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Assembly and Extracellular Release of Chimeric HIV-1 Pr55^{gag} Retrovirus-like Particles

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The HIV-1 Pr55^{gag} precursors were previously shown to assemble and bud from a variety of different cell types as noninfectious virus-like particles (VLPs) resembling immature HIV virions. The use of these VLPs as an immunogenic and autologous carrier component may allow the presentation of defined epitopes deduced from reading frames other than *gag* to the immune system, thereby avoiding the induction of adverse immune responses. In order to identify domains within Pr55^{gag} that can be replaced by immunologically relevant epitopes without affecting its capacity to assemble into VLPs, we deleted three domains of a predicted high surface probability. Deletion of amino acids 211–241 within p24CA and amino acids 436–471 within the p6LI portion of Pr55^{gag} had no effect on the assembly, ultrastructure, biophysical properties, and yields of mutant VLPs when expressed via recombinant vaccinia viruses in mammalian cells. Deletion of amino acids 99–154 overlapping the p17MA/p24CA cleavage site completely abolished the capacity of the *gag* polyprotein to form VLPs and led to a reduction of immature Pr55 VLPs released into the cell-culture supernatants when coexpressed with wild-type Pr55^{gag}. In contrast, assembly and budding of chimeric VLPs could be demonstrated after replacing amino acids 211–241 and 436–471 by immunologically relevant epitopes derived from reading frames other than Pr55^{gag} (e.g., V3 loop; CD4-binding-domain; nef-CTL epitope) or after fusion of these sequences to the carboxy terminus of Pr55^{gag}. The importance of these data for the development of novel HIV candidate vaccines is discussed.

INTRODUCTION

Considerable efforts have been directed in recent years toward developing a safe and effective HIV vaccine. Most approaches tried to closely mimic the pathogen using killed virus preparations or complete subunits of the viral HIV-1 envelope glycoproteins. However, severe immune dysfunctions are suggested to be induced by antibodies either enhancing the ability of HIV to infect macrophages (Robinson *et al.*, 1990; Takeda *et al.*, 1988) or cross-reacting with essential modulators of the immune response, e.g., with defined IgA and IgG subclasses (Maddon *et al.*, 1986) and with class II MHC molecules (reviewed by Habeshaw *et al.*, 1992; Young, 1988; Golding *et al.*, 1989; Blackburn *et al.*, 1991). There is evidence that at least part of the mechanism involved in HIV pathogenesis is associated with the HIV-1 envelope protein gp120/41, which itself can contribute to the breakdown of the immune system either by labeling CD4-positive cells for autoimmune attack or by cross-linking adjacent CD4 molecules, rendering these cells unresponsive to antigenic stimulation and possibly inducing apoptosis (reviewed in Amadori and Chieco-Bianchi, 1990; Amadori *et al.*, 1992; Siliciano *et al.*, 1988; Weinhold *et al.*, 1989; Tyler *et al.*, 1989). Therefore, only immunologically defined epitopes suggested to be involved in eliciting protective immune responses should be presented to the immune system.

A number of different formats have been devised for the presentation of selected epitopes to the immune system including short peptides, particulate carrier systems, or live viruses such as vaccinia or adenoviruses (Macket *et al.*, 1984; Graham and Prevec, 1992). Particulate carrier systems, which are mainly based on the hepatitis B virus (HBV) surface antigen (Michel *et al.*, 1990), the HBV core antigen, or on the yeast transposon TY-A gene product (Kingsman and Kingsman, 1988) appear to evoke strong immune responses, but suffer from the presentation of only a small number of relevant epitopes.

Accordingly, we investigated the possibility of constructing an antigen presentation system based on recombinant HIV-1 Pr55^{gag} retrovirus-like particles. Incorporation of foreign antigens into retroviral particles has been described previously using the gag proteins of Rous sarcoma virus (RSV) and Moloney murine leukemia virus (MLV) as a particulate carrier (Weldon *et al.*, 1990; Jones *et al.*, 1990; Hansen *et al.*, 1990). Inserting immunologically relevant HIV-1 epitopes into the HIV-1 Pr55^{gag} precursor would allow the presentation of selected epitopes by an autologous and highly immunogenic particulate carrier (Haffar *et al.*, 1990; Wagner *et*

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al., 1992a). A series of clinical studies demonstrated that the HIV-1 group specific antigens contribute to the stimulation of the host's immune response (Weber *et al.*, 1987; Cheingsong-Popov *et al.*, 1991; Haist *et al.*, 1992). Additional evidence from *in vitro* studies on virus-inhibiting activities of monoclonal antibodies against $Pr55^{gag}$ (Papsidero *et al.*, 1989; Wolf *et al.*, 1990) and the characterization of *gag*-specific CTLs (cytolytic T-lymphocytes) from AIDS patients (Nixon *et al.*, 1988; Phillips *et al.*, 1991) recommended the $Pr55^{gag}$ precursor as an attractive carrier component for the presentation of additional epitopes.

The formation of noninfectious, morphologically immature HIV-1 retrovirus-like particles (VLPs) solely depends on the expression of the myristoylated HIV-1 *gag* polyproteins (Goettlinger *et al.*, 1989). Accordingly, the production of recombinant VLPs has been demonstrated by transiently (Smith *et al.*, 1990; Mergener *et al.*, 1992) or stably transfected eucaryotic cells (Kräuslich *et al.*, 1993) and after infection of different host cells with recombinant vaccinia (Karacostas *et al.*, 1989; Haffar *et al.*, 1990; Wagner *et al.*, 1991) or baculoviruses (Gheysen *et al.*, 1989; Royer *et al.*, 1991; Wagner *et al.*, 1992a). The molecular mechanisms involved in the assembly and budding of the immature HIV virions is still poorly understood. Very recently, however, we were able to identify two domains located in the p17MA (aa 47–59) and in the p24CA (aa 339–349) portions of $Pr55^{gag}$ essential for the formation of immature HIV virions (Niedrig *et al.*, 1992; von Poblotzki *et al.*, 1993).

Major conformational changes and consecutive loss of the particle-forming capacity would be expected upon random insertion of selected epitopes into $Pr55^{gag}$. In order to identify domains within $Pr55^{gag}$ that can be replaced by immunologically relevant epitopes without affecting its capacity to assemble to VLPs, several deletion mutants were established and functionally characterized. To extend the immunological spectrum of the altered $Pr55^{gag}$ VLPs, a conserved CTL epitope of the nef reading frame (Chenciner *et al.*, 1989; Culmann *et al.*, 1989), a linear portion of the gp120 discontinuous CD4-binding domain (Lasky *et al.*, 1987) and a consensus sequence of the gp120 principal neutralizing domain V3 (Wagner *et al.*, 1992b) were inserted into susceptible sites of $Pr55^{gag}$. The effects of inserting additional epitopes into the $Pr55^{gag}$ precursor on the formation of chimeric VLPs were investigated.

MATERIAL AND METHODS

Cells

CV-1 cells and 143B cells, grown in MEM, 10% FCS (GIBCO) were used to establish recombinant vaccinia viruses. Formation of VLPs was studied in SW480 colonadenocarcinoma cells (L15, 10% FCS, GIBCO).

HIV-sequence

All amino acid positions given in the text refer to the HIV-1 LAI strain.

Monoclonal antibodies

The V3-specific murine monoclonal antibody recognizing a central motive of the V3 loop region (RIQRGPGRAFVTIGKI) was purchased from DuPont (Nea 9301). The $Pr55^{gag}$-specific monoclonal antibodies used have been previously mapped to amino acids 147–154 (mab 13/5) and to amino acids 307–336 within $Pr55^{gag}$ (mab 16/4/2) (Wolf *et al.*, 1990).

Selection of deleted epitopes

Three regions within the $Pr55^{gag}$ precursor located (i) at the MA/CA cleavage site, (ii) within the p24CA domain product, and (iii) within the p6LI portion of $Pr55^{gag}$ were selected for their predicted antigenic indices and their logarithmic surface probabilities using computer-assisted secondary structure analysis of the HIV-1 group-specific antigen (UWGCG software; Modrow *et al.*, 1987).

Construction of chimeric antigens

A new pUC8 derivative, plin8, has been constructed and has been treated as follows. The pUC8 multiple cloning site was replaced by a synthetic linker sequence containing all restriction sites (*Bam*HI, *Hind*III, *Xho*I, *Sac*I, *Pst*I, *Spe*I, *Sal*I) necessary for the construction of the described plasmids (Fig. 1): In order to delete the p17MA/p24CA cleavage site including flanking amino acids (aa 99–154) a 300-bp *Bam*HI/*Hind*III fragment encoding the amino-terminal part of $Pr55^{gag}$ was cloned into the *Bam*HI/*Hind*III site of plin8 (plin8Pr55BH). The 1283-bp *Nsi*I/*Sal*I fragment encoding the carboxy-terminal part of $Pr55^{gag}$ was inserted into the *Pst*I/*Sal*I site of plin8Pr55BH to achieve plin8Pr55Δ1. A stretch of 30 amino acids (aa 211–241) located within p24CA moiety was deleted by subcloning the complete 1752-bp *Bam*HI/*Sal*I fragment of pUC8Pr55 (Wagner *et al.*, 1992) into the *Bam*HI/*Sal*I sites of plin8 (plin8Pr55) and replacing a 90-bp *Pst*I/*Spe*I fragment with a 27-bp *Xho*I/*Sac*I linker fragment (TGCAGCTCGA GAATTCGAGC TCACTAG) (plin8Pr55Δ2). For the construction of deletion mutant Pr55Δ3 (lacking aa 436–471 within p6Li), the original 3′ part of the $Pr55^{gag}$ coding sequence was replaced by a PCR fragment, amplified by using a 64-bp 5′ primer (CGACTCGGAT CCAAGATCTC TCTCGAGAAT TCGAGCTCGA AGAGAGATTC AGGTCTGGGG TAGA) containing four 5′ restriction sites (*Bgl*II, *Xho*I, *Eco*RI, *Sac*I) and a 21-bp 3′ primer

(TTCCAATTAT GTCGACAGGT G) containing a 3′ *Sal*I site. The amplified *Bgl*II/*Sal*I fragment was inserted into the corresponding vector fragment of plin8Pr55 (plin8Pr55Δ3). In order to allow a carboxy-terminal fusion of selected epitopes to the complete $Pr55^{gag}$ precursor, we inserted a *Xho*I/*Sac*I linker fragment between the codon encoding the carboxy-terminal glutamine and the stop codon. For this purpose, a 260-bp DNA fragment was amplified by PCR using a 5′ primer overlapping the *Bgl*II restriction endonuclease site (TAGGGAAGATCTGGCCTT), a 3′ primer to introduce the restriction sites *Xho*I and *Sac*I, and a translation stop codon followed by a *Bcl*I and a *Sal*I site (CCGTTG-CTGG GGAGCAGTGT TCGAGCTCAT CTACTCGAGA TTACTAGTCA GCTGACTG). The *Bgl*II/*Sal*I fragment was used to replace the original 3′ end of the complete $Pr55^{gag}$ coding sequence (plin8Pr55F4).

A synthetic oligonucleotide encoding a composite sequence based on a subset of V3 sequences (V3c-36; Wagner *et al.*, 1992a) was then inserted into the *Xho*I/*Sac*I sites of the above described Pr55 mutants to create the $Pr55^{gag}$/V3c-1, -2, -3, and -4 chimeric genes (Fig. 1). A linear portion of the CD4-binding domain (CD4BR; 196 bp) was amplified from a $HIV\text{-}1_{LAI}$ provirus clone using the 5′ primer GAG GGA TCC GCT CGA GGG TCA AAT AAC ACT GAA GGA AGT and the 3′ primer GAG AAG CTT ATC GAT CCG AGC TCA CCA TCT CTT GTT AAT AGC AGC CC. A 98-bp DNA fragment was amplified from a highly conserved nef CTL epitope using the primer pair ACA GGA TCC GCT CGA GCA CTT GAT CTT GAT CTG TGG ATC (5′ primer) and CAC AAA GCT TCC GAG CTC CCC TGG TGT GTA GTT (3′ primer). All amplified DNA sequences are flanked by a *Xho*I and a *Sac*I site. This allowed a direct subcloning of the selected subgenic fragments into the Pr55 deletion mutants plin8Pr55Δ1-Δ3 and plin8Pr55F4, resulting in the plasmids plin8Pr55V3c-1 to V3c-4, plin8Pr55CD4BR-1 to -CD4BR-4, and plin8Pr55nef-1 to nef-4 (Fig. 1). All subcloned DNA sequences including the flanking regions were verified by double-stranded DNA sequencing.

Recombinant vaccinia viruses

Recombinant vaccinia viruses were established according to standard procedures (Mackett *et al.*, 1984) after subcloning of the *Bam*HI/*Sal*I fragments into the pAvB vaccinia virus transfer vector (von Brunn *et al.*, 1991). Five days after infection (m.o.i. = 1), the cells were harvested, resuspended in phosphate-buffered saline (PBS), and sonified three times for 10 sec at 150 W. The cell debris was discarded and the supernatant containing the enriched virus preparation was centrifuged over a 37.5% sucrose cushion using a Contron TFT41.14 rotor (36000 rpm, 20 min, 4°). The pelleted virus was resuspended in PBS.

Detection and quantification of chimeric proteins

Expression of the chimeric proteins in cells infected with the recombinant vaccinia viruses was tested by Western blot and immunoprecipitation analysis as described (Sambrook *et al.*, 1989). Renaturation of the chimeric proteins following electrotransfer onto a nitrocellulose membrane was achieved by incubation of the Western blots in decreasing urea and DTT concentrations diluted with PBS starting with 250 m*M* DTT and 6 *M* urea at 37°. Yields of the chimeric proteins were determined from crude cell lysates after sonification of the infected cells using a commercial antigen capture assay (Abbott, Chicago).

Electron microscopy

Infected SW480 cells were harvested with a cell scraper, washed with PBS and then fixed for 2 hr with 2.5% glutaraldehyde in PBS. Cells were washed with PBS and postfixed for 1 hr with osmium tetroxide in PBS. After washing in PBS and in distilled water, fixed cells were stained with 1% uranyl acetate in 20% acetone, for 30 min. Following dehydration in a graded series of acetone, cells were embedded in Spurr's low-viscosity resin. Sections of 25 to 75 nm thickness were cut with a diamond knife and mounted on uncoated copper grids. The sections were poststained with 100 mmol lead (II) citrate, pH 12.6. All pictures were taken with a Siemens Elmiskop 101 electron microscope. The magnification was calibrated with a cross line grating replica.

Gradient sedimentation analysis

Supernatants of SW480 cells infected with the described recombinant vaccinia viruses were collected 5 days p.i. (postinfection). Enrichment of virus-like particles from precleared culture supernatant (3300 *g*) was achieved by an Ultrasette-Filtron filter membrane (Satorius, FRG) following the protocol of the manufacturers. Two-milliliter aliquots of the concentrated material were layered onto a 12-ml 10–60% sucrose gradient in 10 m*M* phosphate buffer, pH 7.5, containing 0.15 *M* NaCl and centrifuged at 20,000 rpm for 2 hr in a contron TFT41.14 rotor. Fractions (0.5 ml) were collected, diluted by PBS, and virus-like particles were pelleted at 16,000 rpm for 40 min in an Eppendorf centrifuge at room temperature. The antigen preparations were resuspended in 200 μl 20 m*M* Tris–HCl, pH 7.5, 150 m*M* NaCl, 2 m*M* EDTA and analyzed by conventional Western blot analysis (Sambrook *et al.*, 1989). Antigen from all fractions was quantified exactly using a commercial p24-specific antigen capture assay (Abbott). Triplicate infection experiments followed by sucrose sedimentation analysis of the cell culture super-

natants yielded standard errors which were always less than 8% of the means (pg p24CA).

RESULTS

Pr55^{gag} mutants

The assembly of immature Pr55^{gag} virus-like particles is a complex multistep process that requires the targeting of the myristoylated precursor molecules to the inner face of the plasma membrane followed by some poorly characterized interactions between *gag* precursors which lead to the formation, budding, and final release of immature VLPs from the cell surface. To construct chimeric VLPs, allowing for the presentation of additional epitopes, we first identified domains within Pr55^{gag} which are not involved in the assembly and budding process. We established three deletion mutants selected on the criteria of both their biological function and their logarithmic surface probability predicted by computer-assisted analysis of the Pr55^{gag} secondary structure. Two domains within the carboxy-terminal region of p17MA (aa 47–59) and of p24CA (aa 339–349), which had previously been demonstrated to be involved in the assembly process (Yu *et al.*, 1992b; von Poblotzki *et al.*, 1993; Niedrig *et al.*, 1992), were not impaired by the deletions. Accordingly, 30 to 55 amino acids were deleted in three regions: (i) overlapping the p17CA/p24MA protease cleavage site (aa 99–154), (ii) located within p24CA (aa 211–241), and (iii) located within the p6LI moiety (aa 436–471) of Pr55^{gag}. Moreover, we investigated whether the fusion of foreign amino acids is tolerated by adding a small linker fragment to the authentic Pr55^{gag} carboxy terminus (Fig. 1). DNA sequences encoding the HIV-1 protease have not been expressed by the constructs used.

Analysis of the Pr55^{gag} mutants

The Pr55^{gag} mutants referred to as Pr55Δ1, Pr55Δ2, Pr55Δ3, and Pr55F4 were inserted into a highly attenuated vaccinia virus (strain tien tan) by recombination and expressed in SW480 cells after infection with the recombinant vaccinia viruses (Fig. 1A). Correct expression of the mutant polypeptides was tested by conventional Western blot analysis (Fig. 2A). The Pr55^{gag} derivatives were detected by monoclonal antibody 16/4/2, which specifically recognizes amino acids 307–336 within the p24CA portion of Pr55^{gag} (Wolf *et al.*, 1990). The molecular weight of the recombinant antigens in relation to Pr55^{gag} primarily correlated with the number of deleted or fused amino acids. Some lower bands which were regularly observed are due to a limited degradation of the *gag* derivatives within the infected cells or due to premature translational stops. Using an antigen capture assay, we calculated the amount of antigen to be 1.0–1.2 ng/10^6 infected cells.

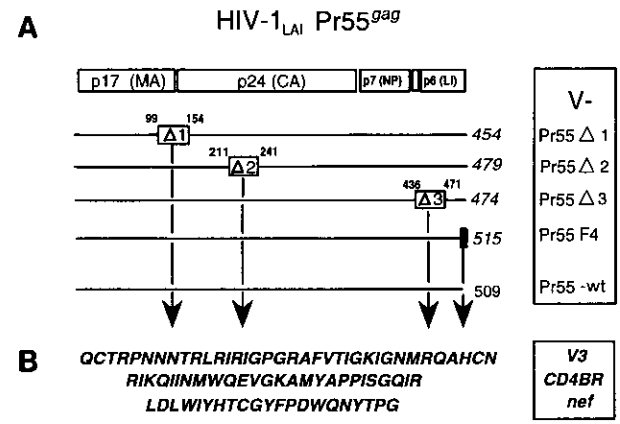

FIG. 1. Schematic drawing of the mutant Pr55^{gag} polypeptides. (A) Deleted amino acids are indicated by open boxes. The associated numbers represent the stretch of deleted amino acids relative to the sequence of the complete HIV-1$_{LAI}$ Pr55^{gag} precursor. A carboxy-terminal fusion of the amino acids ARVDEL resulting from the addition of a polylinker sequence to the 3′ end of the Pr55^{gag} gene is indicated as a black box. The wt-Pr55^{gag} precursor is shown as a reference. The total numbers of amino acids of the mutant Pr55^{gag} polypeptides are given in italic letters. The correct designation of the mutant vaccinia viruses (V–) is given at the right. (B) Three domains derived from reading frames other than Pr55^{gag} were either inserted into the mutant gag polypeptides (Pr55Δ1–Pr55Δ3) to replace the deleted sequences or fused to mutant Pr55F4 to generate carboxy-terminal fusion proteins. V3, composite sequence based on a subset of sequences of the HIV-1 gp120 major neutralizing epitope V3; CD4BR, a linear portion of the discontinuous CD4-binding domain; nef, conserved nef-CTL epitope. The complete amino acid sequence of the selected epitopes is shown at the left and the abbreviations are given at the right side of the figure.

The capacity of the Pr55^{gag} mutants to assemble into VLPs which are capable of budding into the cell-culture supernatant was investigated by analyzing the cell-culture supernatants in 10–65% sucrose velocity gradients (Fig. 2B). Mutants Pr55Δ2, Pr55Δ3, and Pr55F4 demonstrated a maximum of p24CA antigen at a sucrose density around 1.15 to 1.17 g/cm^3, which is typical for HIV-virions (fractions 11–13). Similar results were obtained for the Pr55^{gag}-positive control which has been previously shown to include all essential information for the formation of VLPs. Reduced total amounts of antigen were determined in triplicate infection experiments for mutant Pr55Δ2 compared to Pr55Δ3 and Pr55F4 and only minute amounts of mutant protein could be detected in the corresponding peak fractions of mutant Pr55Δ1. Western blot analysis performed with the identical sucrose fractions confirmed these data (Fig. 2C). The molecular weight of the major protein species detected by monoclonal antibody 16/4/2 corresponded exactly to that calculated for the various Pr55^{gag} derivatives. Some of the minor bands could indicate a limited susceptibility of the proteins to partial cleavage by cellular proteases. These

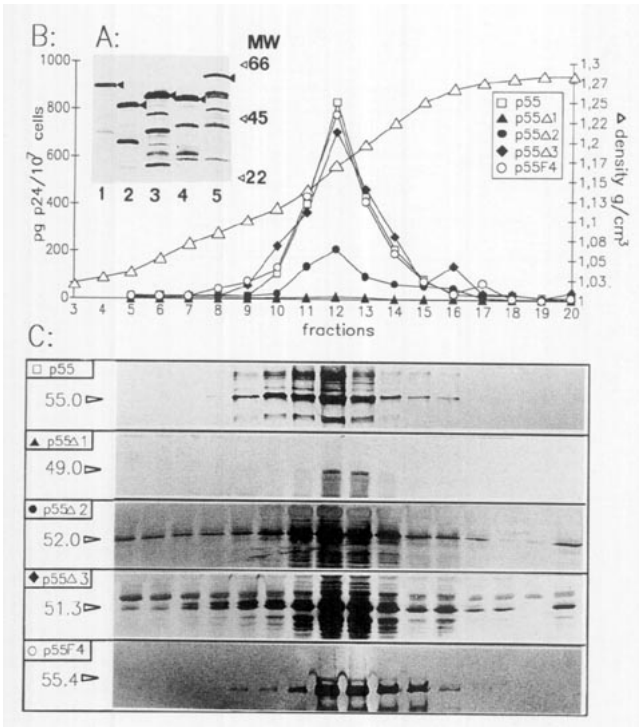

FIG. 2. Biochemical analysis of Pr55^{gag} mutants. (A) Western blot analysis of lysates of SW480 cells (5×10^4) infected with recombinant vaccinia viruses (m.o.i. = 10): v-Pr55 (lane 1), v-Pr55Δ1 (lane 2), v-Pr55Δ2 (lane 3), v-Pr55Δ3 (lane 4), and Pr55F4 (lane 5). Recombinant polypeptides were detected by a p24-specific monoclonal antibody (16/4/2); full-length proteins are marked by arrows. Positions of the molecular weight markers (kDa) are given at the right. (B, C) Analysis of the particle-forming capacity of the mutant polypeptides by sucrose sedimentation analysis. The supernatants of SW480 infected with v-Pr55 (□), v-Pr55Δ1 (▲), v-Pr55Δ2 (●), v-Pr55Δ3 (◆), and Pr55F4 (○) (m.o.i. = 10) were analyzed as described under Material and Methods. 20 fractions were collected. The yields of the mutant proteins determined by a p24 capture assay from single fractions are shown. The indicated values represent the means of three independent infection experiments followed by sedimentation analysis of the cell-culture supernatants. Standard errors of the means (pg p24CA) were always less than 8% of the mean. Antigenic peaks were observed at a density (Δ) of about 1.14–1.18 g/cm^3 (fractions 11–13). Only minute amounts of p24 were detected in supernatants of cells infected with mutant v-Pr55Δ1 (▲) (B). (C) Western blot analysis of fractions 5–20 using a p24CA-specific monoclonal antibody (16/4/2) for the detection of Pr55 derivatives. The molecular weight of the major protein species (indicated at the left) corresponded exactly to that calculated for the analyzed polypeptides.

results suggested that extended deletions within p24CA and within p6LI or the fusion of additional sequences to the carboxy terminus of Pr55^{gag} are tolerated without significantly affecting the assembly and budding of VLPs. In contrast, deletion of a 55-amino-acid region overlapping the p17MA/p24CA protease cleavage site almost completely abolished particle formation.

We were able to confirm these observations by ultrathin-section electron microscopy of SW480 cells infected with the indicated vaccinia viruses. Formation of VLPs budding from the plasma membranes of SW480 expressing mutant Pr55Δ1 was not found. However, *gag* material accumulated in large vacuole-like vesicles in the cytoplasm of the infected cell. These vesicles varied between 2 and 4 μm in diameter and were covered on the inner side by a 15- to 17-nm-thick layer of electron-dense material typically detected at the inner side of the plasma membrane of v-Pr55-infected cells (not shown). Similar structures were found neither in noninfected cells nor in cells infected with wild-type vaccinia virus (v-wt) or v-Pr55. In contrast, abundant formation of VLPs has been demonstrated for mutants Pr55Δ2, Pr55Δ3, and Pr55F4 (Figs. 3A–3F). These mutant VLPs showed a typical doughnut-shaped structure, which is surrounded by a lipid bilayer derived from the plasma membrane of the infected cell. The inner face of the plasma membrane of these particulate structures was coated with a 15–17 nm electron-dense layer of *gag* protein. Occasionally, this layer exhibited another, more electron-dense boarderline surrounding the electron-translucent lumen of the VLPs. The fine structure of VLPs generated by *gag* mutants Pr55Δ2, Pr55Δ3, and Pr55F4 could not be distinguished from the structures generated by the wild-type Pr55^{gag} precursor. Essentially, these VLPs strongly resembled the structures previously described as HIV-1 immature HIV virions (Gelderblom *et al.*, 1989).

Transdominant negative effect of mutant Pr55Δ1

Quantitative analysis of the cell-culture supernatants revealed almost identical yields of recombinant VLPs in the supernatants of SW480 cells expressing mutant Pr55Δ3 and Pr55F4 and a slight reduction in the case of Pr55Δ2 compared to wild-type Pr55^{gag} (Table 1A). For these *gag* mutants, we found 63.9 to 75.8% of the total antigen in fractions 11–13, clearly indicating an antigenic peak at a sucrose density between 1.15 and 1.18 g/cm^3. In contrast only minute amounts of p24CA could be identified in the supernatant of Pr55Δ1-infected cells. In this case, only 27.2% of the total antigen was restricted to fractions 11–13, suggesting a rather equal distribution of the mutant protein over all fractions tested. Coinfection of SW480 cells by v-Pr55^{gag} and v-Pr55Δ1 (m.o.i. = 25 for each virus) led to the expression of similar amounts of wild-type and mutant protein within the cell lysates tested (Fig. 4). Discrimination of both Pr55^{gag} alleles was performed by immunoblotting using two different Pr55^{gag}-specific monoclonal antibodies. Mab 13/5, which has been previously mapped to amino acids 147–154, failed to detect mutant Pr55Δ1 (lacking aa 99–154), whereas antibody 16/4/2, which recognizes aa 307–336, detected both *gag* alleles. Coinfection of SW480 cells by v-wt and v-Pr55^{gag} neither reduced the synthesis of Pr55^{gag} within the infected cells (Fig. 4) nor the number of virus-like particles budding into the cell-culture super-

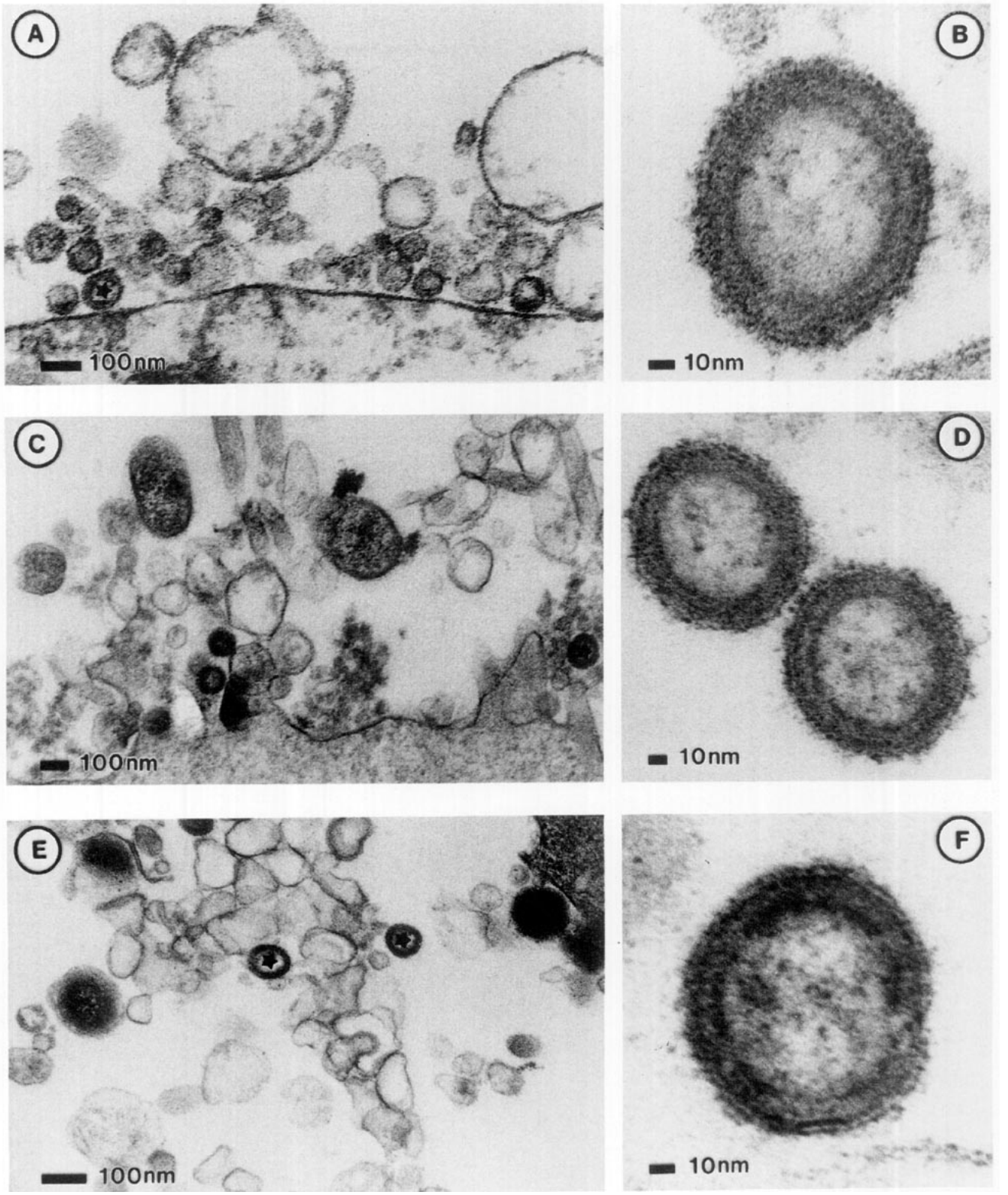

FIG. 3. Ultrathin-section electron micrographs of SW480 cells harvested 2 days after infection with recombinant vaccinia viruses expressing the mutant polypeptides Pr55Δ2 (A, B), Pr55Δ3 (C, D), and Pr55F4 (E, F). Examples of typical retrovirus-like particles are labeled in A, C, and E by asterisks. Details of the ultrastructure of the mutant virus-like particles, which strongly resemble immature HIV virions, are given in B, D, and F.

natant (Table 1). In contrast, coinfection of SW480 cells by v-Pr55^{gag} and v-Pr55Δ1 resulted in a significant reduction of particulate antigen released into the cell-culture supernatants (Table 1). Moreover, for the coinfection experiment only 47.0% of the detected antigen could be correlated to fractions 11–13, suggesting that the antigen released into the cell-culture supernatants only partially consisted of VLPs. Analyzing a series of ultrathin sections of coinfected cells, we could not detect lentivirus-like budding structures. Instead, we found giant vacuole-like vesicular structures previously observed exclusively in v-Pr55Δ1-infected cells. Similar structures were detected neither in noninfected cells nor in cells infected with v-wt or v-Pr55 (Fig. 5). These data suggest a transdominant negative function of Pr55Δ1 over wild-type Pr55^{gag}.

Analysis of Pr55^{gag}/V3 chimeric polypeptides

For the construction of a successful HIV vaccine, it might prove useful to develop rationally designed antigens excluding epitopes known to induce adverse side effects and combining reading frames with epitopes demonstrated to elicit beneficial immune responses. For this purpose, we either replaced the domains deleted in the *gag* constructs described above (Pr55Δ1–Δ3) with a composite epitope based on a subset of variant V3 sequences or inserted this epitope into the linker fragment originally fused to the C-terminus of the complete *gag* precursor (Pr55F4). This domain was previously characterized in its structure and extended biological reactivity (Wagner *et al.*, 1992b; Habazettl *et al.*, submitted), therefore recommending this epitope as an attractive compound for a candidate vaccine.

TABLE 1

ANALYSIS OF CELL-CULTURE SUPERNATANTS

		[pg] p24 f1–f20[a]	[pg] p24 f11–f13[a]	$\frac{f11–f13^b}{f1–f20} \times 100$
A	p55	2192	1662	75.8
	p55Δ1	40	12	27.2
	p55Δ2	678	440	64.9
	p55Δ3	2402	1534	63.9
	p55F4	2251	1617	71.8
B	p55 & p55Δ1[c]	283	134	47
	p55 & v-wt	2084	1621	77.8

[a] [pg p24] Determined from the supernatants of 10^7 SW480 cells infected with the indicated vaccinia viruses after sucrose sedimentation analysis (Abbott, p24 capture assay) (see Fig. 2). Total amounts of antigen in fractions 1–20 (f1–f20) and in the antigenic peak fractions (f11–f13) are indicated.

[b] [pg p24] Determined for fractions 11–13 in relation to the total amount of antigen in all fractions (f1–f20).

[c] [pg p24] Determined from the supernatants of 10^7 SW480 cells coinfected with v-Pr55/v-Pr55Δ1 or v-Pr55/v-wt after sucrose sedimentation analysis (m.o.i. = 25 for each virus).

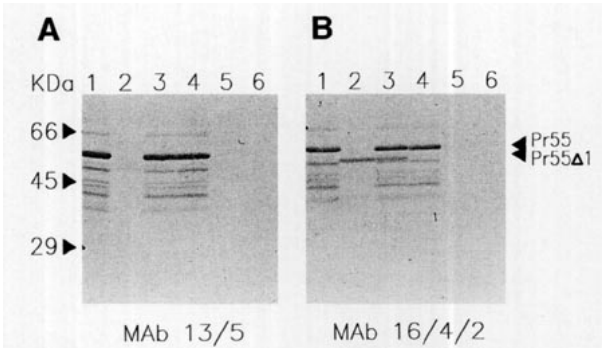

FIG. 4. Analysis of SW480 cells (co)infected with various vaccinia viruses (m.o.i. = 25 for each virus) by SDS–PAGE followed by immunoblotting. Recombinant proteins were detected by two different monoclonal antibodies recognizing amino acids 147–154 (MAb 13/5; A) and amino acids 307–336 (MAb 16/4/2; B) of the HIV-1_{LAI} Pr55^{gag} precursor. Lane 1, v-Pr55; lane 2, v-Pr55Δ2; lane 3, v-Pr55 and v-Pr55Δ2; lane 4, v-Pr55 and wild-type vaccinia virus (v-wt); lane 5, v-wt; lane 6, uninfected SW480 cells. The M_r of the recombinant proteins is indicated to the right. Specifically detected proteins are labeled by arrows. Positions of the M_r markers are given to the left.

The chimeric vaccinia viruses were referred to as v-Pr55^{gag}/V3$_c$-1, -2, -3, and -4 depending on the position of the V3$_c$ domain within the Pr55^{gag} precursor (see Fig. 1B). Correct expression of the chimeric proteins in lysates of infected SW480 cells was tested by renaturing Western blotting and immunoprecipitation analysis. The chimeric proteins were detected by the monoclonal antibody 16/4/2, which specifically recognizes the p24CA portion of Pr55^{gag}. These data demonstrated that the overall amount of the chimeric proteins was comparable in all cell lysates considered (Fig. 6A). The recognition of the V3$_c$ domain within Pr55^{gag} by a V3$_{LAI}$-specific monoclonal antibody differed among the cell lysates considered depending on the position within Pr55^{gag} (Fig. 6B). To confirm these observations under native conditions, we performed an immunoprecipitation analysis from lysates of infected SW480 cells using the V3$_{LAI}$-specific monoclonal antibody. This antibody was not able to precipitate the Pr55V3$_c$-1 chimeric antigen, although the correct sequence of the inserted V3$_c$ domain was verified by double-stranded DNA sequencing. Confirming the Western blot data, the antigenicity improved after insertion of the V3$_c$ domain into position 2, 3, or 4 (Fig. 6C).

Sucrose sedimentation analysis performed with supernatants of the infected cells indicated the formation and budding of chimeric VLPs (Fig. 7A): As demonstrated for the mutant polypeptides Pr55Δ2, Pr55Δ3, and Pr55F4 we identified an antigenic peak at a density of about 1.15–1.18 g/cm^3 for the chimeric polypeptides Pr55V3$_c$-2, -3, and -4. Inserting the V3 domain into position 1 was not sufficient to reconstitute the Pr55 particle-forming capacity. However, the total amounts of chimeric antigens released into the cell-culture supernatants decreased by a factor of 2 after

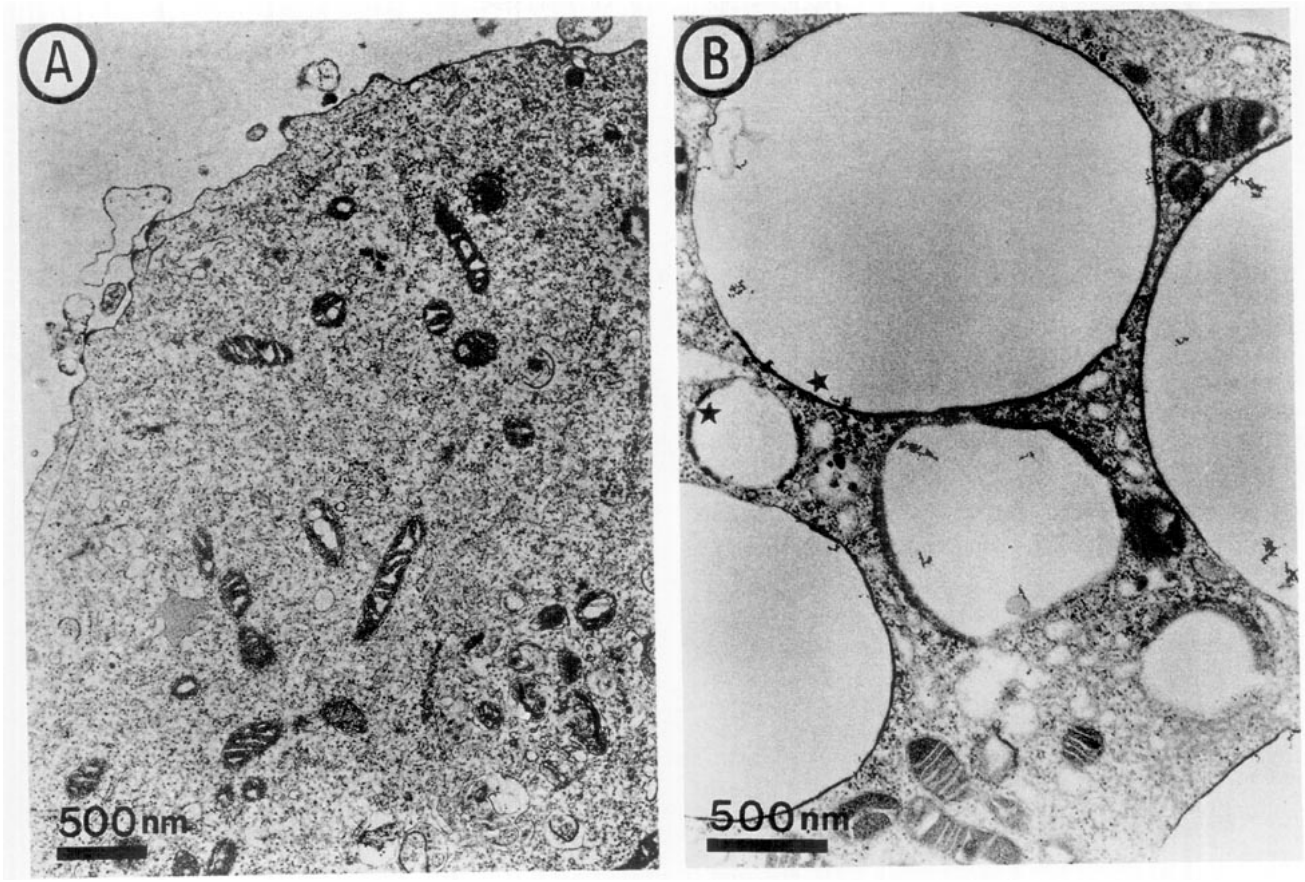

FIG. 5. Ultrathin-section electron micrographs of SW480 cells infected by v-Pr55 (A; m.o.i. = 25) and coinfected by v-Pr55 and v-Pr55Δ1 (B; m.o.i. = 25 for each virus). Budding of typical lentivirus-like structures demonstrated in (A) were not detected in coinfected cells (B). Large vacuole-like vesicles which were only found in v-Pr55Δ1-infected cells (not shown) and in cells coinfected with v-Pr55 and v-Pr55Δ1 (B) are labeled by asterisks.

insertion of the V3 loop into position -2, -3, and -4 compared to the basic constructs Pr55Δ2, Pr55Δ3, and Pr55F4. Western blot analysis of the collected sucrose fractions identified unprocessed Pr55 derivatives of the calculated molecular weights as the major protein species (Fig. 7B). Analysis of the antigenic peak fractions (fraction 12) by immunoblotting confirmed that the recognition of the $V3_c$ domain by a $V3_{LAI}$-specific

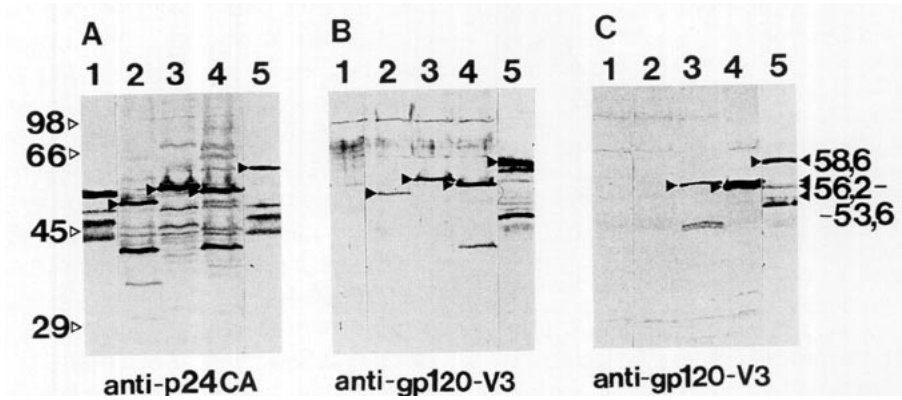

FIG. 6. Western blot (A and B) and immunoprecipitation analysis (C) of lysates of SW480 cells infected with v-Pr55 (lane 1), v-Pr55$V3_c$-1 (lane 2), v-Pr55$V3_c$-2 (lane 3), v-Pr55$V3_c$-3 (lane 4), and v-Pr55$V3_c$-4 (lane 5) (10 PFU/cell). (A and B) Cell lysates were analyzed by SDS–PAGE followed by immunoblotting. The recombinant proteins were detected by a monoclonal antibody to p24CA (anti-p24CA; A) and by a gp120–V3 loop (LAI isolate) specific monoclonal antibody (anti-gp120–V3; B). (C) Immunoprecipitation analysis from cell lysates of SW480 cells after infection with the indicated vaccinia viruses. Pr55V3 chimeric proteins were precipitated by the V3-loop-specific monoclonal antibody. Shifts in the electrophoretic mobility chiefly correlate to the predicted molecular weight of the recombinant proteins indicated to the right. Specifically detected chimeric proteins are labeled by arrows. Positions of the molecular weight marker are given in kDa to the left.

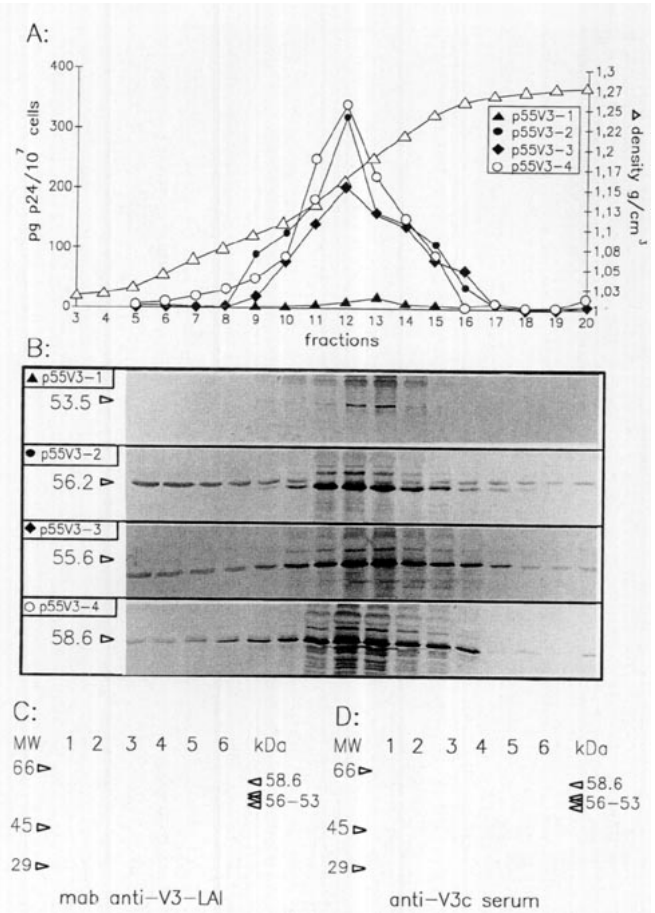

FIG. 7. Analysis of the particle-forming capacity of different Pr55^{gag}/V3 chimeric polypeptides by sucrose sedimentation analysis. The supernatants of SW480 cells infected with v-Pr55V3$_c$-1 (▲), v-Pr55V3$_c$-2 (●), v-Pr55V3$_c$-3 (♦), and Pr55V3$_c$-4 (○) (m.o.i. = 10) were analyzed as described. 20 fractions were collected and the yields of the mutant proteins were determined by a p24 capture assay from single fractions. The indicated values represent the means of three infection experiments followed by sedimentation analysis of the cell-culture supernatants. Standard errors of the means (pg p24CA) were always less than 8% of the mean. (B) Western blot analysis of fractions 5–20 using a p24CA-specific monoclonal antibody (16/4/2) for the detection of Pr55 derivatives. The molecular weight of the major protein species (indicated to the left) corresponded exactly to that calculated for the analyzed polypeptides. (C, D) Fraction 12 of each gradient was further analyzed by immunoblotting; Pr55V3-1 (lane 3), Pr55V3-2 (lane 4), Pr55V3-3 (lane 5), and Pr55V3-4 (lane 6). As a negative control we used corresponding fractions of gradients performed with v-wt- (lane 1) and v-Pr55-infected cells (lane 2). A HIV-1$_{LAI}$ gp120–V3-specific monoclonal antibody (anti-V3$_{LAI}$; C) and a polyclonal antiserum raised in rabbits to a V3 consensus peptide (anti-V3c serum; D) were used to detect the inserted or fused V3 domain of the chimeric polypeptides. Specifically detected polypeptides are marked by open arrows from the right. The molecular weights calculated for the chimeric antigens are indicated. Positions of the M_r markers (kDa) are given to the left.

monoclonal antibody and a polyclonal V3$_c$-specific rabbit serum clearly depends on its position with Pr55^{gag} (Figs. 7C and 7D).

Ultrathin-section electron microscopical analysis of SW480 cells infected with the Pr55V3 recombinant vaccinia viruses confirmed these observations and readily revealed the formation of chimeric retrovirus-like particles in all cases tested except for that of mutant Pr55V3-1 (Fig. 8). Careful analysis of the ultrathin sections occasionally identified the budding of chimeric VLPs strongly resembling immature HIV virions. However, in preparations of v-Pr55V3-2- (Figs. 8A and 8B), v-Pr55V3-3- (8C and 8D), and v-Pr55V3-F4 (8E and 8F) infected SW480 cells, we found representative numbers of core-like particles which significantly deviated from the typical retrovirus-like structures in size and shape. Similar deviations from the regular structure have been observed for the incorporation of RSV *gag* chimeras into VLPs (Weldon *et al.*, 1990). These morphologically irregular particles varied in their diameters between 100 and 250 nm. They were surrounded by a typical plasma membrane, which is covered on the inner site by a 15- to 17-nm electron-dense layer of chimeric Pr55^{gag}. Short stretches were identified in some sections which were characterized by a local absence of Pr55^{gag} precursor on the inner plasma membrane (Figs. 7B, 7D, and 7F).

Pr55^{gag}—a carrier for various epitopes

In order to analyze the general applicability of this Pr55^{gag}-based carrier system for the presentation of epitopes other than V3, we inserted a linear stretch of the CD4-binding domain (CD4BR; aa 419–444) and a conserved nef-CTL epitope (nef; aa 110–130) into the deletion mutants Pr55Δ1, Pr55Δ2, Pr55Δ3, and Pr55F4. The recombinant vaccinia viruses expressing these chimeric polypeptides were referred to as v-Pr55CD4BR1–4 and v-Pr55nef1–4 (see Fig. 1B). As demonstrated by immunoblotting, the mobility of the chimeric proteins expressed in infected SW480 cells chiefly correlated with their calculated molecular weight (not shown). Harvested cell-culture supernatants were analyzed in sucrose sedimentation gradients as described (Table 2). For all constructs tested, antigenic peaks were detected at the expected sucrose density of 1.15–1.18 g/cm^3 (fractions 11–13). The highest yields of VLPs were obtained after fusion of the selected epitopes to the carboxy terminus of the unprocessed Pr55^{gag} precursor (Pr55CD4BR-4 and Pr55nef-4). For all Pr55nef chimeric polypeptides tested, we repeatedly found the amount of antigen detected in fractions 11–13 to correlate with the distance of the inserted or fused nef-CTL epitope from the Pr55^{gag} carboxy terminus. However, a similar correlation could not be demonstrated for the chimeric polypeptides fused with the CD4BR. Electron microscopical analysis of SW480 cells expressing either Pr55nef-2, -3, and -4 or Pr55CD4BR-2 and -4 demonstrated assembly and budding of particulate structures strongly resembling the intact immature retrovirus-like

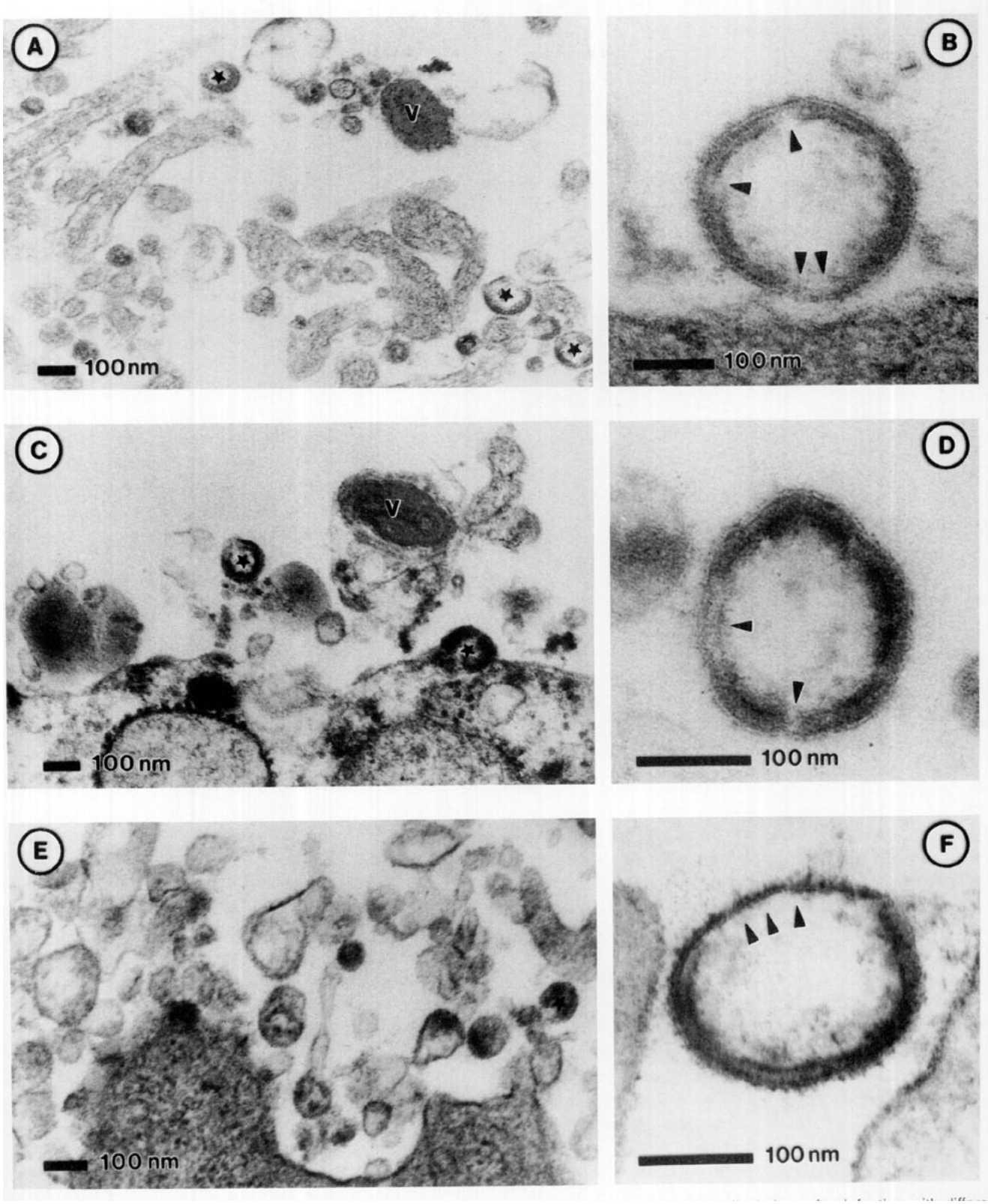

FIG. 8. Ultrathin-section electron micrographs of HIV-1 virus-like particles produced from SW480 cells 2 days after infection with different recombinant vaccinia viruses expressing the chimeric polypeptides $Pr55V3_c$-2 (A, B), $Pr55V3_c$-3 (C, D), and $Pr55V3_c$-4 (E, F). Examples of particulate structures resembling typical retrovirus-like particles are labeled in A, C, and E by asterisks. Due to their size and shape, these virus-like particles can be easily distinguished from vaccinia viruses (200 × 250 nm; V). Details of the ultrastructure of the mutant virus-like particles are given in B, D, and F. Areas within the virus-like particles characterized by a local absence of group-specific antigen are indicated by black arrows.

TABLE 2

CELL-CULTURE SUPERNATANTS

	[pg] p24 f1–f20[a]	[pg] p24 f11–f13[a]	$\frac{f11–f13^b}{f1–f20} \times 100$
Pr55CD4BR-1	795	520	65.4
Pr55CD4BR-2	1190	780	65.5
Pr55CD4BR-3	740	495	66.8
Pr55CD4BR-4	2650	1790	67.5
Pr55nef-1	590	380	64.0
Pr55nef-2	930	670	72.0
Pr55nef-3	1630	1070	65.6
Pr55nef-4	3040	1980	65.1

[a] [pg p24] Determined from the supernatants of 10^7 SW480 cells infected with the indicated vaccinia viruses after sucrose sedimentation analysis (Abbott, p24 capture assay) (see Fig. 2). Total amounts of antigen in fractions 1–20 (f1–f20) and in the antigenic peak fractions (f11–f13) are indicated.

[b] [pg p24] Determined for fractions 11–13 in relation to the total amount of antigen in all fractions (f1–f20).

particles depicted in Fig. 3 (data not shown). Particulate structures detected in preparations of v-Pr55CD4BR-1- and -3-infected cells were slightly aberrant in size and shape, similar to those described as Pr55V3 chimeric particles (Figs. 8B, 8D, and 8F). These data indicate that the length and/or chemical properties of the epitopes inserted into or fused to the Pr55 mutants clearly determine the conformation and capacity of the modified *gag* precursor to assemble and bud as VLPs from eucaryotic cells.

DISCUSSION

In this paper, we describe the effect of three extended deletions (i) overlapping the p17MA/p24CA cleavage site, (ii) located within p24CA, and (iii) within the p6LI portion on the assembly and budding process of the HIV-1 $Pr55^{gag}$ VLPs (Fig. 1).

Deletion of amino acids 99–154 completely abolished the capacity of the mutant polypeptide to assemble into VLPs and resulted in the formation of large cytoplasmic vacuole-like vesicles. These results confirmed previous data obtained for other retroviruses, such as Mason–Pfitzer monkey virus (M-PMV), which indicate that MA genes are very sensitive to deletion mutations (Rhee and Hunter, 1991). Accordingly, we suggest that myristoylation of the amino-terminal glycine is not sufficient to direct the *gag* polypeptides to the correct membrane compartment. Whether the observed transport deficiency is due to the elimination of a specific targeting sequence or rather due to major conformational changes within $Pr55^{gag}$ needs further investigation. Recently, Yu and co-workers described a series of p17MA deletion mutants. Although clearly involved in the process of budding and formation of virus particles, p17MA also seems to play an important role during early steps of the life cycle such as the entry into the target cells (Yu *et al.*, 1992a,b).

Few *gag* proteins have been examined with regard to deletions within the CA region. Different results have been obtained for RSV and MuLV after intensive testing. In the case of MuLV, the CA domain appears to be exceedingly sensitive to even minor deletions and sequence alterations (Schwartzberg *et al.*, 1984). In accordance with RSV, which was shown to tolerate extended deletions within its capsid moiety (Wills and Craven, 1991), we found that a stretch of 30 amino acids within the central region of HIV-1 p24CA can be deleted without significant loss of function. Very recently, others demonstrated that a deletion of 56 amino acids (aa 155–211), which is directly adjacent to the p24CA deletion (aa 211–231) described in this paper, is tolerated without any effect on the morphology of the budding particles (Wang and Barklis, 1993). Taken together, these data suggest that large parts of the capsid proteins of type C retroviruses and of lentiviruses exhibiting a similar type of budding are not essential for the assembly of immature HIV virions.

A 35-amino-acid deletion within the p6LI portion of $Pr55^{gag}$ was demonstrated in our assay system to be well tolerated with respect to the release of immature VLPs. However, our data and similar results by others (Royer *et al.*, 1991; Hoshikawa *et al.*, 1991) deviated from a previous report stating a clear effect of p6LI on the closure and efficient release of immature particles (Goettlinger *et al.*, 1991).

Additionally, we could demonstrate that the fusion of a short polylinker sequence to the $Pr55^{gag}$ carboxy terminus does not alter the functional properties of the *gag* precursor. Taken together, these data indicated that distinct alterations are tolerated without negatively influencing the assembly and release of VLPs from eucaryotic cells.

Based on the mutant $Pr55^{gag}$ polypeptides described we tried to extend the immunological spectrum of the modified VLPs by inserting either the V3 principal neutralizing determinant, the CD4-binding domain, or a nef-CTL epitope into the *gag* mutants. In all cases tested—except in the chimeras based on mutant Pr55Δ1—we found assembly and budding of particulate structures. Depending on the length and amino acid sequence of the inserted epitopes, we detected particulate structures of either identical or slightly altered size and shape compared to immature HIV virions or recombinant $Pr55^{gag}$ VLPs. Accordingly, the yields of budding chimeric VLPs have been demonstrated in some cases to be reduced compared to the wt-$Pr55^{gag}$ precursor or the mutants Pr55Δ1–Pr55Δ3 and Pr55F4.

Different interpretations are possible to explain these alterations. The insertion of foreign amino acids may reduce the ability of the modified *gag* precursor molecules to be involved in protein–protein interactions which are not well characterized so far. As a consequence, a lower packaging density of the chimeric proteins at the plasma membrane might result in an inefficient release of morphologically altered particulate structures. Alternatively, the insertions might alter the flexibility of the chimeric gag precursors, thereby preventing conformational changes which are suggested to lead to the exposure of more hydrophobic residues and consecutive budding of spherical structures (Wills and Craven, 1991; Rhee and Hunter, 1991).

Due to the intrinsic adjuvant properties and high immunogenicity of particulate structures, a series of carrier systems has been developed for the presentation of heterologous epitopes during the past years, mainly based on the HBV surface antigen, its core antigen, and on yeast Ty-A particles (Kingsman and Kingsman, 1989; Michel *et al.*, 1990; Schlienger *et al.*, 1992). However, with respect to a future vaccine development, rationally designed antigens should be as complex as possible and allow the presentation of different immunologically relevant epitopes. This paper described the feasibility of constructing a novel noninfectious, particulate carrier system based on the HIV-1 $Pr55^{gag}$ precursor. Inserting the above-mentioned epitopes into defined positions of the $Pr55^{gag}$ precursor leads in some cases to the generation of chimeric VLPs. Very recently, we demonstrated in a BALB/c mouse model that V3-specific CTLs can be induced by the $Pr55^{gag}$/V3 recombinant vaccinia viruses described above. This indicated that a CTL epitope can replace different regions within an antigenic carrier protein without significant loss of biological activity (Wagner *et al.*, 1992b; Wagner *et al.*, 1993). However, the use of these viruses as a live vaccine in rabbits induced only low titers of V3-specific antibodies (data not shown). This was not unexpected, due to previous studies underlining the low immunogenicity of $Pr55^{gag}$ recombinant vaccinia viruses compared to purified $Pr55^{gag}$ VLPs (Wagner *et al.*, 1992a). Detailed immunological analysis of the above-described chimeric VLPs will give us more information on the possibility of inducing a humoral immune response, on neutralizing activities of antibodies directed toward the inserted V3 loop or the CD4-binding domain, and on the induction of a cell-mediated immune response. Whether the position of the epitopes inserted or added to $Pr55^{gag}$ will influence the immunogenicity of the chimeric VLPs remains to be demonstrated. The possibility of presenting more than one epitope by a single chimeric VLP (unpublished results) appears to be very promising with respect to the induction of a broad immune response.

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