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High efficient production of Pr55^{gag} virus-like particles expressing multiple HIV-1 epitopes, including a gp120 protein derived from an Ugandan HIV-1 isolate of subtype A

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Abstract

The main goal of this study was to investigate a novel approach for an efficient and reproducible production of Virus-Like Particles (VLPs) expressing multiple HIV-1 epitopes. The HIV-1 Pr55^{gag}-based VLPs have been produced in a Baculovirus expression system, using a transfer vector able to support the independent expression of different open reading frames (ORFs). In this regard, the gp120 derived from 94UG018 HIV-1$_A$ isolate, previously studied in our laboratory, has been packaged into the VLPs together with *nef* and *pol* ORFs. In particular, the gp120$_{UG}$ sequence shows a 90% homology in the V3 region compared to African HIV-1 strains of the A-clade. This novel approach is extremely effective for the production of VLPs expressing all the epitopes, as confirmed by Western Blot characterization. Furthermore, the resulting HIV-VLP$_A$s show the expected density (1.14–1.18 g/ml) on a 10–60% sucrose gradient and the morphology of an immature virion at standard transmission electron microscopy. Our results demonstrate that this strategy is highly efficient for expressing a balanced amount of multiple epitopes and their packaging in VLP structures, without affecting the Pr55^{gag} autoassembling capacities. Furthermore, the genetic transposition performed in a modified *E. coli* represents a methodological improvement, allowing a faster and more reproducible identification of recombinant Baculovirus DNA molecules.

Keywords: Vaccine, HIV-1; Clade-A; VLPs; Baculovirus

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1. Introduction

The development of an effective, safe and affordable vaccine strategy represents a crucial goal for both industrialized and developing countries. Different candidate HIV-1 vaccine strategies have been developed since early 90s, including whole inactivated virus (Daniel et al., 1992; Almond et al., 1995; Cranage et al., 1997), recombinant HIV-1 envelope subunits (Berman et al., 1990; Girard et al., 1991; Fultz et al., 1992; el-Amad et al., 1995), live vector-based (Berglund et al., 1997; Girard et al., 1997) and DNA-based vaccines (Boyer et al., 1997; Letvin et al., 1997).

In order to develop a vaccine model characterized by the capacity of inducing a strong humoral as well as cellular immune response, without risks of pathogenicity, we have chosen an approach based on virus-like particles (VLPs). The HIV-1 $Pr55^{gag}$ precursor protein, when expressed in recombinant baculovirus-infected cells, assembles as immature, non-replicating and non-infectious HIV–VLPs (Gheysen et al., 1989; Wagner et al., 1994). This can be used as particulate antigen delivery system, able to induce a significant immune response against epitopes packaged in the resulting particles. In particular, VLPs have been employed to deliver linear epitopes or complete conformational proteins, with an effective induction of both arms of the immune response (Haffar et al., 1991; Rovinski et al., 1992; Griffiths et al., 1993; Wagner et al., 1994; Osterrieder et al., 1995; Deml et al., 1997a; Tobin et al., 1997).

In this perspective, different approaches have been employed to package HIV-1 envelope epitopes into VLPs in order to induce neutralizing antibodies. In particular, immunologically relevant sequences have been introduced into the $Pr55^{gag}$ precursor (Haffar et al., 1990; Wagner et al., 1994) or fused to the 3′ end of the gag ORF, taking advantage of the ribosomal frameshifting signals (Tobin et al., 1997). Moreover, the complete gp120 envelope protein has been packaged into the VLP in order to express conformational epitopes. In particular, the gp120 molecule has been anchored on the $Pr55^{gag}$-VLPs through the trans-membrane (TM) portion of the Epstein–Barr virus (EBV) gp220/350, to increase the expression and stability of the chimeric gp120-TM on the VLPs surface (Deml et al., 1997a). Chemical cross-linking analyses suggest that these gp120-TM molecules retain the ability to form oligomeric structures (Deml et al., 1997a), which seem to be a conformational requirement for the induction of cross-neutralizing antibodies (Sattentau and Moore, 1995; Fouts et al., 1997). Such envelope-presenting HIV–VLPs have shown a significant immunogenicity in Balb/c mouse and Rhesus Macaque models, with the induction of both neutralizing antibodies and CTLs in absence of adjuvants (Deml et al., 1997b; Wagner et al., 1998). Moreover, the VLP model is currently under investigation as potential vaccine for other human and non-human viruses, such as hepatitis, papilloma, pseudorabies and equine herpes viruses (Kirnbauer et al., 1992; Garnier et al., 1995; Osterrieder et al., 1995; Li et al., 1997).

Based on these extensive observations on the feasibility and efficacy of the VLP model as a potential preventive vaccine, we investigated a novel approach to obtain a more efficient packaging of multiple epitopes/proteins in the VLP structures. Our intent was to increase the transposition frequency of multiple sequences on the same baculovirus genomic DNA, in a prokaryotic system, and to find a more reliable strategy of screening the recombinants, in order to avoid the homologous recombination step in insect cells with multiple rounds of plaque purification. The approach described in this study is based on a commercially available baculovirus expression system (Gibco-BRL), which allows the independent expression of multiple genes from the same transfer vector. A site-specific transposition of viral genes into the baculovirus DNA is then obtained within a modified bacterial strain and the generation of recombinant baculovirus DNA is coupled to the identification of colorless bacterial colonies.

The latest data produced by the 'UNAIDS HIV Network' indicate that the A- and C-clades account for 73% (25 and 48%, respectively) of all HIV-1 isolates identified worldwide, implying that, in order to have better chances of efficacy, future vaccine strategies should be based on a cocktail of antigens derived from such clades.

This prompted us to package into the VLP a $gp120_{UG}$ derived from an Ugandan HIV-1 isolate of the A-clade (UG5.94UG018), previously characterized in our Laboratory (Buonaguro et al., 1995, 1998a,b).

Furthermore, the possibility has been verified of packaging into the VLPs the chimeric $gp120_{UG}$-TM molecule as well as the Nef and Pol proteins which show a much lower divergence among HIV-1 strains and could induce cross-clade immunity.

Our results clearly indicate that multiple epitope/proteins can be included into the VLP structure without affecting the $Pr55^{gag}$ auto-assembling properties and, therefore, that $Pr55^{gag}$-based VLPs can provide a ductile antigenic presentation model for developing candidate anti-HIV-1 vaccines.

2. Materials and methods

2.1. Cloning of virus genes in the transfer vector

The trans-membrane (TM) fragment of the EBV glycoprotein gp220/350, consisting of 22 aa of the trans-membrane domain and 27 aa of the cytoplasmic region, was linked to the C-terminus of the gp120 coding region via a Ser-Gly-Ser-Gly-Ala-Gly flexible hinge, as previously described (Deml et al., 1997a). In particular, the gp120 reading frame from the 94UG018 Ugandan HIV-1 A-clade isolate (Buonaguro et al., 1998b) was PCR amplified by the primer set A (5′-CGTCGCGAATTCAGAAGACAGTGGCAATGA-3′) and B (5′-ATATTATCCGGACACCACTCT-TCTCTTTGC-3′), where the underlined nucleotides indicate the *Eco*RI and *Mro*I sites, respectively, and cloned into a modified pUC8 vector, upstream of the hinge-TM fragment (Deml et al., 1997a). The resulting 120-TM fusion gene was PCR amplified by the primer set C (5′-GCCTTAGCTAGCAGAAGACAGTGGCAATGA- 3′) and D (5′-GCCTTAGCTAGCTTATACATAGGTCTCGGCCTC-3′), where the underlined nucleotides indicate the *Nhe*I site. The amplified fragment was subcloned in the corresponding site, downstream of the p10 promoter, and positive clones were screened for the correct orientation relative to the driving promoter.

2.2. Production of recombinant baculovirus

Recombinant bacmids were obtained, in the DH10Bac™ *E. coli*, by site-specific transposition of the mini-Tn7 element from the recombinant pFastBac Dual vector to the mini-*att*Tn7 attachment site on the bacmid, with the Tn7 transposition functions provided *in trans* by the pMON7124 helper plasmid. The transposition of viral genes on the bacmid disrupts the *lac*Zα reading frame, resulting in white bacterial colonies over a background of blue colonies that harbor the parental bacmid. The recombinant bacmid DNA was isolated from bacteria following a protocol specifically developed for large plasmids (>100 kb) (Ioannou et al., 1994). The insertion of viral genes was verified by the polymerase chain reactions (PCR) performed with pUC/M13 universal primers, located on either side of the mini-*att*Tn7 sequence of the bacmid, paired with internal primers specific for the transposed sequences:

(5′-GGCCCCTAGGAAAAAGGGCT-GTTGG-3′) for the $Pr55^{gag}$ sequence, and (5′-TGTAAAACGACGGCCAAGTCTGTTAAATGGCAGTCTAGC-3′) for the gp120 sequence. All PCR amplifications were performed in 1.5 mM $MgCl_2$ and were based on 30 cycles with an elongation step of 5 min at 72°C.

2.3. Insect cell cultures and VLPs production

Spodoptera frugiperda Sf-9 insect cells were propagated in TC100 medium supplemented with 10% FCS (Gibco-BRL) and 9×10^5 cells seeded in a 6-well plate were transfected with 10–20 μg of recombinant bacmid DNA, by the Cellfectin method (Gibco-BRL). For large-scale preparations of VLPs, HighFive cells, derived from *Trichoplusia ni* egg cell homogenates (Invitrogen Inc.) and propagated in serum-free SF900 medium (Gibco-BRL), were infected with the recombinant baculovirus, released in the Sf9 cell culture supernatants, at a multiplicity of infection (m.o.i.) of 5. Both insect cell lines were propagated at 28°C in the absence of CO_2.

2.4. Analysis of virus-like particles protein expression

Cells and supernatants from Sf9 or HighFive cell cultures were collected 4 days post-transfection/infection. Lysates were prepared by boiling cellular pellets for 5 min in a buffer containing 5% β-mercaptoethanol, 2% SDS, 10% glycerol and 0.01% bromophenol blue. In parallel, supernatants were clarified by centrifugation at 2000 × *g* for 15 min at 4°C and VLPs were pelleted by ultra-centrifugation at 100 000 × *g* for 75 min through a 25% sucrose cushion, as previously described (Rovinski et al., 1992). Samples were resuspended in 40 μl of TNE buffer (10 mM Tris–HCl, pH 8.0, 100 mM NaCl and 1 mM EDTA), mixed with 10 μl of 5 × Laemmli sample buffer (Laemmli, 1970) and boiled for 3 min. Viral proteins were separated by SDS-PAGE and analyzed by conventional Western Blot technique (Sambrook et al., 1989; Buonaguro et al., 1992). Immobilized proteins were incubated with a 1:200 dilution of mouse anti-gp120/V3 or anti-p24 MAb (Intracel Co.) and, subsequently, with biotin-conjugated anti-mouse IgG antibodies (1:1500 dilution). The bound molecules were visualized by the addition of horseradish peroxidase (HRPO)-conjugated streptavidine and the chromogen 4-Chloro-1-Naphtol.

2.5. Sucrose gradient fractionation analysis

VLPs pelleted from cell culture supernatants by ultra-centrifugation were resuspended in TNE solution, layered onto a continuous sucrose gradient (10–60%) and centrifuged at 100 000 × *g* for 1.5 h at 4°C in a Beckman SW41 rotor. Gradient fractions were collected from the bottom of the tubes in 500 μl aliquots and the VLPs quantified by a commercial p24-antigen capture assay (NEN-Dupont).

2.6. Chemical cross-linking

The VLPs purified through the sucrose gradient were treated with 0.5–2 mM of bis(Sulfosuccinimidyl) Suberate (BS^3), incubated at room temperature for 1 h and quenched for 15 min with 50 mM Tris, pH 7.5. Samples were resuspended in a solution containing 4% SDS, 100 mM Tris, pH 6.8, 10% Glycerin, 10% 2-mercaptoethanol, 0.01% bromophenol blue and boiled for 10 min. Samples were then subjected to electrophoresis on 5% SDS polyacrylamide gel under reducing buffer conditions. Proteins were transferred to nitrocellulose, immunoblotted with monoclonal antibodies directed against gp120 (Intracel Co.) and probed with a biotin-conjugated secondary antibody. The bound molecules were visualized by the addition of horseradish peroxidase (HRPO)-conjugated streptavidine and the chromogen 4-Chloro-1-Naphtol.

2.7. Electron microscopy

The p24 reactive fractions were pooled and analyzed for morphology. The VLPs (approximately 100 ng) were washed in PBS and fixed in 6.4% glutaraldehyde in PBS. Particles were then post-fixed with 2% osmium tetroxide for 1 h, embedded in Araldite and adsorbed to grids. Grids were stained with 2.6% alkaline lead citrate and 0.5% uranyl acetate in 50% ethanol. The analyses were performed by standard transmission electron microscopy technique and the pictures were taken with an electron microscope operated at 80 kV.

3. Results

3.1. V3 amino acid sequence of the 94UG018 isolate

The 94UG018 strain has been chosen among 10 HIV-1 Ugandan isolates, previously identified by us in a rural area of Northern Uganda within a cohort of pregnant women (Buonaguro et al., 1995).

The 94UG018 shows a 12.6% average nucleotide divergence with gp120s of the A-clade and clusters with Ugandan/Rwandan samples in a major subgroup with a very strong phylogenetic relationship (97% of bootstrap value) (Buonaguro et al., 1998b).

Furthermore, the V3 loop amino acid sequence shows an 82.9–100% range of homology (88.5% on average) with isolates of clade-A and a 94.3% homology (33 out of 35 aa) with the clade-specific Consensus sequence. In particular, the lack of mismatches between the 94UG018 and the IBNG isolate, identified in Nigeria in 1994 (Howard et al., 1994), indicates the presence of identical antigenic epitopes in virus isolates from unrelated countries (Fig. 1).

3.2. Construction of the HIV-1 $Pr55^{gag}$-pol-nef/$gp120_{UG}$-TM transfer vector

The pGag-Pol-Nef/Fast Bac vector was obtained by cloning a *gag-pol-nef* polycistronic cDNA into the MCS of the pFastBac Dual (Gibco-BRL) baculovirus transfer vector (plasmid), downstream of the pPolh promoter. The *nef* reading frame has been linked to the 3′ end of the *gag-pol* precursor (at the RNase H/Integrase proteolytic site) by a $(Gly\text{-}Ser)_3$ flexible hinge; the protease and reverse transcriptase enzymatic activities were knocked out by site directed mutagenesis (Wagner R. et al., unpublished results) (Fig. 2A).

The $gp120_{UG}$-TM fusion gene, was obtained by cloning the gp120 reading frame of the 94UG018 isolate into the *Eco*RI and *Mro*I sites of a modified pUC8 vector, upstream of the hinge-TM_{EBV} fragment (Deml et al., 1997a) (Fig. 2B).

Consensus	C T R P N N N T R K s V r I G P G Q a F Y A T G D I I G D I R Q A H C
UG94018	C T R P N N N T R K G V H I G P G Q A F Y A T G D I I G D I R Q A H C
IBNG	. . . . .
1703	. . . . . S . R . . . . . Y .
DJ264	. . . . . S . R . . . . . T . . . . . K . . .
RW20.5	. . . . . R . . . . . G . . . . .
RW9.15	. S . . . . . S . . . . . V . . . . . Y .
UG273	. . . . G . . . . T S . R . . . . .
UG275	. . . . . T S . R . . . . . S . . . . . K . . .
UG31.7	S . I . . . . . S . R . . . . . N . . . . .
UG37.8	. . . . . S . R . . . . . T . . . . .
VI191	. . . . S . . . . . G I . . . . . R . . . . . Q . T . . . . .
Z321	T . M . . . . . I S . . . . R . . F . . . . .

Fig. 1. Alignment of amino acid sequences from V3 regions of 94UG018 isolate and 11 reference strains of the A-clade. Nucleotide sequences were translated, aligned and compared by Clustal method using the MegAlign option of the Lasergene software package (DNASTAR Inc.). Sequence identity is represented by dots. The amino acid residues in capital letters of the Consensus sequence indicate a residue conservation in that position $>80\%$. The underlined sequence represent the tip of the V3 loop.

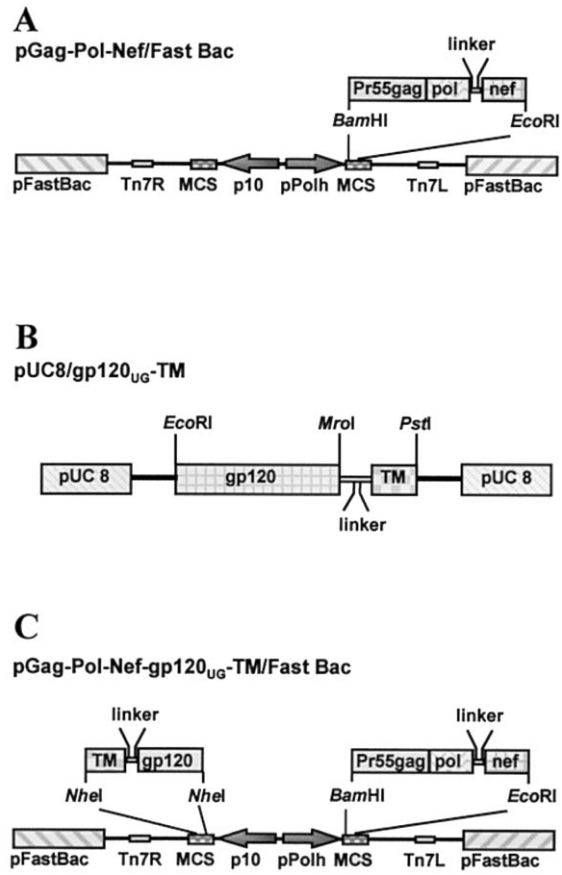

Fig. 2. (A) Schematic representation of the pGag-Pol-Nef/Fast Bac vector, which contains a *gag-pol-nef* polycistronic cDNA into the MCS of the pFastBac Dual (Gibco-BRL) baculovirus transfer vector, downstream of the pPolh promoter. The *nef* reading frame is linked to the 3′ end of the *gag-pol* precursor (at the RNase H/Integrase proteolytic site) by a $(Gly\text{-}Ser)_3$ flexible hinge. (B) The chimeric $gp120_{UG}$-TM glycoprotein is generated in a modified pUC8 vector, sub-cloning the gp120 coding sequence at the 5′ end of the $(Gly\text{-}Ser)_3$ flexible hinge-TM fragment. (C) Schematic representation of the final recombinant transfer vector containing the $gp120_{UG}$-TM fusion gene as well as the *gag*, *pol*, *nef* polycistronic cDNA. The two multiple cloning sites (MCS), located downstream of the p10 and pPolh Baculovirus late promoters, are flanked by Tn7 elements (L and R) for site-specific transposition into the Baculovirus shuttle vector (Bacmid).

The entire $gp120_{UG}$-TM sequence (1.7 Kb) was then PCR amplified and cloned into the *Nhe*I site position of the pGag-Pol-Nef/Fast Bac vector MCS, downstream of the p10 promoter (Fig. 2C). The final resulting vector (pGag-Pol-Nef-

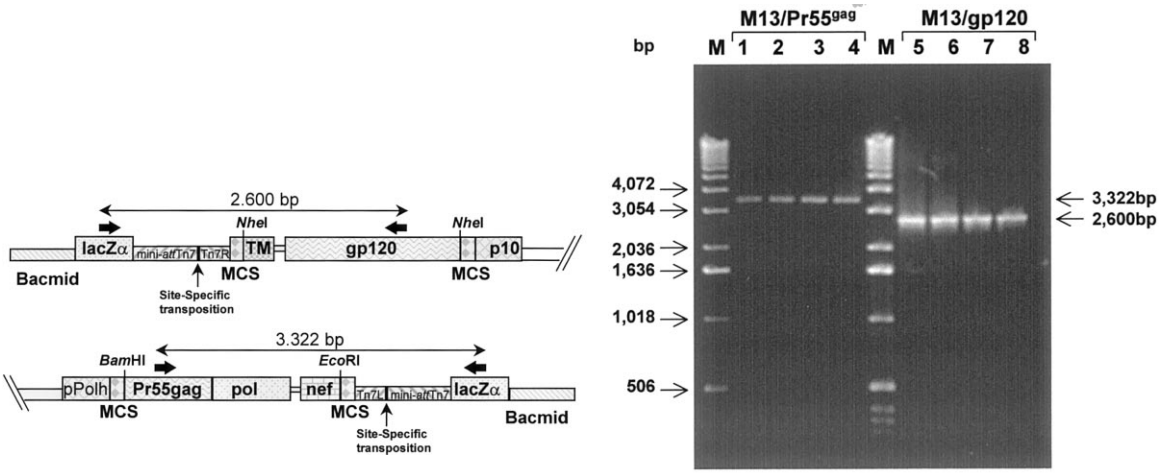

Fig. 3. (A) Schematic representation of the recombinant Bacmid resulting from the site-specific transposition of HIV-1 viral genes at the mini-*att*Tn7 sites. The transposed whole region breaks the *lac*Zα ORF with a loss of the β-galactosidase enzymatic activity. The amplification products, indicated with the corresponding size, are delimited by the two primer sets used for PCR screening. (B) Agarose gel analysis of PCR products obtained from four randomly-selected colorless colonies isolated after transformation of DH10Bac cells with the recombinant transfer vector. Amplification products of the expected molecular size (expressed in bp) were obtained with both primer sets from each individual colony (lanes 1 and 5, 2 and 6, 3 and 7, 4 and 8). The M lane contains the molecular weight marker 1 Kb DNA Ladder (Gibco BRL) and the size of corresponding bands (expressed in bp) is indicated on the left side of the figure.

$gp120_{UG}$-TM/Fast Bac) contains, therefore, the coding sequences for all the HIV-1 proteins to be packaged in the HIV–VLPs.

3.3. Generation of recombinant bacmid shuttle vector

The pGag-Pol-Nef-$gp120_{UG}$-TM/Fast Bac transfer vector has been subsequently used to transform DH10Bac™ *E. coli* bacterial strain (Gibco-BRL) modified to propagate both a baculovirus shuttle vector (bacmid), which contains mini-*att*Tn7 attachment sites, and the pMON7124 helper plasmid, which provides *in trans* the Tn7 transposition functions. The effective transposition of both $Pr55^{gag}$-*pol-nef* and $gp120_{UG}$-TM ORFs into the bacmid generates colorless bacterial colonies by disruption of the *lac*Zα reading frame (Fig. 3A). In our experiment, the colorless phenotype occurred in 10–20% of the antibiotic-resistant colonies and consistently correlated with the genetic transposition event, as confirmed by PCR. The predicted molecular size of amplification products was 3322 base pairs (bp) for the $Pr55^{gag}$-*pol-nef* fragment and 2600 bp for the $gp120_{UG}$-TM region (Fig. 3A), and results obtained on 10 randomly-selected colorless colonies showed both full length products (Fig. 3B).

3.4. Expression of HIV-1 viral proteins in insect cells

Sf9 insect cells were transfected with the recombinant bacmid DNA and the correct intracellular expression of HIV-1 proteins was investigated by immunoblotting, using monoclonal antibodies specific for p24 or V3 epitopes (Intracel Co.). Equivalent levels of $Pr55^{gag}$ and gp120-TM proteins were identified in cellular lysates from Sf9 cells, 96 h post-transfection (Fig. 4, lanes 1 and 2). These results suggest a comparable gene expression driven by the pPolh and p10 baculovirus late promoters, as well as an efficient post-transcriptional processing. A less intense band with a molecular weight of approximately 160 Kd was identified by the anti-p24 MAb, indicating the presence of the Gag-Pol-Nef fusion protein derived from the low-frequent

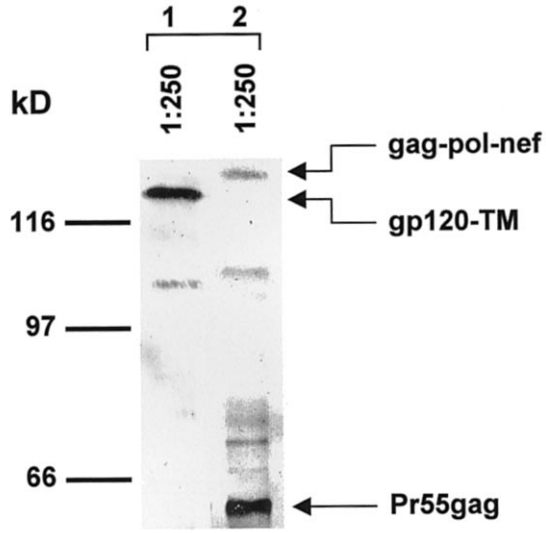

Fig. 4. Immunoblot analysis of Pr55^{gag}-*pol-nef* /gp120-TM VLPs produced in transfected Sf9 insect cells. Cell lysates were prepared 96 h post-transfection; proteins were separated on 12% SDS-PAGE, transferred to a nitrocellulose membrane and reacted with a 1:250 dilution of anti-gp120/V3 or anti-p24 MAbs (Intracel Co.) (lanes 1 and 2, respectively). Arrows indicate the specific reactive HIV-1 viral proteins; molecular weight marker positions are shown on the left of the figure and expressed in kilodaltons (kD).

readthrough of the *gag-pol-nef* polycystronic cDNA (Fig. 4, lane 2). All other bands of intermediate molecular sizes probably represent products of protein degradation.

3.5. Characterization of the HIV–VLPs released by insect cells

Cellular supernatants from recombinant bacmid-transfected Sf9 cells were used to infect the highly expressing High Five™ insect cells. Culture supernatants were collected 96 h post-infection and clarified at low-gravity centrifugation; the cell-free VLPs were further purified and concentrated by ultra-centrifugation through a 25% sucrose cushion. The pelleted VLPs were layered onto a 10–60% continuous sucrose gradient, to evaluate the sedimentation profile of the assembled particles, and 500 μl aliquots were collected to measure the p24 content by a commercial capture assay (NEN-duPont). The antigenic peak was detected in the gradient fractions corresponding to a density of about 1.14–1.18 g/ml, in accordance with previous data reported for Pr55^{gag}-based VLPs (Wagner et al., 1994; Rovinski et al., 1995) (Fig. 5A). The pool of p24-reactive gradient fractions were investigated by WB analysis as described above, confirming the presence of all viral proteins packaged in the assembled VLPs (Fig. 5B). In particular, the anti-V3 MAb specifically recognizes a single band showing a molecular weight compatible with the chimeric gp120-TM protein (Fig. 5, lane 1) and the anti-p24 MAb specifically recognizes two bands showing molecular weights compatible with the Pr55^{gag} and the Gag-Pol-Nef fusion protein (Fig. 5, lane 2). In parallel, the same pooled fractions were examined by standard transmission electron microscopy analysis. The samples showed 100–120 nm particles closely resembling immature HIV virions (Gelderblom et al., 1987; Katsumoto et al., 1987; Deml et al., 1997a), characterized by a light gray, translucent center encircled by an electron-dense ring and surrounded by a lipid bilayer (Fig. 6). Altogether, these results strongly indicate that multiple proteins can be packaged into the VLPs without affecting the assembling of particles.

3.6. Oligomeric organization of the VLP-associated chimeric gp120-TM glycoproteins

It has previously been shown that the external envelope glycoprotein is expressed on the virion surface in oligomeric forms, in association with the trans-membrane glycoprotein gp41 (Earl et al., 1990; Schawaller et al., 1990). In order to investigate the oligomeric structure of the VLP-associated chimeric gp120-TM glycoproteins, sucrose gradient purified VLPs have been mixed with the bis(Sulfosuccinimidyl) Suberate (BS^3) cross-linking reagent at concentrations ranging from 0.5 to 2 mM. Following a 30 min incubation at room temperature, the reaction products were separated by low percentage SDS-PAGE and detected by immunoblotting with anti-V3 monoclonal antibody (Intracel Co.). The results show that increasing concentrations of (BS^3) allows the identification of additional reactive slow-migrat-

ing bands (Fig. 7). In particular, with 2 mM three high molecular weight bands are visible possibly corresponding to dimers, trimers and tetramers of the chimeric gp120-TM glycoproteins (Fig. 7, lanes 2-4). The same bands were not visualized by using a p24-specific monoclonal antibody, indicating that they represent pure oligomers of the chimeric gp120-TM glycoproteins (data not shown).

4. Discussion

The main objective of this study was to engineer a Virus-like Particle model, based on the HIV-1 $Pr55^{gag}$ and produced in a Baculovirus expression system, presenting a broad spectrum of HIV-1 epitopes/proteins. The nucleotide sequence of an entire gp120, derived from an Ugandan HIV-1 isolate of the A-clade (94UG018), has been analyzed in our laboratory and the derived amino acid sequence of the V3 region shows an 88.5% average homology with isolates of clade-A and a 94.3% homology (33 out of 35 aa) with the clade-specific consensus sequence. Furthermore, the 100% homology with the Nigerian IBNG isolate (Howard et al., 1994), indicates the presence of identical antigenic epitopes in unrelated countries and suggests that HIV-1 field isolates of the A-clade may have a limited range of diversification in the Ugandan region.

The $gp120_{UG}$ has been linked, by a flexible hinge, to the trans-membrane domain of the EBV gp220/350 ($gp120_{UG}$-TM), since this has been previously shown to be an efficient strategy to increase the number of glycoproteins on the VLPs surface (Deml et al., 1997a) and to eliminate the immunosuppressive effects induced by the gp41 trans-membrane envelope protein (Ruegg et al., 1989; Denner et al., 1994). In order to test the possibility of packaging multiple HIV-1 antigens relevant for the induction of both humoral and cellular immunity, the *nef* and *pol* coding sequences have been included in this HIV–VLP structure as a *gag-pol-nef* polycistronic cDNA. Such proteins, in fact, show a much lower divergence than Env among HIV-1 strains and could induce a cross-clade immunity also with a potential therapeutic effect. To ensure a complete safety of the VLP structure, the protease and the reverse transcriptase enzymatic activities were knocked out by site directed mutagenesis targeted at the *pol* ORF.

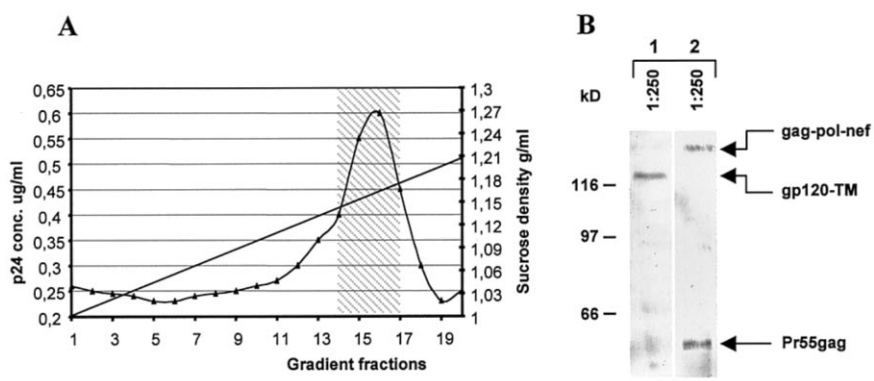

Fig. 5. (A) Evaluation of the $Pr55^{gag}$-*pol-nef*/gp120-TM VLPs density. Supernatants from cultures of HighFive insect cells, infected with recombinant baculovirus (M.O.I. = 5), were harvested 96 h post-infection. VLPs were sedimented through a continuous sucrose gradient (10–60%) and 20 aliquots of 500 μl were collected. The $Pr55^{gag}$ content was measured by a commercial p24 capture assay and antigenic peaks were observed in the fractions 14–17 spanning the 1.14–1.18 g/ml density interval. (B) The p24 reactive fractions were pooled and characterized by Western Blot analysis. Proteins were separated on 12% SDS-PAGE, transferred to a nitrocellulose membrane and reacted with a 1:250 dilution of anti-gp120/V3 or anti-p24 MAb (Intracel Co.) (lanes 1 and 2, respectively). Arrows indicate the specific reactive HIV-1 viral proteins; molecular weight marker positions are shown on the left and expressed in kilodaltons (kD).

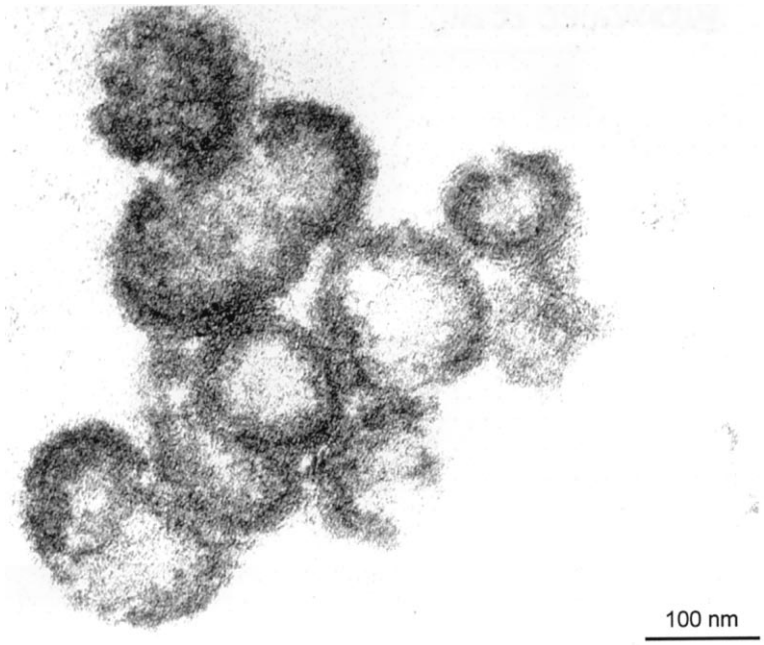

Fig. 6. Standard transmission electron microscopy (EM) analysis of the HIV–VLP_As produced by HighFive cells. Approximately 100 ng of VLPs in PBS were fixed in 6.4% glutaraldehyde, post-fixed with 2% osmium tetroxide for 1 h and embedded in Araldite. Grids were stained with 2.6% alkaline lead citrate and 0.5% uranyl acetate in 50% ethanol. The magnification bar represents 100 nm.

The coding sequences of the HIV-1 genes have been all cloned in the same transfer vector (pFastBac Dual, Gibco-BRL), downstream of the baculovirus pPolh and p10 late promoters; the insertion of HIV-1 genes into the Baculovirus DNA has been obtained by a transposition mechanism in a modified *E. coli* bacterial strain. The genetic transposition is 100% correlated with a colorless phenotype and the effective presence of all HIV-1 genes in the recombinant baculovirus DNA has been verified by PCR in randomly-selected bacterial colonies. In our experimental model, this approach is extremely efficient for the insertion of multiple coding sequences into the same recombinant baculovirus DNA molecule, which is otherwise ruled by the low efficiency of co-transfection. This approach, therefore, represents a convincing and feasible alternative to the homologous recombination, which has to be performed in insect cells and requires multiple rounds of plaque purification.

Equivalent expression levels of HIV-1 Env and Gag proteins have been observed in SF9 insect cells, confirming that the pPolh and p10 promoters have a comparable strength. As expected, the levels of the Gag-Pol-Nef fusion protein with a molecular weight of approximately 160 Kd, were significantly lower than that of $Pr55^{gag}$, given that the readthrough occurs at the frameshift signals present at the 3′ end of the *gag* reading frame, with a frequency of approximately 5%.

The HIV–VLPs assembled and released into the insect cell culture supernatant, show a pattern of reactive bands at the Western Blot analysis indicating the preferential incorporation of full-length HIV-1 proteins and the absence of degradation products observed in the cellular lysates. Furthermore, the cell-free VLPs show a typical retrovirus-like structure as indicated by sedimentation on a sucrose gradient and confirmed by standard transmission electron microscopy. The inclusion of additional viral proteins (such as Pol and Nef) in the HIV–VLPs structure, therefore, does not affect the $Pr55^{gag}$ auto-assembling properties and represents a feasible strategy to broaden the spectrum of HIV-1 immunogenic antigens presented by VLPs.

In addition, the incorporation of gp120 molecules via the EBV TM domain on the particle surface does not impair the formation of oligomeric structures; cross-linking analyses, indeed, indicate that the VLP-associated chimeric gp120-TM glycoproteins are structured as dimers and higher order oligomers. This confirms previous results described for a VLP expressing the chimeric gp120-TM protein, where the envelope protein was derived from the HIV-1_{MN} strain (Deml et al., 1997a). This feature is highly relevant because the envelope oligomerization is a property common to HIV as well as other enveloped retroviruses (Rey et al., 1990; Weiss et al., 1990) and has great biological implications for the induction of the cross-neutralizing humoral response (Sattentau and Moore, 1995; Fouts et al., 1997). Monoclonal antibodies (MAbs) raised against epitopes presented by a rgp120 monomer, on the contrary, have an extremely poor neutralization potency against primary isolates (Moore et al., 1995). The most accredited explanation is that antigenic sites, involved in the neutralization process, are more efficiently presented on conformational structures acquired by oligomeric gp120s (Earl et al., 1994).

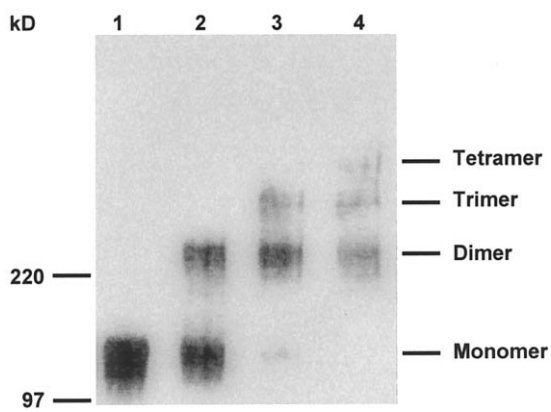

Fig. 7. Cross-linking of VLP-associated chimeric gp120-TM glycoprotein. Sucrose gradient purified VLPs were incubated with the bis(Sulfosuccinimidyl) Suberate (BS^3) cross-linking reagent, at concentrations of 0.5 (lane 2), 1 (lane 3) and 2 mM (lane 4); untreated VLPs were loaded in lane 1. Reaction were quenched with 50 mM Tris, pH 7,5 and subjected to electrophoresis separation in a 5% SDS-Polyacrylamide gel. The transferred proteins were reacted with a 1:250 dilution of anti-gp120/V3 MAb (Intracel Co.). Arrows indicate the chimeric gp120-TM reactive bands, interpreted as monomers, dimers, trimers and tetramers.

The development of a candidate HIV-1 preventive/therapeutic vaccine specific for the A-clade has been prompted by the evidence that this clade accounts for 25% of all HIV-1 isolates identified worldwide and is predominant in many developing countries, including Uganda where a prevalent A and D 'bi-clade' HIV-1 epidemic has been described (Albert et al., 1992; Bruce et al., 1994; Buonaguro et al., 1995; Brennan et al., 1997; Rayfield et al., 1998). Therefore, in the perspective of a global HIV-1 preventive vaccine program, candidate vaccines based on B-clade as well as non-B-clade derived antigens, should be developed. Vaccination strategies, thereafter, should include a cocktail of vaccines presenting epitopes from different HIV-1 clades, in order to induce a hopefully broader protection.

In conclusion, our results show that the approach employed in this study significantly allows the efficient packaging of multiple epitopes/proteins in the VLP structure, offering a feasible opportunity to induce a broad immune response. Furthermore, the genetic transposition performed in a modified *E. coli* bacterial strain represents a methodological improvement which allows a faster and more reproducible identification of recombinant Baculovirus DNA molecules.

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