J. Microbiol. Biotechnol. (2005), **15**(6), 1229–1239

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Mature HIV-like Particles Produced from Single Semliki Forest Virus-Derived Expression Vector

KIM, EUN[1], HARYOUNG POO[2], MOON-HEE SUNG[3], AND CHUL-JOONG KIM[1]*

[1]*Laboratory of Infectious Disease, College of Veterinary Medicine, Chungnam National University, Daejeon, Korea*
[2]*Proteome Research Lab., Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea*
[3]*Department of Bio & Nanochemisty, College of Natural Sciences, Kookmin University, Seoul, Korea*

Received: January 29, 2005
Accepted: February 17, 2005

Abstract Human immunodeficiency virus-like particles (HIV-VLPs) with native conformations similar to that of the wild-type virion could be valid candidates for vaccine development. To this end, we used a Semliki Forest Virus (SFV) expression system to produce HIV-VLPs containing high quantities of native envelope proteins. Here, we described a single SFV replicon containing the HIV *gagpol* and *env* genes under the control of separate subgenomic promoters. Mature VLPs incorporating the Gag and Env proteins were detected in the supernatant of replicon-expressing cells by Western blot analysis. The HIV-VLPs showed the expected molecular density (1.14–1.18 g/ml) on a 20-60% sucrose gradient; the particles were 100–120 nm in diameter and Env proteins were observed on their surfaces by immunogold electron microscopy. RT-PCR analysis of VLP-associated RNAs in mature HIV-VLPs revealed two SFV-derived RNA species (full-length and subgenomic). Immunization studies in Balb/c mice showed that these HIV-VLPs were capable of inducing both HIV-specific antibodies and cell-mediated immune responses. Taken together, our results indicate that the SFV replicon system is useful for the production of HIV-VLPs, which may be valuable candidates for an HIV vaccine.

Key words: HIV-1, Semliki Forest Virus vector, virus-like particle, vaccine

Developing an effective vaccine against human immunodeficiency virus (HIV) has proven to be a formidable challenge because a successful vaccine must induce both neutralizing antibodies targeted to the viral envelope and cytotoxic T-lymphocytes targeted to a variety of viral antigens. Numerous strategies have been undertaken to present viral antigens to the immune system, including live attenuated virus [1, 17, 18], the presentation of viral polypeptides [10, 16, 20], DNA vaccination [9, 12, 13, 23], and live recombinant viruses [14, 26, 29, 32, 35, 36].

Some recent attempts have focused on the generation of virus-like particles (VLPs), as these may present an attractive vaccine strategy capable of inducing both systemic and mucosal immune responses [40] without the risk of pathogenicity. Previous studies have used the recombinant baculovirus expression system to produce VLPs composed of the HIV-1 $Pr55^{gag}$ precursor protein and envelope epitopes. These were introduced into VLPs by fusion of the coding sequences to the 3' end of the *gag* ORF or anchoring through the transmembrane portion of the Epstein-Barr virus [6]. However, these vaccine constructs are unlikely to be totally effective in the prevention of HIV infection, because the baculovirus expression system is unable to produce mature VLPs that strongly resemble the native HIV virion [2].

The Semliki Forest Virus (SFV) belongs to the family *Togaviridae* and contains a single RNA molecule of positive polarity. SFV replicates exclusively in the cytoplasm and has been used as a model organism for studying the molecular biology of RNA virus multiplication and packaging. The pSFV1 plasmid vector contains the cDNA equivalent of the viral nonstructural genes, flanked upstream by an SP6 promoter and a polylinker site downstream of the subgenomic promoter, which allows the insertion of heterologous genes [25]. Replication of this plasmid occurs in the cytoplasm, so use of the SFV system should remove the risk of chromosomal integration and permit expression of heterologous HIV genes without requiring the presence of other HIV-1 proteins, such as Rev. Furthermore, SFV induces apoptosis of infected cells, so the viral genome will not persist in infected cells [14]. Experimentally, recombinant SFV induced better protective responses than plasmid DNA in mice [8]

*Corresponding author
Phone: 82-42-821-6783; Fax: 82-42-823-9382;
E-mail: cjkim@cnu.ac.kr

and was immunogenic in primates both in a combined immunization protocol [15, 30, 33] and alone [3].

Previously, an SFV expression system was used to produce recombinant Moloney murine leukemia virus particles [24]. However, we found that incorporation of the glycoproteins into virus particles after transient transfection was relatively inefficient, and the co-transfection methods used for production of the HIV-VLP required many steps. Here, we sought to co-express the $Pr55^{gag}$ and Env proteins by combining the *gag* and *env* genes into a single SFV replicon under the control of separate subgenomic promoters. We then replaced the *gag* gene with the *gagpol* gene, to allow for the efficient processing of the Gag and Env proteins by the necessary protease, thus creating a single SFV replicon capable of producing mature HIV-VLPs. Production of mature, non-replicating HIV-VLPs incorporating intact Env, which could induce infection in host cells, may represent an attractive strategy for presenting the antigenic epitopes of the native HIV virion. Moreover, proteins that faithfully represent the antigenic structure of the virion-associated envelope glycoprotein complex may be worth evaluating as vaccine immunogens [6, 7, 38, 39]. Thus, our study provides a new, attractive strategy for the production and isolation of non-replicating mature HIV-VLPs and may form the basis of new candidate HIV vaccine work.

MATERIALS AND METHODS

Cell Culture

Baby hamster kidney (BHK-21) cells were grown to confluence in 150-cm^2 TC dishes (SPL) using alpha-minimum essential medium (α-MEM), supplemented with 10% tryptose phosphate broth, 5% fetal bovine serum (FBS), 20 mM HEPES, and 2 mM glutamine (Gibco BRL), and incubated at 37°C in 5% CO_2 for 1–2 days [22].

Construction of SFV Recombinants

SFV recombinants carrying the full-length *gag* (832–2,328 bp), *gagpol* (832–5,132 bp), and *env* (6,264–8,879 bp) genes from a primary HIV-1 Clade E, 90CR402 (GenBank acc. no. U51188) were constructed. *Gag* and *gagpol* were amplified using forward primer *Bam*HIGAG-S (5'-TTA*GGATCC*ATGGGTGCGAGAGCGTCA-3') and two reverse primers, *Bam*HIGAG-AS (5'CGC*GGATCC*CTGTTACTGTGACAAGGGGTCGTTGCCAAA-3') and *Bam*HIGPOL-AS (5'-CGCGGATCCCTAATCCTCATTCTGTCTACC-3'), respectively. For amplification of *env*, primers *Bam*HIENV-S (5'-CGCGCCTCGAGCG*GGATCC*CATGAGAGTGAAGGGGACACGGA-3') and *Bam*HIENV-AS (5'-AAT*GGATCC*TATAGCAAAGCCCTTTCCAAGCCC-3') were used. The amplified cDNAs were ligated into pSFV1 [5] via the *Bam*HI site. The resulting recombinant plasmids were designated pSFV/gag, pSFV/gagpol, and pSFV/env, respectively. Recombinant SFVs carrying *gag* and *env*, or *gagpol* and *env*, were constructed by amplifying the *env* gene and the 26S subgenomic promoter (ACCTCTACGGCGGTCCTAGATTGG) of SFV from pSFV/env using primers *Eco*RV-26S-S (5'-CCG*GATATC*ACCTCTACGGCGGTCCTA-3') and *Eco*RVENV-AS (5'-AAT*GATATC*TATAGCAAAGCCCTTTCCAAGCCC-3'). The amplicon was digested with *Eco*RV, and then inserted into the *Sma*I site of pSFV/gag and pSFV/gagpol, resulting in pSFV/gag-env and pSFV/gagpol-env, respectively.

Immunocytochemistry

RNA transcripts were generated *in vitro* from linearized SFV recombinants using SP6 RNA polymerase (Roche, Mannheim, Germany) according the manufacturer's instructions, and electroporated into BHK-21 cells (10^7 cells/cuvette) using a Bio-Rad Gene Pulser. At 36 h post-transfection, the cell monolayers were washed twice with cold PBS [pH 7.4] then fixed with cold methanol for 10 min at 4°C. Cells were washed again with PBS, and then mixed with the following primary antibodies: anti-gp160 mouse monoclonal antibody, anti-p24 rabbit antiserum, anti-protease sheep antiserum, and serum from a human AIDS patient. The antigen-antibody complexes were detected with the appropriate secondary antibodies (biotinylated anti-mouse, anti-rabbit, anti-sheep, or anti-human IgG) followed by streptavidin-biotinylated horseradish peroxidase. Samples were then visualized with the Vectastain DAB substrate (Vector, Burlingame, CA, U.S.A.) according to the manufacturer's instructions.

Production of HIV-VLPs

Transfection of BHK-21 with SFV transcripts was carried out as above. At 48 h post-transfection, HIV-VLPs were recovered from culture supernatants by centrifugation at 1,250 ×*g* for 10 min followed by ultracentrifugation through a 20% (w/v) sucrose cushion in TE buffer (10 mM Tris-Cl [pH 7.4], 1 mM EDTA) at 100,000 ×*g* at 4°C for 3 h. The viral pellets were resuspended in 100 ml TNE buffer (20 mM Tris-HCl [pH 7.4], 100 mM NaCl, and 0.5 mM EDTA) and stored at −70°C.

Density Gradient Centrifugation

The purified HIV-VLP suspension was layered on top of a 20–60% (w/v) linear sucrose gradient, centrifuged at 100,000 ×*g* for 12 h at 4°C, and then separated into 1 ml fractions. Each fraction was diluted to 5 ml with TNE buffer and centrifuged at 150,000 ×*g* for 1 h at 4°C. The resulting precipitates were resuspended in 100 μl of TNE buffer and screened for the presence of HIV-VLPs by detection of the p24 protein using the Retro-Tek HIV-1 p24 Antigen ELISA kit (Zeptometrix, Buffalo, NY, U.S.A.). HIV-VLP-containing fractions were electrophoresed on 4–12% or 4–20% polyacrylamide gels (Invitrogen, Carlsbad, CA,

U.S.A.) and transferred to polyvinylidene difluoride (PVDF) membranes. Immobilized proteins were probed with rabbit polyclonal anti-p24 and monoclonal anti-env (Chemicon, Temecula, CA, U.S.A.) antibodies followed by biotinylated anti-rabbit or anti-mouse IgG. Samples were then visualized with the SuperSignal chemiluminescence detection kit (Pierce, Rockford, IL, U.S.A.).

Electron Microscopy

Purified HIV-VLPs were adsorbed onto carbon-coated nickel grids (Ted Pella, Redding, CA, U.S.A.) and blocked with 0.1% gelatin (w/v) and 0.8% FBS (v/v) in PBS. The HIV-VLP-coated grids were incubated with anti-env antibody for 1 h and then labeled with gold-conjugated goat anti-mouse IgG for 1 h. The nickel grids were washed thoroughly with PBS, negatively stained with 2% phosphotungstic acid solution, and viewed by transmission electron microscopy following standard procedures.

Analysis of HIV-VLP-Associated RNA

Non-HIV-VLP-associated nucleic acids were removed from sucrose-purified HIV-VLPs by treatment with RNase A and DNase I at room temperature for 1 h. Viral RNA was extracted using the Viral RNA purification kit (Qiagen, Crawley, U.K.) and then reverse-transcribed with p24 antisense primer (5'-CCCAAGCTTTTAGCATGCTGTCATCATTTC-3'), gag500 antisense (5'-ACCCTTCTCTTCTACTACTT-3'), or gp41 antisense (5'-GGTTCTGCAGAAGCTTCCTTGT-TATTTCAAACCA-3') and Superscript II™ RT (Invitrogen) at 42°C for 60 min according to the manufacturer's instructions. To identify HIV-VLP-associated RNAs, the resulting cDNA was PCR amplified with Taq polymerase (Takara, Tokyo, Japan) and the following gene-specific primers: *gag*, p24 sense (5'-CCCAAGCTTCATCAGGCCTTATCACCT-3') and p24 antisense; SFV genomic RNA, SFV7200 sense (5'-GACGAGGTTAGCAAGTGGTT-3') and gag500 antisense; *env*, gp41 sense (5'-TCCCCCGGGAAGCTTGCGCAGC-AGCATCTGTTG-3') and gp41 antisense. Thermocycling was carried out with an initial denaturation at 94°C for 5 min, followed by 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. A final extension was carried out for 7 min at 72°C. The amplicons were electrophoresed in a 1% agarose gel in TAE (w/v), and then stained with ethidium bromide and UV transilluminated at 320 nm [21].

Mouse Immunization

Twenty μg of Gag, Gagpol, GagEnv, and GagpolEnv HIV-VLPs were emulsified separately with 0.2 ml complete Freund's adjuvant and administered to three female BALB/c mice (6–8 weeks of age) in each group via subcutaneous injection. Boosters containing the same amount of antigen emulsified with 0.2 ml incomplete Freund's adjuvant were administered twice at 2-week intervals. Serum samples and spleens were collected for immunological assays two weeks after the final immunization

Detection of Anti-HIV Antibodies

Serum samples from immunized mice were tested for anti-Gag and anti-Env antibodies by Western blot analysis. Purified $Pr55^{gag}$ or purified gp140 proteins were immobilized onto PVDF membranes, which were then cut into strips. Sera from immunized mice (1:500) were reacted with the immobilized proteins for 1 h at room temperature, followed by treatment with biotin-conjugated goat anti-mouse antibody (Invitrogen; diluted 1:1,000) for 1 h at room temperature. Bound antibodies were visualized by adding avidin-biotin solution followed by chemiluminescence and autoradiography.

ELISPOT Assay

IFN-γ-secreting T cells were quantified by enzyme-linked immunospot assay (ELISPOT; BD Pharmingen, Franklin Lakes, NJ, U.S.A.) according to the manufacturer's protocol. Briefly, ELISPOT plates (Whatman Polyfiltronics, Clifton, NJ, U.S.A.) were coated with 10 μg anti-mouse IFN-γ IgG per well and filled with 200 μl RPMI supplemented with 10% FBS and incubated at 37°C in 5% CO_2. Fresh splenocytes from the immunized mice were harvested and resuspended in RPMI supplemented with 10% FBS. ELISPOT wells were loaded with 100 μl splenocytes (2×10^6, 1×10^6, or 5×10^5 cells/well), followed by 2 μg of $H\text{-}2^d$-restricted CTL epitope-encoding [11, 28] Gag (AMQILKDTI, TTSTLHEQI and EPFRDYVDRF) and Env (GPGRVFHTT) peptides to each well together. For control wells, medium without peptide was added and incubated for 48 h at 37°C in 5% CO_2. Bound IFN-γ was visualized by reacting with biotinylated rat anti-mouse IFN-γ, followed by addition of streptavidin-horseradish peroxidase and the peroxidase membrane substrate (Sigma, St. Louis, MO, U.S.A.). The enzyme-substrate reaction was allowed to proceed at room temperature for 30 min or until spots formed, and then terminated by washing the samples with distilled water. The plates were air dried overnight and examined the following day.

RESULTS

Expression of HIV-1 Structural Proteins using the pSFV System

Recombinant plasmids harboring the HIV-1 *gag*, *gagpol*, and *env* genes were constructed by inserting the *gag* and *gagpol* genes after the 26S subgenomic promoter of the pSFV1 expression vector, with the *env* gene inserted further downstream under the control of a second subgenomic promoter (Fig. 1). To express the HIV-1 structural genes in mammalian cells, BHK-21 cells were transfected with *in vitro* RNA transcripts of pSFV/gag-env and pSFV/gagpol-env. Expression of the pSFV/gag-env and pSFV/gagpol-

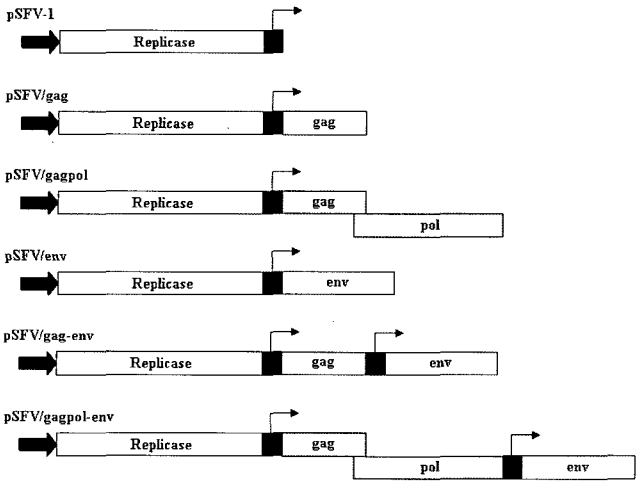

Fig. 1. Construction of different recombinant SFV vectors expressing HIV-1 Gag, Gagpol, Gag and Env, or Gagpol and Env.

The pSFV-1 was used for cloning of the HIV-1 *gag* (pSFV/gag), the HIV-1 *gagpol* (pSFV/gagpol), and the HIV-1 *env* (pSFV/env). pSFV/gag and pSFV/gagpol were the target to insert the subgenomic promoter and *env*, resulting in a recombinant SFV expressing HIV-1 Gag and Env, or HIV-1 Gagpol and Env. The bold arrow indicates the SP6 promoter for the transcription *in vitro* and the narrow arrow denotes the promoter, which is recognized by the viral replicase for transcription of the subgenomic RNA encoding the foreign genes.

env transcripts was confirmed by immunocytochemistry 36 h after electroporation. To check the expression of HIV-1 recombinant proteins, control BHK-21 cells and BHK-21 cells transfected with *gag-env* and *gagpol-env* transcripts were observed by immunocytochemistry with HIV-specific antibodies (Fig. 2). Human serum (from an AIDS positive patient) was used to detect expression of HIV-1 (Fig. 2A). Gag expression was confirmed by polyclonal anti-p24 antibody (Fig. 2B), Pol expression by polyclonal anti-protease antibody (Fig. 2C), and Env expression by anti-gp160 antibody (Fig. 2D). BHK-21 cells transfected with *gag-env* and *gagpol-env* transcripts expressed both Gag and Env, whereas only Gagpol was expressed in BHK-21 cells transfected with *gagpol-env*, and control BHK-21 cells expressed none of the HIV-1 proteins. Relatively fewer cells expressed *gagpol* than expressed *gag*, perhaps due to the "ribosomal frameshift" phenomenon, which is generally observed in 5% of HIV RNA transcripts [19]. The number of BHK-21 cells expressing *env* was higher in cells transfected with *gag-env* transcripts compared with those transfected with *gagpol-env*, possibly suggesting that the efficiency of a second subgenomic promoter directing the expression of *env* may be affected by its relative distance from the SP6 promoter region of pSFV1.

HIV-VLP Formation from a Single SFV Replicon

HIV-VLPs were harvested from BHK-21 cells at 36 h post-transfection with pSFV/gag, pSFV/gag-env, and pSFV/gagpol-env RNA transcripts. The virions were purified by ultracentrifugation on a 20% sucrose cushion and then analyzed by Western blotting. Figure 3 shows HIV proteins expressed in HIV-VLPs recovered from transfected BHK-21 cells. Expression of the $Pr55^{gag}$ precursor protein (~55 kDa) was observed in HIV-VLPs recovered from *gag*-transfected BHK-21 cells; in these samples, the protein detected by the anti-p24 antibody was approximately 50 kDa, perhaps indicating a partially degraded form of $Pr55^{gag}$. HIV-VLPs recovered from BHK-21 transfected with the *gagpol-env* transcript were found to express $Pr55^{gag}$, as well as the 41 kDa processing intermediate and the 24 kDa final cleavage product of p24 (Fig. 3A). In addition, the HIV Env protein (160 kDa) was observed in HIV-VLPs recovered from *gag-env*- and *gagpol-env*-transfected BHK-21 cells, suggesting that the Env protein was successfully incorporated into the host membrane preceding virus release (Fig. 3B).

To confirm that the Env protein was specifically found on the surface of the virion and to characterize the general morphology of GagEnv and GagpolEnv VLPs, we purified the VLPs and examined them by electron microscopy. The virions were adsorbed onto carbon-coated nickel grids, incubated with mouse anti-*env* antibody, labeled with gold-conjugated goat anti-mouse IgG, negatively stained, and examined by transmission electron microscopy. Figure 4 shows that most of the virus-like particles were circular, with diameters of approximately 100 nm. GagEnv consisted of an electron-dense spherical shell with a translucent interior core region similar to that of immature virions. We also observed that GagpolEnv was a subset of virus-like particles containing irregular cores surrounded by an electrolucent shell. In both types, abundant amounts of Env protein were observed on the virion surfaces.

Analysis of Gag-Env and Gagpol-Env VLPs by Sucrose Density Gradient

To determine whether HIV-VLPs were similar to wild-type virions in terms of particle density, the VLPs were concentrated by ultracentrifugation through a 20% equilibrium density sucrose gradient, followed by equilibrium density centrifugation on a 20–60% linear sucrose gradient. Fractions (1 ml) were collected and the presence or absence of viral particles in each fraction was determined by HIV-1 p24 ELISA and Western blot analyses.

As shown in Fig. 5A, GagEnv VLPs were found in the 1.16–1.18 g/ml density fraction. In contrast, the GagpolEnv VLPs had a density of 1.14–1.16 g/ml, which was similar to that previously reported for wild-type HIV-1 particles [38], although the density of the immature HIV-VLPs may be a little higher than that of mature HIV-VLPs. Expression of Gag and Env was confirmed by Western blot analysis of the gradient fractions using polyclonal anti-p24 and monoclonal anti-env antibodies (Fig. 5B). HIV-VLPs from cell culture supernatants transfected with pSFV/gag-env transcripts

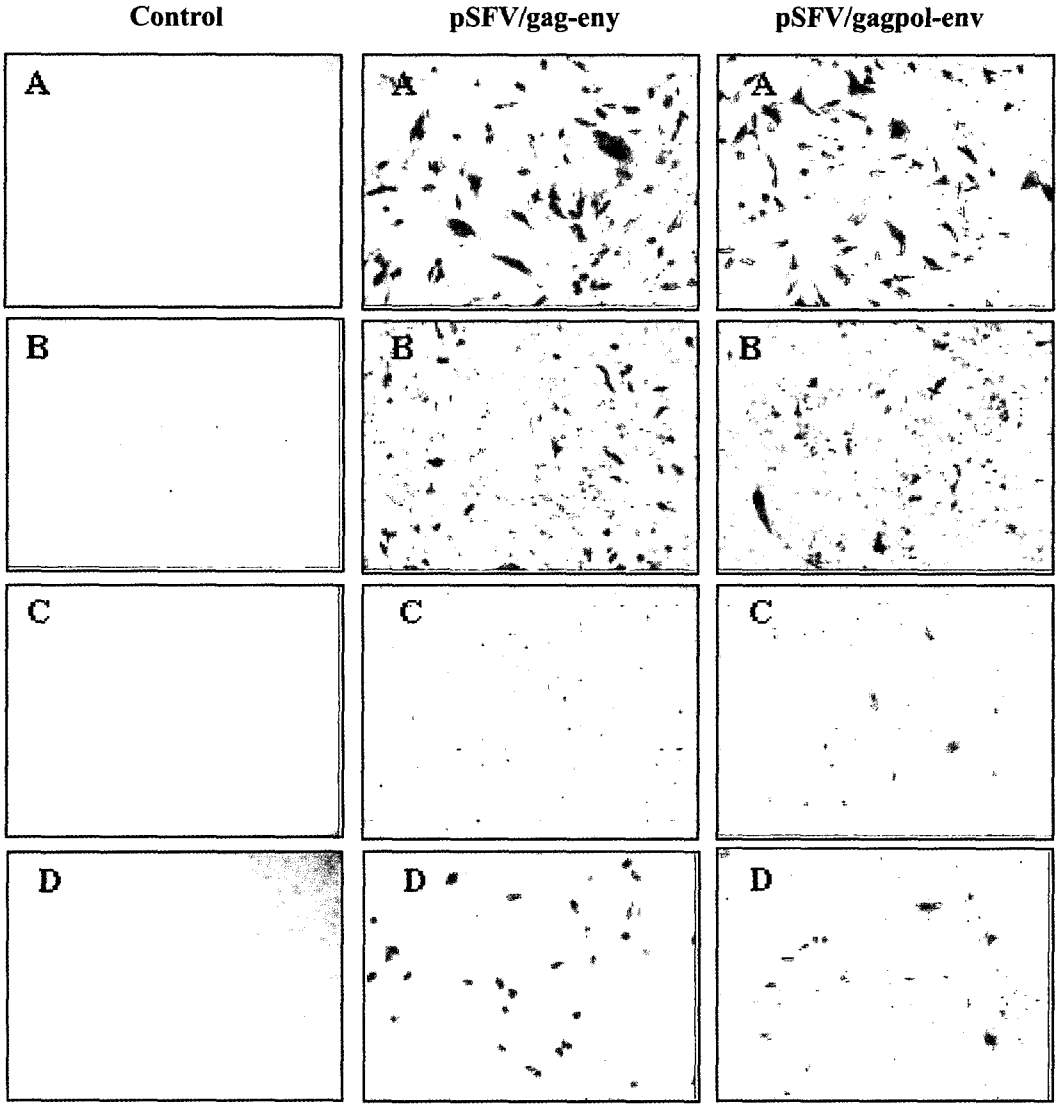

Fig. 2. Expression of HIV structural proteins on BHK-21 cells.
BHK-21 cells were transfected with RNA transcripts from control (left), pSFV/gag-env (middle), or pSFV/gagpol-env (right) vectors and cultured for 36 h. The cells were then fixed and Gag and Env expression was detected with human AIDS patient serum (**A**), anti-p24 Ab (**B**), anti-protease Ab (**C**), and anti-gp160 MAb (**D**).

expressed only $Pr55^{gag}$, whereas VLPs harvested from cells transfected with pSFV/gagpol-env transcripts consisted of proteolytically processed p24, two intermediate precursor species (p45 and p39), as well as $Pr55^{gag}$, as previously observed (Fig. 3). The amount of $Pr55^{gag}$ protein peaked in fraction 6 for GagEnv VLPs, whereas in the case of GagpolEnv VLPs, the $Pr55^{gag}$ and p24 protein expressions peaked in fraction 7. In GagEnv VLPs, there was not much proteolytic cleavage of the gp160 Env protein precursors, as indicated by detection of large amounts of gp160. In contrast, the gp160 Env precursor protein and an abundance of its proteolytic processing products (gp120 and gp41) were detected in GagpolEnv VLPs. These results indicate that both cellular and viral proteases were necessary to cleave the Env and Gag proteins efficiently where appropriate. We could also detect the oligomeric forms of Env protein in the fraction numbers 6 to 8 of GagpolEnv VLPs (Fig. 5B).

RNA of HIV-VLPs

SFV-derived vector containing *gag* and *env* genes produced three species of RNAs; a full-length genomic RNA and two subgenomic RNAs (Fig. 6A). We performed RT-PCR with the viral genome isolated from HIV-VLP using three different sets of primers to assay what kind of RNAs were packaged during viral assembly. As shown in Fig. 6B, more *p24* gene transcripts (650 bp) were detected in comparison with those of SFV-*gag* (700 bp), suggesting that HIV-VLPs may be packaged more efficiently with subgenomic RNA

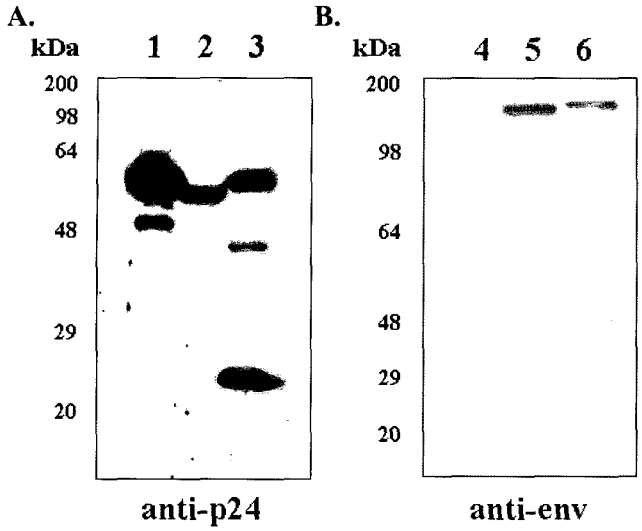

Fig. 3. Western blot analysis of HIV-VLPs.
BHK-21 cells were transfected with RNA transcripts of pSFV/gag (lane 1), pSFV/gag-env (lane 2), and pSFV/gagpol-env (lane 3), and HIV-VLPs produced in the cell culture supernatants were collected by ultracentrifugation 36 h later. The HIV-VLPs were then separated by SDS-PAGE and analyzed by Western blotting using anti-p24 (**A**) and monoclonal anti-gp160 (**B**) antibodies.

than with the full recombinant SFV replicon. We also tested the possibility that the smaller subgenomic RNA encoding Env protein was involved in the packaging into the VLPs. The amount of amplified *gp41* gene was a little larger than those of *p24* (Fig. 6B, lanes 2 and 4, respectively). This result showed that the smaller subgenomic RNA transcripts were also included nonspecifically into the VLPs for the composition of the structure of the particles.

HIV-VLPs Elicit Both Humoral and Cellular Immune Responses in Mice

To determine whether the generated HIV-VLPs are capable of stimulating host immune responses, female Balb/c mice were immunized with purified Gag, Gagpol, GagEnv, and GagpolEnv VLPs. Serum samples from the immunized mice were tested for anti-HIV antibodies by Western blotting. All four species of HIV-VLPs were able to induce production of antibodies against the $Pr55^{gag}$ protein (Fig. 7A). In addition, HIV-VLPs containing the Env protein on their surfaces also induced anti-gp140 antibody production.

Next, we investigated CTL activity due to HIV-VLP immunization. Fresh spleens from immunized mice were collected and passed thru a 70 μm sieve to generate single

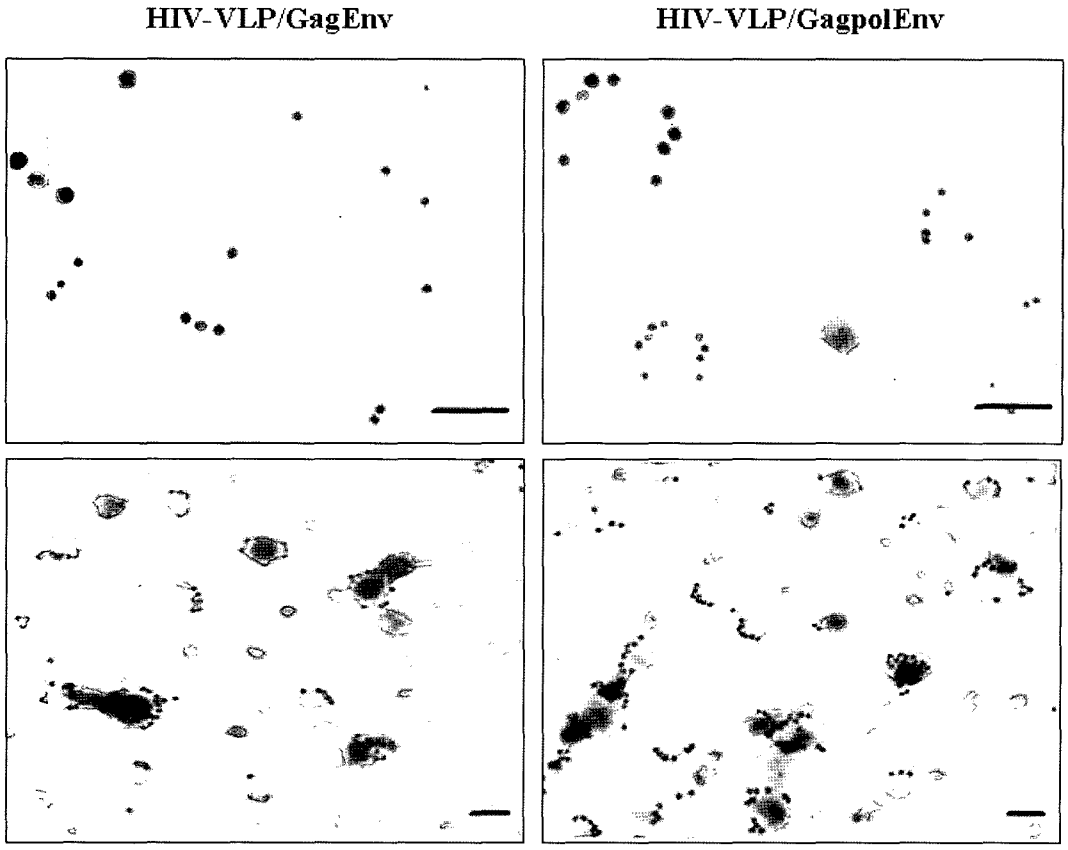

Fig. 4. Electron micrographs of purified HIV-VLPs.
HIV-VLPs composed of GagEnv (A) and GagpolEnv (B) were absorbed to grids and incubated with anti-gp160 MAb. Bound MAbs were detected with an anti-mouse IgG-gold conjugate and analyzed by negative-staining electron microscopy. Bar=100 nm.

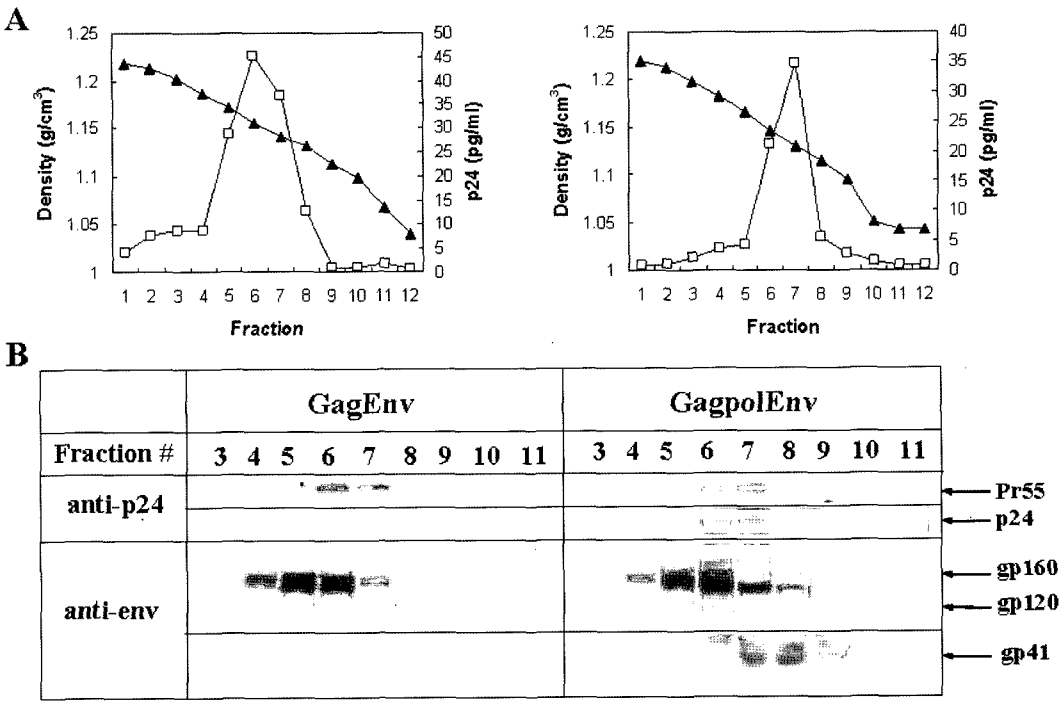

Fig. 5. Characterization of HIV-VLP prepared by sucrose density gradients ultracentrifugation.
HIV-VLPs released from BHK-21 cells transfected with pSFV/gag-env (Fig. 5A left) or pSFV/gagpol-env (Fig. 5A right) RNA transcripts were harvested and pelleted through a 20% sucrose cushion. The HIV-VLPs were resuspended, layered on top of a linear 20–60% sucrose gradient, and subjected to centrifugation at 35,000 rpm in an SW41Ti rotor for 18 h at 4°C. Each gradient fraction was assayed by p24 ELISA (**A**) and Western blotting with polyclonal rabbit anti-p24 (upper panel) and monoclonal anti-gp160 (lower panel) antibodies (**B**). Symbols. ▲, the sucrose gradient; □, the p24 concentration of each fraction.

cells, which were then subjected to ELISPOT analysis for IFN-γ. We counted the mean number of IFN-γ-secreting cells per million splenocytes after specific stimulation with HIV-1 Gag and Env MHC class I CD8-restricted peptides. All groups immunized with mature HIV-VLPs were found to have a higher number of IFN-γ-producing cells as compared with groups immunized with immature HIV-VLPs (Fig. 7B). Splenocytes from mice immunized with Env-containing HIV-VLPs produced substantially more IFN-γ spots compared with those from mice immunized with HIV-VLPs lacking Env. These results indicate that the Env protein is beneficial to increasing cell-mediated immunity.

Discussion

An ideal AIDS vaccine candidate should be reasonably safe and capable of eliciting strong, long-lasting humoral and CTL responses. Thus, many researchers have currently focused on the use of VLPs or recombinant viral vectors presenting HIV antigens in ways that mimic the native virion. In order to produce HIV-VLPs incorporating the relevant structural proteins, we cloned the *gag* and *env* genes downstream of the subgenomic promoter of an SFV replicon, separated by a second subgenomic promoter. We were able to obtain HIV-VLPs expressing *gag/env* or *gag/pol/env* by transfection of a single SFV transcript into mammalian BHK-21 cells. Previously, both the HIV *gag* and *env* genes had been simultaneously expressed from a single rhabdovirus-based vaccine vector genome [29]. Although the GagpolEnv pseudovirions generated by this system elicited a vigorous cytotoxic T-cell response in mice, bullet-shaped rabies virus particles were also produced along with the pseudovirion, raising the question of harmful effects due to preexisting immune responses in the human population. In another study, a human adenovirus vector was used to generate chimeric GagEnv VLPs that were able to induce humoral responses in mice [27]. However, adenovirus proteins were displayed on the chimeric virus; this could decrease the efficacy of the vaccine since most humans already have immunities against adenoviruses. Thus, we sought to generate an HIV-VLP that did not contain viral vector antigens. To this end, we used a SFV-derived expression system without the accompanying helper plasmid to produce HIV-VLPs incorporating only HIV-1 proteins.

Newly budded virus-like particles have an immature morphology. Gag proteins surround the periphery of the particle just underneath the plasma membrane-derived lipid envelope, giving it a doughnut-shaped appearance [34]. After budding, the viral protease (PR) is activated and

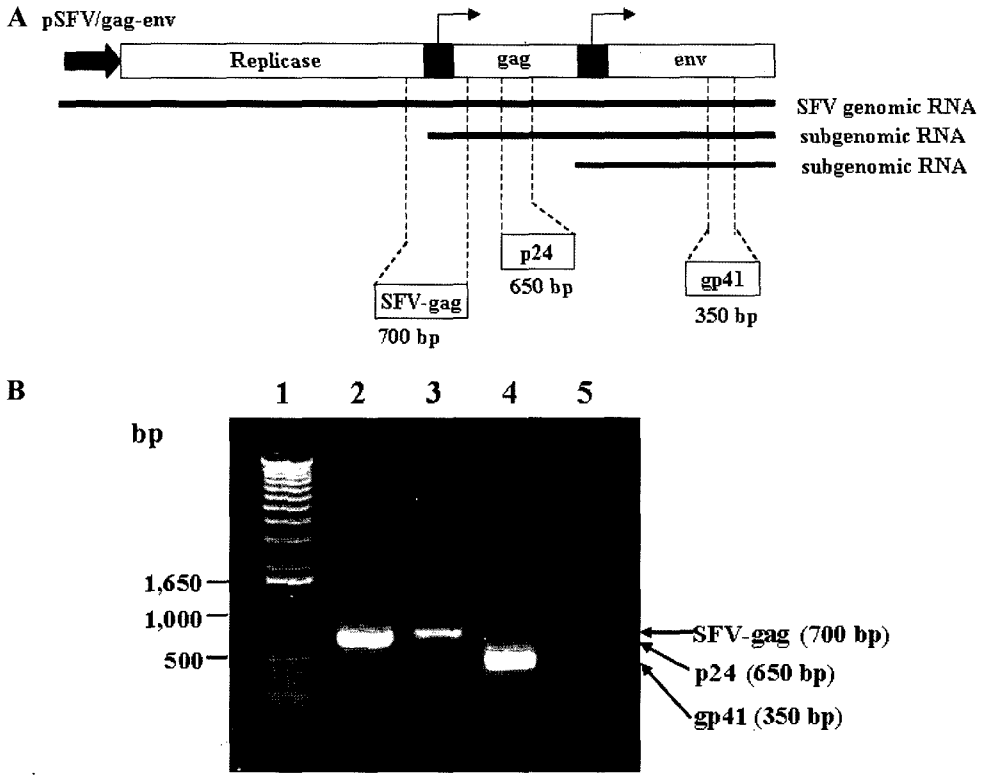

Fig. 6. Detection of RNAs in HIV-VLPs composed of GagEnv proteins.
(A) The schematic diagram shows the position of the oligonucleotide. (B) RT-PCR was performed with viral RNA of HIV-VLPs. (lane 1, 1 kb plus DNA ladder; lane 2, p24 (650 bp); lane 3, SFV-gag (700 bp); lane 4, gp41 (350 bp); lane 5, control).

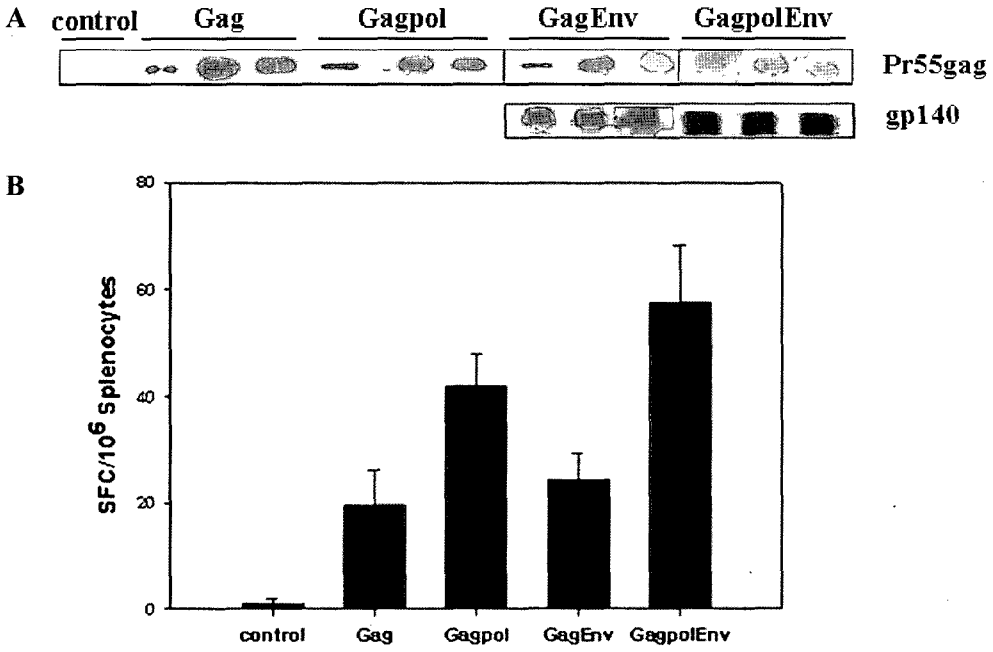

Fig. 7. Humoral and cell-mediated immune responses in mice immunized with purified Gag, Gagpol, GagEnv, and GagpolEnv HIV-VLPs.
For the humoral immune response, serum samples from the immunized mice were examined for reactivity to the HIV structural protein using Western blot strips containing purified $Pr55^{gag}$ and gp140 proteins (**A**). IFN-γ secretion was measured in splenocytes obtained from mice immunized with HIV-VLPs and stimulated with peptides. The graph shows averaged values from ELISPOT wells normalized to numbers of IFN-γ spot forming cells per 1×10^6 splenocytes; error bars represent the standard error of the mean (**B**).

some of the peripheral material is condensed into the cone-shaped core that characterizes mature HIV-1 virions [4]. EM analysis of the HIV-VLPs produced in our study showed that the HIV-VLPs were approximately 100–120 nm in diameter, which is similar to the HIV-1 virion. There were morphological differences between GagEnv and GagpolEnv VLPs because of the viral protease encoded by the *pol* gene. The former exhibited the thickened layers of protein at the membrane and the circular internal dense molecules. However, the latter contained the irregular conical cores similar to the characteristic of mature HIV-1 particles. The HIV-VLPs produced using our SFV expression system contained two subgenomic RNAs and genomic RNA even though the packaging signal of HIV-1 was not included. The amount of subgenomic RNAs in HIV-VLPs was more abundant than full-length SFV-derived RNA. This observation was in agreement with previous work [31] showing that SFV contained 2.4 kb subgenomic RNAs more than full 13 kb genomic RNAs.

In a previous study, naked SFV RNA containing the NP protein gene of the influenza virus successfully induced humoral and cell-mediated immunity against influenza NP proteins and reduced the viral load in the lungs after a challenge infection with mouse-adapted influenza [37]. However, since RNA is unstable, the use of recombinant viral particles or DNA vectors may form more practical approaches to the vaccine development. In macaque monkeys, recombinant SFV particles expressing the envelope glycoproteins of the human and simian immunodeficiency viruses (SIV) have been shown to induce protective immunity [30, 33]. Although the significance of VLP formation in the induction of host immune responses has not yet been fully established, HIV-VLPs produced in a single SFV-derived expression system induced specific humoral and cell-mediated immune responses in mice. This system could be very convenient to produce non-replicating HIV-VLPs presenting antigens from different HIV-1 clades in order to induce broader protection. This result indicates that HIV-VLPs produced with our single SFV-derived vector may be promising candidates for further development into a possible HIV vaccine.

Acknowledgment

This work was supported by National Research Laboratory Program grant (2000-N-NL-01-C-171) from the Korea Ministry of Science and Technology.

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