl aliquots. RT activity was measured in each fraction as previously described (28).

Immunoprecipitations. Two days after transfection, cells were washed in phosphate-buffered saline (PBS) and disrupted in 0.5 ml of lysis buffer (50 mM Tris-HCl [pH 8.0] containing 0.5% Nonidet P-40, 5 mM EDTA, 150 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride) for 15 to 30 min at

4°C. The lysates were adsorbed for 1 h at room temperature with 30 μl of a 50% suspension of protein G-agarose (BRL) and clarified by centrifugation. Immunoprecipitation analyses were performed by adding 1 μg of antibody and 30 μl of a 50% suspension of recombinant protein G-agarose to clarified extracts. After 16 h of incubation at 4°C, immunoprecipitates were collected by centrifugation at 14,000 × *g*, washed three times in lysis buffer, resuspended in Laemmli sample buffer (37), boiled for 2 min, and fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). To detect the presence of proteins in the culture medium of cells transfected with recombinant vectors encoding chimeric gp120(LAI/MN) proteins, the cell supernatants were filtered through a 0.45-μm-pore-size filter, and 1 μg of the human 268-11D monoclonal antibody (22) was added to the medium as described above. Immunoprecipitates were then washed, pelleted, resuspended in Laemmli sample buffer, fractionated by SDS-PAGE, and analyzed by immunoblotting with the neutralizing mouse anti-gp120(III_B) monoclonal antibody 5023 (14).

Western immunoblot analysis. Pelleted particles were suspended in 40 μl of TNE, mixed with 10 μl of 5× Laemmli sample buffer (37), and boiled for 3 min. Viral proteins were then separated by SDS-PAGE and transferred to Immobilon membranes (Millipore, Bedford, Mass.) (63). Membranes were blocked with BLOTTO buffer (PBS containing 5% Carnation instant nonfat dry milk, 0.0001% wt/vol thimerosal, and 0.01% vol/vol antifoam A emulsion) for 2 h at 25°C and then incubated with appropriate dilutions of antibodies overnight at 4°C. Filters were then incubated with a goat anti-mouse immunoglobulin G antibody conjugated to alkaline phosphatase (Promega, Madison, Wis.) and reacted with the alkaline phosphatase chromogenic substrates nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyphosphate *p*-toluidine salt (BRL).

CD4-binding assays. $CD4^+$ HeLa cells (41) were transfected with the recombinant expression plasmid vectors as described above. Cells were induced with 5 μM $CdCl_2$ 24 h after transfection, and cell lysates were prepared 24 h after induction. All lysates were adsorbed for 1 h at room temperature with 30 μl of a 50% suspension of recombinant protein G-agarose and clarified by centrifugation. The precleared cell lysates were then immunoprecipitated with the monoclonal antibody OKT4. Immunoprecipitated material containing *env* protein-CD4 complexes was then resolved by SDS-PAGE and analyzed by immunoblotting with the mouse anti-gp120(III_B) monoclonal antibody 5023. This assay only detects envelope-CD4 protein complexes and is not quantitative.

Immunogenicity studies and measurement of antibody responses. Fully assembled envelope-containing particles were isolated from the supernatants of stably engineered Vero cells transfected with plasmids pMTHIVd25, pMTHIVST, and pMTHIVBG, respectively, by ultracentrifugation through a glycerol cushion and purified by sucrose gradient fractionation (28). The p24 content of the various particle species was determined by a p24-specific enzyme immunoassay (Coulter Immunology, Hialeah, Fla.). All stable cell lines secreted approximately 500 μg of p24 per liter.

Female SJL/J mice (Charles River, Montreal, Quebec, Canada) between 6 and 8 weeks of age were immunized subcutaneously with doses of purified particles corresponding to 10 μg of p24 antigen emulsified in Freund's complete adjuvant. A booster injection equivalent to 5 μg of p24 antigen was given 3 weeks later in Freund's incomplete adjuvant. Mice were sacrificed 9 days after the second immunization, and sera were collected and heat inactivated at 56°C for 30 min. The presence of antibodies to HIV-1 antigens was determined by antigen-specific enzyme-linked immunosorbent assay (ELISA). ELISA plates (LKELKAY plates; Lab Systems, Shrewsbury, Mass.) were coated at 20°C for 18 h with 100 μl of a solution containing either recombinant proteins at 1 μg/μl or peptides at 10 μg/ml in 50 mM carbonate buffer, pH 9.6. The recombinant gp120 was obtained from American Bio-Technologies, Inc., Cambridge, Mass., and p24 was obtained from Dupont Canada, Inc., Markham, Ontario, Canada. Synthetic peptides corresponding to the neutralizing determinant found in the V3 loops of gp120 from HIV-1 strains HXB2, MN, and ELI (Table 1) were purchased from American Bio-Technologies, Inc., Cambridge, Mass. Plates were blocked at room temperature for 1 h with 200 μl of 2% gelatin in PBS and washed three times with PBS containing 0.05% Tween 20. Serum samples were serially diluted in PBS-Tween 20 and added to individual wells for 1.5 h at room temperature. The plates were then washed three times with PBS-Tween 20, and a goat anti-mouse immunoglobulin G-horseradish peroxidase enzyme conjugate (Amersham Canada Ltd, Oakville, Ontario, Canada) diluted 1:5,000 in PBS-Tween 20 was added for 30 min at 37°C. After an additional washing step, the color was developed by using 0.1% tetramethylbenzidine–0.004% hydrogen peroxide (ADI Diagnostics, Willowdale, Ontario, Canada). Optical density was read at 450 nm by using a Titertek Multiskan MCC/340 plate reader (Flow Laboratories, McLean, Va.). Endpoint titers were defined as the highest serum dilution which resulted in optical density readings at least twofold greater than the baseline absorbance established for normal mouse serum controls.

Determination of cross-neutralizing antibodies in immunized guinea pigs by a cell fusion blockade assay. Guinea pigs were immunized subcutaneously with purified HIV-like particles corresponding to 10 μg of p24 antigen emulsified in Freund's complete adjuvant. Booster immunizations with doses corresponding to 5 μg of p24 were given intramuscularly every 2 weeks in Freund's incomplete adjuvant. Blood was collected 14 days after each booster immunization, and the serum fraction was isolated. The ability of immune guinea pig antisera to neutralize the HIV-1_{LAI} and HIV-1_{MN} strains was assessed after four booster immunizations by determining the presence of cell fusion-blocking antibodies in the sera as previously described (59). Prior to the assay, all antisera were adsorbed with monkey Vero cells to remove cross-reactive anti-primate antibodies.

RESULTS

Recombinant plasmid vectors used to study the expression of HIV-like particles with modified envelope glycoproteins. A set of plasmid vectors was constructed to study the expression of noninfectious viruslike particles with structurally modified *env* proteins. We previously used the inducible human MT promoter to produce immunogenic HIV-like particles in stably engineered primate cell lines (28). The original expression vector (pMTHIV) was further modified to construct pMTHIVd25 by deleting the 25-bp DNA fragment known to contain viral RNA packaging sequences (1, 39) from HIV-1_{LAI} proviral DNA (Fig. 1A). This deletion did not significantly affect particle assembly. In addition, a collection of pMTHIVd25-based expression vectors with genetic modifications in the *env* coding sequence was constructed (Fig. 1B). These vectors were engineered to produce HIV-1_{LAI}-like particles with chimeric envelope glyco-

proteins containing the same heterologous V3(MN) loop epitope inserted at different positions.

Expression of HIV-like particles with chimeric MN/LAI envelope proteins. To broaden and enhance the immunogenicity of the HIV-1_{LAI}-like particles, we engineered chimeric envelopes and investigated whether alterations in the *env* gene would affect particle assembly. To this end, we constructed four pMTHIVd25-based expression vectors in which a synthetic oligomer encoding gp120 residues 306 to 328 (amino acids YNKRKRIHIGPGRAFYTTKNIIG) from the V3 loop of the MN viral isolate (47) was inserted at various positions within the HIV-1_{LAI} *env* gene (Fig. 1B). These mutations should result in insertion of the V3(MN) neutralization epitope(s) either into the first conserved region (C1) of gp120 approximately 12 residues away from the N terminus of the mature glycoprotein (pMTHIVKP) or into the second conserved region (C2) of the *env* protein 173 (pMTHIVST) and 242 (pMTHIVBG) residues away from the N terminus, respectively. In plasmid pMTHIVSB, the mutation was designed to replace 69 amino acids in the C2 region with the V3(MN) segment.

To characterize and compare the products of both native and mutated *env* genes, COS-7 cells were transfected with the various recombinant expression vectors. The transfectants produced between 400 to 500 μg of particle-associated p24 per liter, upon induction with heavy metals. Particles pelleted from the culture supernatants of induced cells were analyzed by immunoblotting 48 h after transfection. *env* proteins were visualized by using the mouse anti-gp120 monoclonal antibody 5023, which recognizes the *env* proteins of the LAI and, to a lesser extent, of the MN strains in Western blots (14). Protein bands with apparent molecular weights consistent with those of native gp120 and gp160 were observed in pelleted material derived from the supernatants of cells transfected with plasmids pMTHIVd25, pMTHIVST, and pMTHIVBG, respectively (Fig. 2A). However, *env* glycoproteins were not expressed from vectors pMTHIVKP and pMTHIVSB. Immunoblot analysis of these samples with a mouse anti-gp41 monoclonal antibody confirmed that the transmembrane protein was only detected in the three samples in which gp120 was present (Fig. 2B). Taken together, these data demonstrate that, of the four chimeric *env* gene constructs, only the pMTHIVST and pMTHIVBG vectors were able to produce viruslike particles containing surface- and membrane-associated envelope subunits when transfected into COS-7 cells. Therefore, insertion of the heterologous V3(MN) loop segment at two different positions within the C2 region of gp120, either 173 (pMTHIVST) or 242 (pMTHIVBG) residues from the N terminus, resulted in expression of structurally modified *env* proteins. However, insertion of the V3(MN) loop segment into the C1 region of gp120 approximately 12 residues away from the N terminus of the mature glycoprotein (pMTHIVKP) or replacement of 69 amino acids in the C2 region with the heterologous segment (pMTHIVSB) resulted in structural modifications that affected the expression of the *env* proteins.

To determine whether the lack of *env* protein expression in the supernatants of cells transfected with constructs pMTHIVSB and pMTHIVKP was due to a defect in particle assembly and production or to the inability of transfected cells to synthesize gp160 precursor protein, we first assessed the presence of RT in pelleted material by immunoblot analysis, using a mouse monoclonal anti-RT antibody. Similar steady-state levels of both forms of the RT enzyme (51 and 66 kDa, respectively) were observed in all particle species analyzed (data not shown). Since the presence of RT in pelleted material is indicative of viral assembly and virus release into the culture medium, these results demonstrate that all constructs expressed comparable amounts of viruslike particles in response to $CdCl_2$ induction. In addition, the ability of nonsecreting transfectants to synthesize the *env* glycoprotein precursor intracellularly was demonstrated by immunoblot analysis of cell lysates probed with the anti-gp120 monoclonal antibody 5023. The gp160 glycoprotein was detected in all cell lysates (Fig. 2C). The truncated *env* glycoprotein precursor expressed from pMTHIVSB which lacks 69 amino acids had, as expected, a lower apparent molecular mass relative to the other precursor proteins. The variations in steady-state levels of the various precursor proteins after $CdCl_2$ induction probably reflect differences in

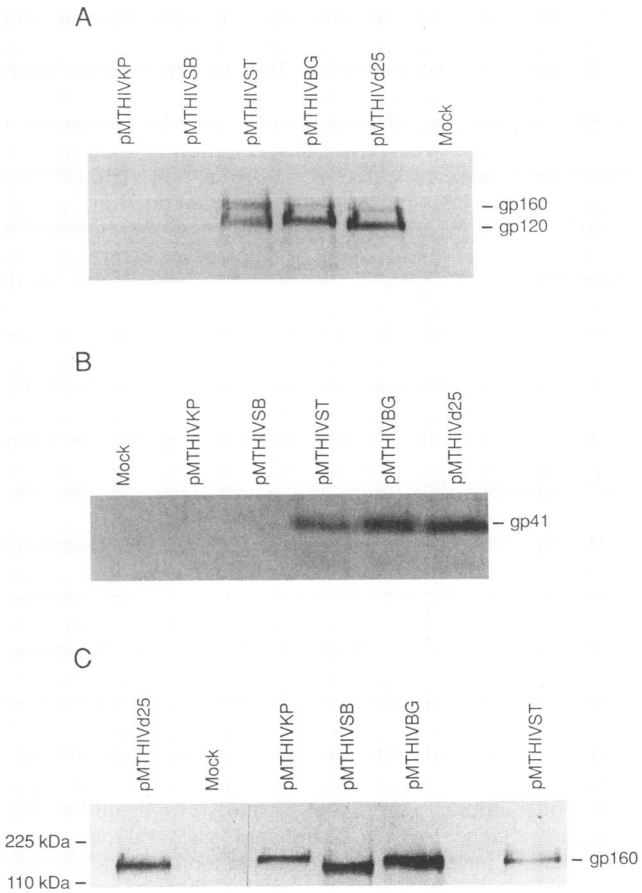

FIG. 2. Immunoblot analysis of envelope glycoproteins present in cell lysates or associated with HIV-like particles expressed by transfected COS-7 cells. Cells were transfected with either control (pMTHIVd25) or mutated (pMTHIVST, pMTHIVBG, pMTHIVSB, and pMTHIVKP) plasmid vectors. (A and B) Viruslike particles were pelleted from the supernatants (10 ml) of COS-7 cells induced with 5 μM $CdCl_2$ at 48 h after transfection. The pellet was analyzed by SDS-PAGE and immunoblotting, using either the mouse anti-gp120 monoclonal antibody 5023 (Dupont) (A) or a mouse anti-gp41 monoclonal antibody (B). (C) Lysates prepared by treating 5×10^5 transfected and $CdCl_2$-induced COS-7 cells with lysis buffer were resolved by SDS-PAGE and analyzed for the presence of precursor gp160 protein by immunoblotting with the mouse anti-gp120 monoclonal antibody 5023 (Dupont). Electrophoretic mobilities of native gp120, gp160, and gp41 glycoproteins are indicated. Mock, pelleted material obtained from mock-transfected COS-7 cells.

their intracellular stability. However, these results confirmed the ability of all constructs to express gp160. The absence of detectable gp120 in cell lysates may be due to rapid intracellular degradation or transport to and release from the cell surface. Furthermore, only a small fraction of gp160 is processed into the gp120 subunit (67).

To establish that the gp120 subunits produced by pMTHIVST and pMTHIVBG contained the heterologous V3(MN) loop epitope(s), the envelope proteins were immunoprecipitated in the absence of any detergents or denaturing agents with the human monoclonal antibody 268-11D directed against a neutralization epitope of the V3(MN) loop (22). Immunoprecipitates were then resolved by SDS-PAGE and analyzed by immunoblotting with the mouse anti-gp120(III_B) monoclonal antibody 5023 (14). Immunoblot analysis (Fig. 3) revealed that antibody 5023 specifically recognized a single band corresponding to gp120 only in immunoprecipitates from the supernatants of cells transfected with the pMTHIVST and pMTHIVBG constructs. These results clearly indicate that the processed *env* glycoproteins expressed from these vectors contain the heterologous V3(MN) loop segment.

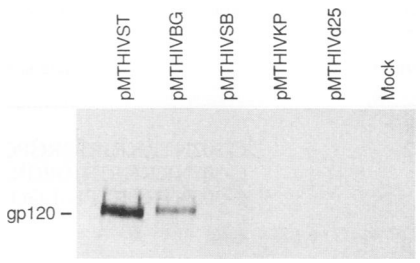

FIG. 3. Immunoblot analysis of material immunoprecipitated from supernatants of cells transfected with control (pMTHIVd25) and mutated (pMTHIVST, pMTHIVBG, pMTHIVSB, and pMTHIBKP) expression vectors. Culture supernatants of cells transfected with the various recombinant plasmid constructs were first immunoprecipitated in the absence of any detergent or denaturing agents with the human monoclonal antibody 268-11D which specifically recognizes a neutralization epitope within the V3 loop of an HIV-1_{MN} envelope (22). Immunoprecipitates were then resolved by SDS-PAGE and analyzed by immunoblotting with the mouse anti-gp120(III_B) monoclonal antibody 5023 (Dupont). Mock, immunoprecipitate of mock-transfected cell supernatant.

Influence of structural modifications of the *env* glycoprotein on CD4 binding. Insertion of the V3(MN) loop-coding sequence into the *env* gene did not affect the DNA sequences encoding a region critical for interaction with the CD4 receptor in any of the four mutated constructs (38) (Fig. 1B). However, we investigated whether these structural modifications had any effect on the ability of the altered *env* proteins to interact with CD4. Since all the mutated constructs express a precursor *env* glycoprotein, $CD4^+$ HeLa cells (41) were transfected with the mutated vectors or with the control plasmid pMTHIVd25 and CD4 binding was assayed on cell lysates at 48 h posttransfection. The lysates were first immunoprecipitated with the monoclonal antibody OKT4 which binds to a portion of CD4 not masked by gp160 binding and therefore recognizes complexes between CD4 and the HIV-1 glycoprotein (10, 35, 41). The immunoprecipitated material was then resolved by SDS-PAGE and analyzed by immunoblotting with the mouse anti-gp120 monoclonal antibody 5023. The precursor *env* glycoprotein present in the lysates of cells transfected with pMTHIVd25, pMTHIVST, and pMTHIVKP was precipitated with OKT4 and is thus capable of binding to CD4 (Fig. 4). In contrast, precursor *env* proteins expressed by the constructs pMTHIVBG and pMTHIVSB were not immunoprecipitated by OKT4, suggesting that they did not interact with CD4.

Immunogenicity of HIV-like particles with chimeric envelope glycoproteins. Fully assembled viruslike particles were purified by ultracentrifugation through a glycerol cushion and then by sucrose gradient fractionation from the supernatants of stably engineered Vero cells transfected with plasmids pMTHIVd25, pMTHIVST, and pMTHIVBG, respectively (see Materials and Methods). Groups of two to four mice received two injections (10 and 5 μg of p24 equivalent per dose, respectively) of particles with modified *env* proteins to evaluate their respective immunogenicities. The antibody response to envelope and core antigens was analyzed by antigen-specific ELISA, and the results obtained are presented in Table 1. Envelope- and core-specific antibody titers were similar in the three groups of mice immunized with particles isolated from stably engineered Vero cells transfected with constructs pMTHIVd25, pMTHIVST, and pMTHIVBG, respectively. These antibody responses were of the same order of magnitude as those previously reported for HIV-like particles secreted by stably engineered primate cell lines (28).

The antisera were further tested for their reactivities with synthetic epitopes from the V3 loops of three different HIV-1 isolates. These peptides consist of amino acid residues 302 to 322, 307 to 325, and 303 to 323 of gp120 from the HXB2, MN, and ELI viral strains, respectively (47). Since the three expression constructs used to produce the immunogens contain the *env* coding sequences of the HIV-1_{LAI} isolate,

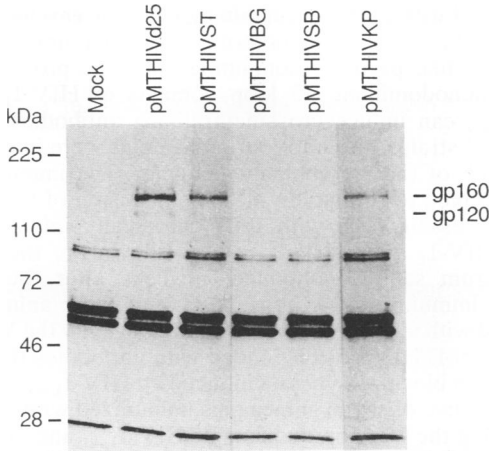

FIG. 4. Immunoblot analysis of CD4-envelope complexes immunoprecipitated with monoclonal antibody OKT4. $CD4^+$ HeLa cells (41) were transfected with control (pMTHIVd25) and mutated (pMTHIVST, pMTHIVBG, pMTHIVSB, and pMTHIVKP) expression plasmid vectors. Cells were induced with 5 μM $CdCl_2$. Forty-eight hours after transfection, cells were treated with lysis buffer and lysates (1 ml) were immunoprecipitated with the mouse anti-CD4 monoclonal antibody OKT4 (Ortho). Immunoprecipitates containing *env* protein-CD4 complexes were then resolved by SDS-PAGE and analyzed by immunoblotting with the mouse anti-gp120 monoclonal antibody 5023 (Dupont). Mock, immunoprecipitate from mock-transfected $CD4^+$ cells. Some of the bands with electrophoretic mobilities with apparent molecular masses lower than 110 kDa correspond to the heavy and light chains of the murine OKT4 antibody.

TABLE 1. Antibody responses after immunization with HIV-1 viruslike particles containing modified envelope glycoproteins

Immunoreactivity to:	Amino acid sequence[a]	ELISA titer (reciprocal dilution)		
		pMTHIVd25	pMTHIVST	pMTHIVBG
Peptides				
HXB2	C-302NTRKRIRIQRGPGRAFVTIGK-322	2,500	2,500	2,500
MN	C-307NKRKRIHIGPGRAFYTTKN-325	2,500	12,500	12,500
ELI	C-303NTRQRTPIGLGQSLYTTRSRS-323	<100	<100	<100
Proteins				
rgp120		>62,500	>62,500	>62,500
rp24		>62,500	>62,500	>62,500

[a] The second and last amino acid of each peptide is numbered as to its position in the V3 loop, according to the system of Myers et al. (47).

we first tested the reactivity of the antisera with a peptide containing most of the V3(HXB2) loop residues but differing from the corresponding V3(LAI) sequence by only one amino acid at position 306 (Table 1). HXB2 peptide-specific titers were similar in all three groups of mice, suggesting that the introduction of the heterologous V3(MN) loop segment did not affect the humoral response against the endogenous V3(LAI) loop. Interestingly, the particles produced by cells transfected with pMTHIVd25 that lacked the V3(MN) domain also elicited cross-reacting anti-V3(MN) antibodies (1/2,500), suggesting that these antibodies recognize an epitope shared by the two V3 loop peptide sequences which are 65% similar. However, the expression vectors pMTHIVST and pMTHIVBG which contained the V3(MN) loop coding sequences produced viruslike particles which induced a markedly enhanced (1/12,500) and specific antibody response against the V3(MN) loop peptide (Table 1). The lack of antibody response to the divergent V3(ELI) loop peptide served as a control for the specificity of the antibody response.

Generation of cross-neutralizing antibodies in guinea pigs immunized with particles containing chimeric envelope glycoproteins. In an effort to determine whether immunization with HIV-like particles containing envelope proteins with the immunodominant V3 loop domains of HIV-1_{LAI} and HIV-1_{MN} can induce cross-neutralizing antibodies against both viral strains, three to four guinea pigs were immunized with each of the recombinant antigens. The immune sera were assayed for the ability to prevent fusion of uninfected CD4-expressing cells with cells chronically infected with either HIV-1_{LAI} or HIV-1_{MN} (59). Shown are the results with serum samples obtained 2 weeks after the fourth booster immunization (Table 2). Two of three animals immunized with viruslike particles containing only the V3(LAI) domain (pMTHIVd25) responded with antibodies that were effective in blocking syncytia induced by HIV-1_{LAI}. Immune sera from five of seven guinea pigs immunized with particles containing the V3 loop domains of HIV-1_{LAI} and HIV-1_{MN} (pMTHIVST and pMTHIVBG) also blocked fusion of CD4-expressing cells with cells chronically infected with HIV-1_{LAI}. In addition, immunization with HIV-like particles containing chimeric envelopes was very effective in inducing cross-neutralizing antibody responses since six of seven serum samples from immunized animals were able to block syncytia induced by either HIV-1_{LAI} or HIV-1_{MN}. Cross-neutralizing activity was also observed in the serum of one of three guinea pigs immunized with viruslike particles containing only the V3(LAI) loop domain. Some of the serum samples were also checked for the ability to blockade HIV-1_{RF} or HIV-2Z, and none of the tested samples prevented syncytia induced by these viral strains (data not shown).

DISCUSSION

We have previously established mammalian cell lines which produce noninfectious and immunogenic HIV-like particles (28). The main objective of this study was to genetically engineer viruslike particles with additional modifications to optimize their safety and develop a cross-protective AIDS vaccine candidate. Recent experiments suggest that candidate AIDS vaccines should include the HIV-1 *env* glycoprotein, since neutralizing antibodies and protective immune responses were observed in animals immunized with various *env* antigen species (6, 21, 23, 48, 50, 51, 54). Therefore, an essential feature that must be incorporated into the design of a safe and efficacious particle-based AIDS vaccine candidate is the insertion of immunogenic determinants that promote neutralizing and cross-protective immune responses.

In this study we tested the feasibility of this approach by engineering chimeric envelope proteins which contain the V3 neutralization epitopes of two of the most predominant HIV-1 isolates in North America. To establish whether HIV-like particles can express *env* proteins with additional heterologous epitopes, we engineered four HIV-1_{LAI}-based expression constructs in which the *env* gene was modified by inserting heterologous DNA sequences coding for the neutralization epitope (YNKRKRIHIGPGRAFYTTKNIIG) of the V3(MN) loop (47). Insertion of the heterologous V3(MN) loop segment into the conserved C2 region of the *env* protein either 173 (pMTHIVST) or 242 (pMTHIVBG) amino acid residues away from the N terminus of mature gp120 (HIV-1_{LAI}) did not affect the processing of chimeric *env* glycopro-

TABLE 2. Cell fusion blockade

Serum sample	Antigen	Fusion inhibition[a]	
		HIV-1_{LAI}	HIV-1_{MN}
11	pMTHIVd25	+	−
12	pMTHIVd25	−	−
13	pMTHIVd25	+	+
15	pMTHIVST	−	+
16	pMTHIVST	+	+
17	pMTHIVST	+	+
19	pMTHIVBG	+	+
20	pMTHIVBG	−	−
21	pMTHIVBG	+	+
22	pMTHIVBG	+	+

[a] Adsorbed serum samples were tested at a final dilution of 1/10 to block syncytium formation induced by CEM cells chronically infected with either HIV-1_{LAI} or HIV-1_{MN} (59). Numbers of syncytia in uninhibited wells (preimmune or normal sera) were greater than 50 for each virus. A negative (−) score indicates no inhibition and a positive score (+) indicates fusion inhibition by the test serum, with five or less syncytia per well.

teins or their association with HIV-like particles. In contrast, insertion of the V3(MN) epitope into the conserved C1 region of gp120 (HIV-1_{LAI}) near the N terminus of the mature glycoprotein (pMTHIVKP) or replacement of 69 amino acids in the C2 region with this epitope (pMTHIVSB) resulted in the expression of viruslike particles devoid of *env* glycoproteins. These modifications, however, did not affect the synthesis of precursor glycoproteins. These observations suggest that the mutations likely resulted in an incorrect folding of the chimeric gp160, hindering its proteolytic cleavage site. Previous studies have also demonstrated that mutations in these two regions of gp120 either abolished or significantly reduced the processing of gp160 (29, 49).

The site of insertion of the heterologous V3(MN) epitope determined the ability of the four chimeric *env* glycoprotein precursors to bind to CD4. It has been shown that gp120 residues located in the fourth conserved region (C4) are critical for CD4 binding (38), although other gp120 amino acid residues have also been implicated in gp120-CD4 interactions (36, 49, 62). None of the V3(MN) insertions affected the critical gp120 C4 region. However, only two of the four chimeric envelope precursors were able to bind to CD4. The fact that these unprocessed *env* glycoproteins were shown to associate with CD4 is consistent with the recent observation that cleavage of gp160 is not required for CD4 binding (15). Our results also confirm that mutations outside the critical region required for CD4 binding may indirectly affect the conformation or the accessibility of the CD4 binding site (9, 13, 36, 49, 62, 64).

The two processed chimeric *env* proteins produced by the pMTHIVST and pMTHIVBG expression vectors expressed the heterologous V3(MN) loop epitope, and the *env* proteins were immunoprecipitated with an anti-gp120 monoclonal antibody which specifically recognizes a neutralization determinant of the V3(MN) loop (22). This finding suggests that additional neutralization epitopes can be inserted into the envelope of chimeric viruslike particles and remain antigenic regardless of significant conformational changes in gp120 tertiary structure as exemplified by the loss of CD4 binding activity observed for one of the antigenic constructs (pMTHIVBG).

For immunogenicity studies, viruslike particles containing chimeric *env* proteins were expressed in Vero cell lines stably transfected with expression plasmids pMTHIVd25, pMTHIVST, and pMTHIVBG, respectively. All secreted HIV-like particles elicited strong envelope- and core-specific humoral responses in mice immunized with these particles. Nonchimeric particles induced cross-reacting antibodies recognizing the V3(MN) segment. This observation may be explained by the fact that the sequences of V3(LAI) and V3(MN) epitopes are 65% similar and that both peptides share the highly conserved GPGRAF motif. However, chimeric particles induced a markedly enhanced anti-V3(MN)-specific antibody response as judged by a sixfold increase in antibody titer. We have therefore identified two specific endogenous restriction sites in the HIV-1_{LAI} *env* gene that can be utilized to insert DNA sequences coding for immunogenic heterologous V3 loop epitopes without affecting *env* protein processing and assembly into viruslike particles. Structural modifications of this nature could be incorporated into candidate HIV vaccines to broaden the specificity of cross-neutralizing antibodies.

Indeed, our results demonstrated that cross-neutralizing antibodies could be elicited in guinea pigs by immunization with chimeric HIV-like particles containing the immunodominant V3 loop epitopes of HIV-1_{LAI} and HIV-1_{MN}. The ability of chimeric particles to induce HIV-1_{MN} neutralization is likely due to the presence of the heterologous V3(MN) epitope, because six of seven animals immunized with these particles responded with antibodies that blocked HIV-1_{MN}-specific cell fusion. Although the V3 region of gp120 generates predominantly isolate-specific neutralizing antibodies, the highly conserved GPGRAF motif within the core of the V3 loop can generate broadly neutralizing antibodies (32). Since this motif is shared by the V3(LAI) and V3(MN) loops, this could partially explain the cross-neutralization observed with the serum from one of three guinea pigs immunized with nonchimeric HIV-1_{LAI}-like particles. However, other factors such as proper presentation of conserved conformational epitopes could contribute to the induction of cross-neutralizing antibodies (27).

In conclusion, we have shown that *env* genes can be manipulated to design structurally modified viruslike particles capable of inducing cross-neutralizing antibodies. We suggest that a major advantage in using genetically engineered HIV-like particles as candidate AIDS vaccines is the degree of flexibility conferred on the rational design of these particles. The safety and immunogenicity of particle-based vaccines can be optimized by introducing structural modifications in selected viral antigens. Although the clinical efficacy of HIV-like particles with chimeric *env* proteins remains to be determined, vaccination with these engineered immunogens may provide an alternative strategy for the prevention of disease and prove to be of immunotherapeutic value for the treatment of HIV-infected patients.

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