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Studies on processing, particle formation, and immunogenicity of the HIV-1 *gag* gene product: a possible component of a HIV vaccine

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Summary. Antigens in a particulate conformation were shown to be highly immunogenic in mammals. For this reason, the particle forming capacity of derivatives of the HIV-1 group specific core antigen p 55 *gag* was assayed and compared dependent on various expression systems: recombinant bacteria, vaccinia- and baculoviruses were established encoding the entire core protein p 55 either in its authentic sequence or lacking the myristylation consensus signal. Moreover, p 55 *gag* was expressed in combination with the protease (p 55-PR) or with the entire polymerase (p 55-*pol*), respectively. Budding of 100–160 nm p 55 core particles, resembling immature HIV-virions, was observed in the eucaryotic expression systems only. In comparison to the vaccinia virus driven expression of p 55 in mammalian cells, considerably higher yields of particulate core antigen were obtained by infection of *Spodoptera frugiperda* (Sf 9) insect cells with the recombinant *Autographa californica* nuclear polyhedrosis (AcMNPV) baculovirus. Mutation of the NH_2-terminal myristylation signal sequence prevented budding of the immature core particles. Expression of the HIV p 55-PR gene construct by recombinant baculovirus resulted in complete processing of the p 55 *gag* precursor molecule in this system. The introduction of an artificial frameshift near the natural frameshift site resulted in constitutive expression of the viral protease and complete processing of p 55, both in *Escherichia coli* and in vaccinia virus infected cells. Interestingly, significant processing of p 55 resembling that of HIV infected H 9 cells could also be achieved in the vaccinia system by fusing the entire *pol* gene to the *gag* gene. Moreover,

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processing was not found to be dependent on amino-terminal myristylation of the *gag* procursor molecule, which is in contrast to observations with type C and type D retrovirus. However, complete processing of p 55 into p 24, p 17, p 9 and p 6 abolished particle formation. Purified immature HIV-virus like particles were highly immunogenic in rabbits, leading to a strong humoral immune response after immunization. Empty immature p 55 *gag* particles represent a noninfectious and attractive candidate for a basic vaccine component.

Introduction

The medical importance of HIV-1 and HIV-2 infections accelerated the detailed biochemical and immunological definition of structural and regulatory proteins of lentiviruses. Intensive work focused on the molecular process leading to the assembly of HIV-virions as one of the key mechanisms in the life cycle of HIV. Increasing attention has been paid to the group specific antigens of HIV-1. The HIV-1 *gag* open reading frame encodes a 55 kDa polyprotein which is subsequently processed into the mature, nonglycosylated matrix protein p 17 (MA), the capsid protein p 24 (CA); the nucleoprotein p 9 (NP) and a link protein p 6 (LI) [25, 31, 41]. Cleavage of the *gag* precursor to yield the mature capsid proteins is at least in part specified by the viral protease (PR) [2, 19, 20, 23]. The viral protease is encoded by the NH_2-terminal region of the *pol* open reading frame which contains precursor forms necessary for viral replication. The *pol* gene products are initially synthesized as a 160 kDa *gagpol* fusion protein, which results from a (− 1) translational frameshift occurring at a relatively low frequency in the *gagpol* overlapping region [14]. This polyprotein precursor molecule may be autocatalytically processed into the 9 kDa protease (PR), the reverse transcriptase (RT), the integrase (IN) and mature core proteins. As for other retroviruses [13, 41] the NH_2-termini of both, the 55 kDa and the 160 kDa precursor proteins are myristylated.

The exact role of the myristyl-moiety and the NH_2-terminus of the capsid presursor molecule is not clear to date, although it has been identified at the amino-terminus of a number of retroviral *gag* products [35, 41]. Myristylation of the *gag* precursor typically occurs cotranslationally [43] and is specified by the NH_2-terminal glycine residue next to the methionine derived from the initiation codon [15]. Obviously, the myristic acid moiety at the amino terminus of p 17 is essential for the release of HIV virus particles [13]. Functions as membrane anchor [14] or signal for intracellular transport [33] have been suggested. As has been also shown in type C Moloney murine leukemia viruses (Mo-MuLV), myristylation of the HIV-1 p 55 *gag* polyprotein precursor is essential for its membrane localization and assembly of capsid protein into discrete particles at the plasma membrane. Changes in the p 17 amino-terminal glycine of provirus clones abolish myristylation of the HIV-1 precursor proteins and prevent virus particle release from transfected cells. In contrast to Mo-MuLV and type D Mason-Pfitzer monkey virus (MPMV), processing of the p 55 capsid precursor is not inhibited in myristylation deficient variants of HIV-1 proviral

clones [9]. Several groups investigated the role of p 55 in the assembly of virus particles when expressed in eucaryotic cells such as yeast [19] and mammalian cells using plasmid vectors [37] or recombinant vaccinia virus [11, 29, 36]. Budding of virus-like particles has recently been shown from a baculovirus vector in insect cells [3, 6].

Due to its group specificity and its particle forming capacity, the HIV core antigen is a promising candidate for vaccine development. As known from other systems such as hepatitis B virus, particulate antigens are highly immunogenic and possess intrinsic adjuvant properties [26]. High antibody titers to p 55 during early stages of infection, the inhibiting activity of reactive monoclonal antibodies in vitro [30] and the significance of core proteins in the elimination of infected cells by cytolytic T-cells [28] support the role of p 55 *gag* in future candidate vaccines. For this reason we conducted a detailed comparative analysis of the particle forming capacity of the HIV-1 core antigen. Identical DNA-constructs containing the entire *gag* in its natural configuration were established and altered (*i*) into a myristylation deficient form, (*ii*) combined with the protease, (*iii*) either in its natural configuration or in frame to p 17/p 24 and (*iv*) fused to the entire *pol* coding sequence. The different polypeptides were expressed in *E. coli* as well as by recombinant vaccinia- and baculoviruses in mammalian and insect cells, respectively. The experiments reported here compare the influence of myristylation and protease activity on HIV-1 core expression and particle assembly in various expression systems in order to identify optimal conditions for the formation of recombinant core particles. Evidence is presented for the induction of a core specific humoral immune response after immunisation of rabbits with purified core particles.

Materials and methods

Plasmid DNA

A DNA clone containing HIV-1 sequences, pBH 102, was kindly provided by Prof. Gallo, NIH, Bethesda, MD, U.S.A. The baculovirus expression vector pVL 941 was provided by Dr. Summers, Texas A & M University, College Station, TX, U.S.A. All reported were propagated in *Eschericha coli* strain JM 109.

Cells

Green monkey kidney CV-1 cells, SW 480 colon carcinoma cells and B 143 cells were grown in Eagles minimal essential medium (MEM) containing 5% fetal calf serum. *Spodoptera frugiperda* (Sf 9) insect cells were propagated in TC 100 medium (GIBCO/BRL, Federal Republic of Germany) supplemented with 10% fetal calf serum.

Sera and monoclonal antibodies

Production and mapping of p 24 specific mouse monoclonal antibody 16/4/2 used for immunoblotting was previously described by Wolf et al. [26]. A p 17 specific mouse monoclonal antibody was kindly provided by Prof. Chandra, University of Frankfurt, Federal Republic of Germany. A rabbit serum directed against the HIV-1 protease was obtained

by Prof. von der Helm, Max von Pettenkofer Institute, Munich, Federal Republic of Germany.

Construction of recombinant plasmids

In order to obtain precise NH_2-termini of the capsid precursor we inserted a 62 bp oligonucleotide,
GATCC*ATG*G GTGCGAGAGC GTCAGTATTA AGCGGGGGAG AATTAGATCG ATGTCGACCT GCA,
encoding the 15 NH_2-terminal amino acids of p 55*gag* into the *Bam* HI/*Hind* III site of the pUC 8 vector. The restriction sites *Cla* I and *Sal* I in the 3′ position within the oligonucleotide were used to complete the *gag* reading frame by insertion of a 1694 bp *Cla* I/*Hinc* II fragment resulting in plasmid pUC8 p55.

For expression of the myristylation deficient capsid precursor, we replaced the 47 bp *Bam* HI/*Cla* I fragment encoding the amino-terminal part of p 17 by a corresponding DNA fragment,
GATTCGTGCA CGCC*ATG*GCT GCGAGAGCGT CAGTATTAAG CGGGGGAGAA TTAGAT.
A G to C transition alters the NH_2-terminal glycine residue into an alanine thereby destroying the myristylation signal sequence (pUC8 p55 M^-). The correct sequence was verified by double strand sequencing using a T 4 polymerase sequencing kit (BRL).

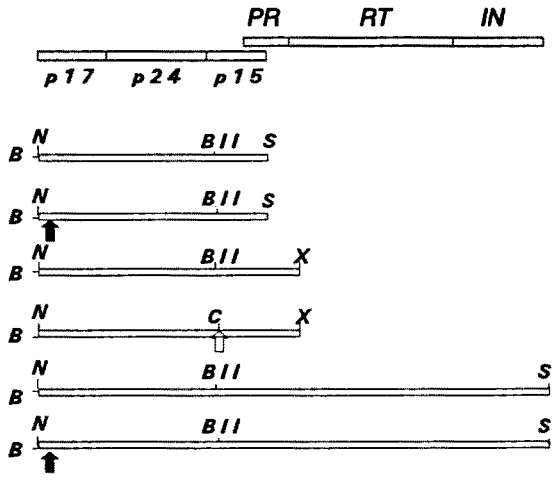
To express p 55 *gag* with the exact protease polypeptide (PR) without additional carboxy-terminal polymerase (*pol*) sequences we inserted a synthetic polylinker sequence,
AATTCGGATT CGTTGACCTG CAG*TAATTAA* T*TAG*ATCTCG A*GCCCGCC*TA ATGA*GCGGGC* TTTTTTGAGC TCA
into the *Eco* RI/*Hind* III restriction sites of pUC 8. This linker fragment contained stop codons in all reading frames (italics) and a procaryotic *rho* independent transcription termination signal (pUC 8 stop) to increase the production of recombinant proteins in *E. coli*. A 402 bp *Nla* IV/*Bal* I fragment including the entire protease reading frame was inserted into the *Hinc* II site of pUC 8 stop to yield pUC 8 PR stop. For insertion of the p 55 *gag* coding sequence, we fused the pUC8 p55 derived *Bam* HI/*Bcl* I fragment with the *Bam* HI/*Bcl* I fragment of pUC 8 PR stop to create pUC8 p55 PR.

To combine the *gag* and the protease gene in the same open reading frame, the *Bgl* II restriction site of pUC8 p55 PR was opened, filled enzymatically using the Klenow fragment and religated, thereby creating an additional *Cla* I site. The resulting plasmid was called pUC8 p55 PR-FS. The enforced frameshift replaced the carboxy terminal 63 amino acids of p 15 by the HIV-1 protease. The regular ribosomal frameshift site is located 6 bp upstream of the generated *Cla* I site.

To align both the myristylation competent and incompetent forms of the *gag* gene with the entire *pol* gene, we replaced the 437 bp *Bgl* II/*Sal* I fragment of pUC8 p55 and of pUC8 p55 M^- by a 3727 bp *Bgl* II/*Sal* I fragment including p 15 and the complete *pol* gene to create pUC8 p55 Pol and pUC8 p55 M^- Pol.

For production of the recombinant proteins and following characterization in *E.coli*, we subcloned the coding sequences into a *Nco* I/*Sal* I digested pTRC 99 ATG-vector to avoid expression of disturbing LacZ derived NH_2-terminal sequences.

To establish recombinant vaccinia viruses, the pUC derived *gag*-gene constructs were subcloned into the *Bgl* II/*Sal* I digested pAvB vaccinia transfer vector [27] to create pAvBp 55, pAvB 55 M^-, pAvBp 55 PR, pAvBp 55 Pol, pAvBp 55 M^- Pol and pAvBPR-FS. For expression by recombinant baculovirus, we subcloned two relevant inserts, encoding p 55 and for comparison p55 PR (Fig. 1), into the *Bam* HI site of the transfer vector pVL 941 [37]. The basic gene constructs and the expression of the polypeptides in the respective expression systems are summarized in Fig. 1.



pTRC E. coli	pAvB Vaccinia (v)	pVL941 Baculo (b)
p55	p55	p55
p55M⁻	p55M⁻	N.D.
p55PR	p55PR	p55PR
p55PR-FS	p55PR-FS	N.D.
p55Pol	p55Pol	N.D.
p55M⁻ Pol	p55M⁻ Pol	N.D.

Fig. 1. Schematic reconstruction of the p 55 and Pol coding sequence. Genomic regions, which were included into the basic constructs are depicted as open bars. Abbreviations of the plasmids and recombinant viruses, described in the text are shown on the right. Restriction sites essential for subcloning are indicated (B *Bam* HI; N *Nco* I; BII *Bgl* II; S *Sal* I; X *Xho* I; C *Cla* I). Mutations introduced to change functional properties of the depicted reading frames are indicated by an open arrow (− 1 frame shift induced by the Klenow fragment at the *Bgl* II restriction site, resulting in a new *Cla* I site) and solid arrows (myristylation signal, Gly-Ala). Biological properties of the polyproteins such as processing of precursor molecules (▲) and particle forming capacity (●) are indicated

Construction of recombinant vaccinia viruses

Transfer of the HIV-1 genes from the recombinant plasmids pAvBp 55, pAvBp 55 M⁻, pAvBp 55 PR, pAvBp 55 PR-FS, pAvBp 55 Pol and pAvBp 55 M⁻ Pol into vaccinia virus Tien Tan (vTT) was achieved by a standard homologous recombination method using CV-1 cells [24]. The recombinant vaccinia viruses were designated as v-p 55, v-p 55 M⁻, v-p 55 PR, v-p 55 PR-FS, v-p 55 Pol and v-p 55 M⁻ Pol, respectively.

Construction of recombinant baculovirus

Sf 9 cells were cotransfected with mixtures of infectious *Autographa califonica* nuclear polyhedrosis virus (AcMNPV) DNA and CsCl purified [34] transfer vector DNA as described by Smith et al. Resultant progeny baculovirus was used to produce plaques in Sf 9 cell monolayers. Plaques lacking polyhedrin structures were selected, purified by further plaque assays and used to produce virus stocks.

Analysis of recombinant polypeptides

IPTG induced recombinant bacteria were harvested and resuspended in lysis buffer as described by Sambrook et al. [34]. CV-1 cells were infected with wild type vTT or recombinant vaccinia virus at 10–20 pfu per cell and incubated at 37 °C for 24 h in serum free MEM. Infected cells were harvested and resuspended in boiling mix. Monolayers of Sf 9 cells were infected with wild type AcMNPV or recombinant baculovirus at a multiplicity of 5–10 pfu/cell and incubated at 28 °C for 1–5 days. Cells were harvested and suspended in the lysis buffer. Supernatant was, without concentration of the recombinant protein, directly mixed with lysis buffer. Protein extracts were boiled for 5 min at 100 °C and analyzed

by SDS-PAGE and Western blotting [21]. Immunological detection was performed using monoclonal antibodies directed to p 24 [44], p 17 and a pool of AIDS patient sera.

Electron microscopy

Infected SW 480 and Sf 9 cells were harvested with a cell scraper, washed with phosphate buffered saline (PBS) and then fixed for 2 h with 2.5% glutaraldehyde in PBS. Cells were washed with PBS and postfixed for 1 h 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 Pb-citrate pH 12.6. All pictures were taken with Siemens Elmiskop 101 electron microscope. The magnification was calibrated with a cross line grating replica.

Gradient sedimentation analysis

Supernatants of Sf 9 cells infected with the recombinant baculoviruses b-p55 and b-p55 PR were collected 5 days p.i. from synthetic medium lacking FCS. Enrichment of core particles from precleared culture supernatant (3300 × **g**) was achieved by a Ultrasette-Filtron filter membrane (Satorius, Federal Republic of Germany) after the protocol of the manufacturers. 2 ml aliquots of the concentrated material was layered onto a 12 ml 10–60% sucrose gradient in 10 mM phosphate buffer pH 7.5 containing 0.15 M NaCl and run at 20,000 rpm for 2 h in a Kontron TFT 41.14 rotor. Fractions (0.7 ml) were collected and directly assayed by conventional Western blot analysis. The yield of recombinant *gag* particles was determined using a commercial p 24/p 55 specific antigen capture assay (Abbot).

Reverse transcriptase assay

Supernatants of uninfected SW 480 cells and cells infected by the recombinant vaccinia viruses v-p55, v-p55 M^- Pol and v-p55 Pol (10 pfu/cell) were collected 2 days p.i. and precleared in a Kontron A 8.24 rotor for 15 min with 8000 rpm. The supernatant was layered onto a 2 ml 37% sucrose cushion and ultracentrifuged at 5 °C for 2 h at 20,000 rpm in a Kontron TFT 41.14 rotor. The supernatant was discarded and the pellet was assayed for RT activity in a reverse transcriptase test using poly rA : dT and Mg^{++} as described previously [10].

Results

Basic constructs and concepts

In order to conduct a detailed comparative analysis of the p 55 particle forming capacity in different eucaryotic expression systems, we established a series of *gag*-gene constructs in the pUC vector system. In addition to the authentic p 55 *gag* gene (pUC8 p55), a mutant form of the *gag* precursor lacking the NH_2-terminal myristylation signal sequence was constructed altering the glycine residue at position 2 of the amino acid sequence to alanine (gly to ala, pUC8 p55 M^-). The effect of proteolytic processing on *gag* expression and particle formation was investigated by adding the protease (PR) coding sequence to the *gag* gene (*i*) in its natural configuration (pUC8 p55 PR) and (*ii*) in frame to p 55 (pUC8 p55 PR-FS). Finally, we added the entire *pol* gene to the "p 55" and "p55 M^-" gene-constructs to reveal the proteolytic capacity of the HIV-

1 protease in the complete 160 kDa *gagpol* polyprotein and to investigate the effect of myristylation on the processing of the precursor molecules. Using identical *gag*-gene constructs the level of HIV-core expression, proteolytic events and efficiency of particle formation could be determined and directly compared in different vector systems. Basic constructs and results described in the results are summarized in Fig. 1.

Expression and biochemical analysis of p 55 and p 55-derived polypeptides in E. coli

After subcloning of the *gag*-gene constructs into the ATG-vector pTRC 99, recombinant proteins were expressed in *E. coli* (Fig. 1). Immunoblot analysis using different p 24 and p 17 specific monoclonal antibodies revealed that all bacterial clones except pTRC p55 PR-FS expressed the authentic p 55 kDa *gag* precursor. Significant, but not complete processing was observed in lysates of p55 PR-, p55 Pol- and p55 M^- Pol-recombinant bacteria (data not shown). Reverse transcriptase activity was measured in both bacterial clones, containing the entire polymerase gene (p55 Pol; p55 M^- Pol).

In contrast, complete processing of the 65 kDa p55 PR precursor was achieved expressing the PR-gene in frame with the *gag* gene (pTRC p55 PR-FS). The p 24/25 cleavage products were clearly visible in a SDS-PAGE stained with coomassie brilliant blue and could easily be detected by conventional Western blot analysis using p 24 specific monoclonal antibodies. Further detection of p 17 and the protease (9 kDa) by a monoclonal antibody directed to p 17 and a monospecific peptide serum directed to protease sequences confirmed the complete processing (data not shown).

Alteration of the myristylation signal sequence did not affect p 55 expression in *E. coli*. Neither sucrose gradient sedimentation analysis performed with culture supernatants of recombinant bacteria or cell extracts, nor electron microscopy analysis performed with recombinant bacteria gave any hint on HIV-core particle formation in *E.coli* (data not shown).

Expression and biochemical analysis of p 55 and related constructs by recombinant vaccinia virus

Expression of the described constructs in CV-1 cells by recombinant vaccinia viruses was a fast approach to evaluate parameters involved in processing and core particle assembly (Fig. 1). The recombinant vaccinia viruses (v-p55, v-p55 M^-, v-p55 PR, v-p55 PRFS, v-p55 Pol and v-p55 M^- Pol) correspond exactly to the bacterial expression clones mentioned earlier. As shown by Western blot analysis (Fig. 2 A), infection of CV-1 cells with v-p55, v-p55 M^- and v-p55 PR resulted in the expression of a 55 kDa and, to a smaller extent, of a 41 kDa protein. Addition of the exact HIV-1 protease coding sequence (v-p55 PR) was not sufficient for detectable processing of p 55 *gag* in CV-1 cells (Fig. 2 A, lanes 5 and 12). The v-p55 PR derived 41 kDa protein was clearly

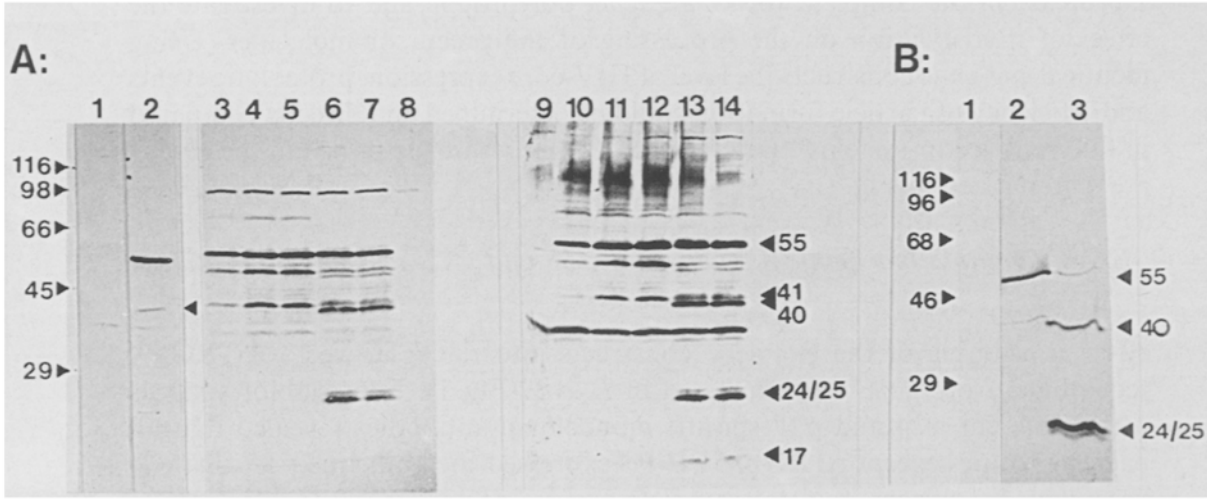


Fig. 2. A Pattern of proteins synthesized in CV-1 cells (2×10^5) after infection with different recombinant vaccinia viruses (10 pfu/cell). Recombinant polypeptides were specifically detected by Western blot analysis using murine monoclonal antibodies directed to p 17 (*1* and *2*), p 24 (*3–8*) and by an AIDS patient serum pool (*9–14*). For negative control, CV-1 cells were infected by vTT wild type vaccinia virus (*1, 8,* and *9*). To compare the influence of myristylation and proteolytic processing on p 55 expression, cells were infected by v-p55 (*3* and *10*), v-p55 M^- (*4* and *11*), v-p55 PR (*2, 5,* and *12*), v-p55 Pol (*6* and *13*) and by v-p55 M^- Pol (*7* and *14*). **B** Complete processing in the vaccinia systems is shown in CV-1 cells infected with v-p55 PR-FS (*3*) as compared to CV-1 cells infected with v-p55 (*2*) and wild type vaccinia virus vTT. Molecular weight markers are indicated (kDa)

recognized by a p 17 specific monoclonal antibody (Fig. 2 A, lane 2). For this reason, the production of p 41 was, at least partially, rather due to a suggested premature translational stop signal near the COOH-terminal part of the p 24 coding sequence [9] than to HIV-mediated proteolytic events. Kinetical studies, following the infection over a period of 48 h did not show any specific, protease mediated processing, although the PR coding sequence was verified by DNA sequencing. In contrast to some previous reports [4, 36], we found significant processing when the entire *pol* reading frame was added to the *gag* gene. Unprocessed p 55 *gag*, p 24/25 and different *gag*-processing intermediates with an estimated molecular weight of 40–41 kDa could now easily be detected in cell lysates (Fig. 2 A, lanes 6 and 7). Using a pool of sera from AIDS patients, p 17 could be shown in addition to the polypeptides mentioned earlier (Fig. 2 A, lanes 13 and 14). As clearly demonstrated by analyzing extracts of v-p55 M^- Pol infected CV-1 cells, processing was not dependent on previous myristylation of the *gag* precursor molecule. For comparison expression of p55 PR-FS resulted in an almost efficient processing of the core precursor in the vaccinia expression system (Fig. 2 B).

Sedimentation studies performed with culture supernatants and following Western blot analysis of the precipitated antigens gave identical results as

described for the cellular extracts in the case of v-p55-, v-p55 PR and v-p55 Pol infected cells. No antigen was detectable in culture supernatants of cells infected with the myristylation deficient *gag*-constructs (v-p55 M^-, v-p55 M^- Pol) and with v-p55 PR-FS (data not shown). 2 days following infection with recombinant vaccinia viruses supernatants of SW 480 cells were analyzed for reverse transcriptase (RT) activity as described in Materials and methods. RT activity could be precipitated only from the culture supernatant of v-p55 Pol infected cells, not from supernatants of cells infected with the analogous myristylation deficient form (v-p55 M^- Pol) (Fig. 3). These results gave first evidence (*i*) for the release of particulate aggregates, that (*ii*) need NH_2-terminal myristylation for the budding from the infected cells into the culture supernatant.

Requirements for budding of immature particles from mammalian cells infected by recombinant vaccinia virus

Budding of p 55 core particles (100–160 nm in diameter) from the plasma membrane and release into the culture medium was detected by electron microscopy after infection of SW 480 cells with v-p55, v-p55 PR and v-p55 Pol (Figs. 1 and 4). The lack of myristylation (v-p55 M^-, v-p55 M^- Pol) at the NH_2-termini apparently prevented targeting of the precursor polypeptides to the plasma membrane, confirming that myristylation of the polyprotein precursor is essential for its membrane localization and consecutive particle formation. Assembly was also abolished when the HIV-protease was expressed in an artificial construct in frame with p 55 (v-p55 PR-FS; data not shown). Analysing v-p55 Pol infected cells morphologically, we clearly detected p 55 particle formation and budding of recombinant particles into the culture supernatant. However, using the conventional vaccinia expression system, the yield of recombinant immature core particles was not sufficient for preparative scale fermentation.

The shape of p 55 *gag* particles produced by v-p55 and v-p55 Pol infected SW 480 cells is spherical, ovoidal or ellipsoid. The budding particles strongly resemble immature virus-like particles released from HIV-1 infected cell cultures [5, 16]. There was no difference in a number of ultrathin sections detectable between v-p55 and v-p55 Pol derived core particles. Although we clearly found processing of the *gag* precursor in v-p55 Pol infected cells, we could neither find maturation of immature virus-like particles nor condensation of p 24 to an

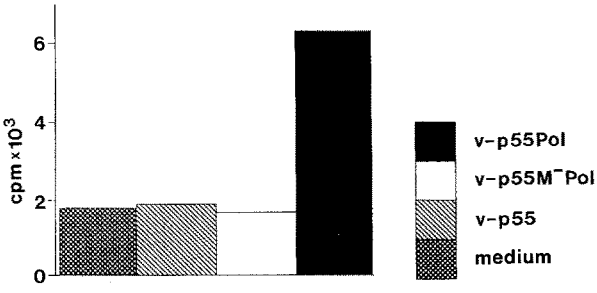
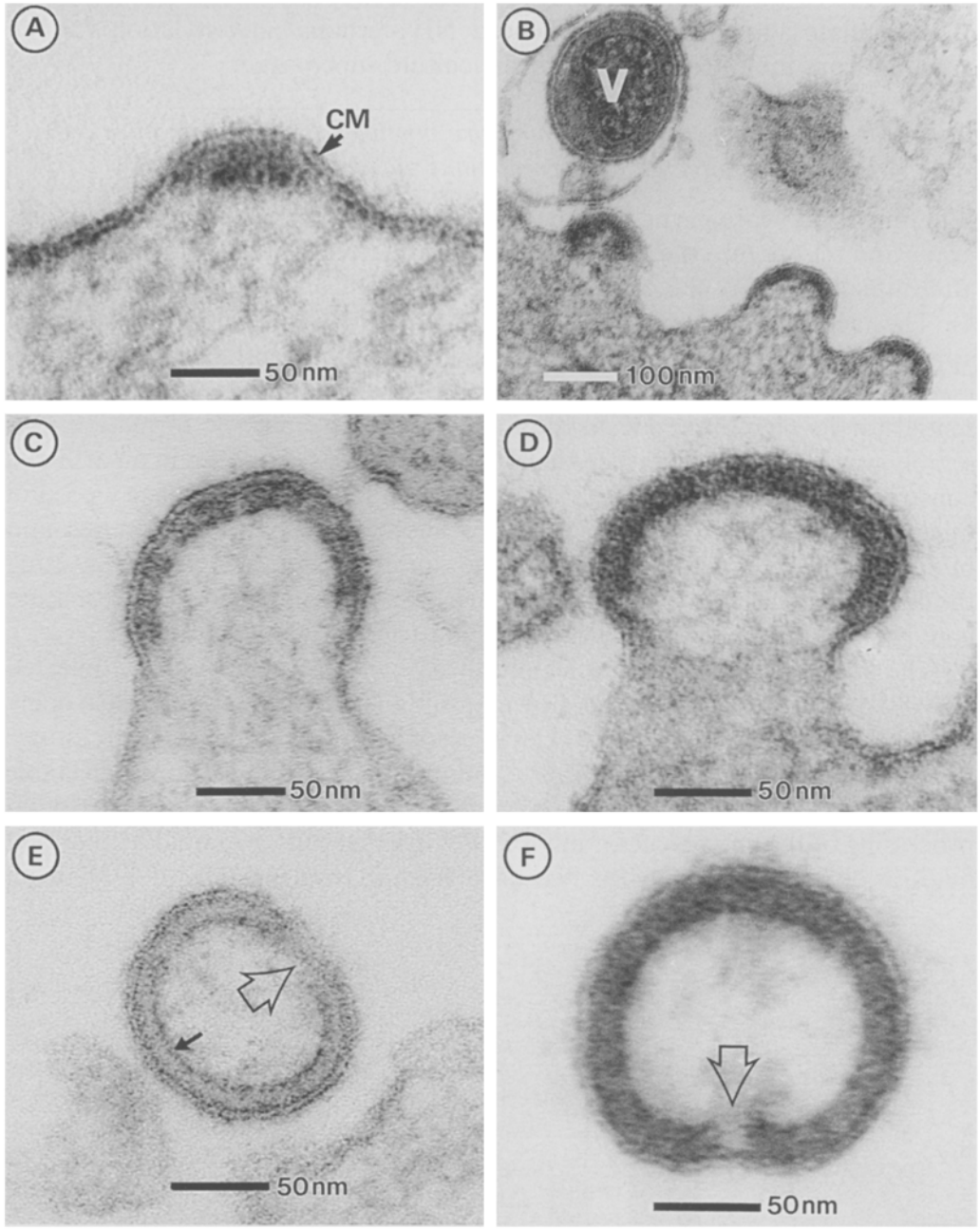


Fig. 3. Reverse transcriptase activity profile of supernatants from uninfected SW 480 cells and from SW 480 cells infected with the recombinant vaccinia viruses v-p55, v-p55 M^- Pol and v-p55 Pol

electron dense conical capsid known from mature HIV virions. The diameters of the budding structures show variations from 100 to 160 nm, whereas a mean diameter of 130 nm is most frequently observed. Vaccinia virus particles were larger (290 × 195 nm) and could be easily distinguished from p55 *gag* particles (Fig. 4 B). Plasma membranes with a thickness of 6–7 nm (Fig. 4 A) formed spherical vesicles, the cytoplasmic site was coated by an electron-dense layer



with a thickness of 14–17 nm. Occasionally, this layer exhibited an additional, more electron dense border line (Fig. 4 E). The electron dense "15 nm" layer was typically interrupted at the site of budding (Fig. 4 E and F). Essentially, all p55 *gag* particles produced in the vaccinia system showed an electron-translucent lumen which exhibited by far less granular components that the cytoplasm. The content of fibrous material was similar to that of the cytoplasm.

Expression and biochemical analysis of gag and related constructs in the baculovirus expression system

To increase the yields of recombinant *gag*-particles and to analyze adverse effects of HIV protease mediated processing on particle formation, two of the basic constructs, pVL p55 and pVL p55 PR, were expressed by a recombinant baculovirus in insect cells. Massive production of recombinant p 55 was achieved by infection of Sf 9 cells with b-p55 recombinant baculovirus (Fig. 5). It is noteworthy that unprocessed capsid precursor could be easily detected on immunoblots analyzing infected cells and in culture supernatants harvested 5 days p.i. without prior concentration. In parallel to high expression, proteolytic digestion of the capsid precursor by cellular or AcMNPV specific proteases could be observed in cellular extracts. In contrast to the vaccinia expression system (v-p55 PR; Fig. 2 A, B), addition of the HIV-1 protease gene (b-p55 PR) resulted in complete processing of the p 55 precursor molecule in the baculovirus vector system.

Budding of immature p55 gag particles following expression of the capsid precursor by recombinant baculovirus

For analysis of the p 55 particle forming capacity, Sf 9 insect cells were infected with b-p55 and, for comparison, with b-p55 PR. Infected cells were examined by electron microscopy (Fig. 6).

Fig. 4. Ultrathin sections of SW 480 cells infected by v-p55 and v-p55 Pol recombinant vaccinia virus illustrating the typical budding mechanism. p55 *gag* precursor molecules accumulate, directed by amino-terminal myristylation, at the cytoplasmic site of the plasma membrane (*CM*) forming electron dense hot spots (**A**). Proceeding aggregation of the capsid precursor forces the plasma membrane to form first hemispheral structures (**B** and **C**), followed up by more spherical vesicular particles (**D**). Dissection of the immature p55 *gag* particles is managed by fusion of the plasma membrane. Appropriate section identifies the site of budding (open arrow), which is characterized by a local absence of the precursor molecule at the inner plasma membrane (**E** and **F**). The architecture and the budding mechanism of both, v-p55 (**C** and **E**) and v-p55 Pol (**D** and **F**) seems to be identical. An internal osmiophilic borderline (**E**, small arrow) can be visualized in very thin sections (35 nm) as compared to 60 nm sections (**F**). **B** Due to its size and shape (290 × 195 nm), recombinant vaccinia virus (*V*) can be easily distinguished from the typical lentivirus-like budding core particles

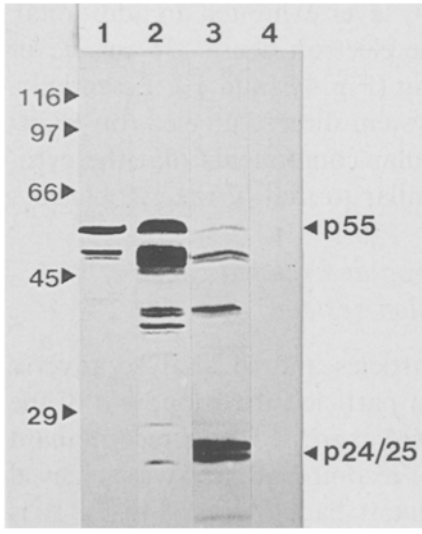


Fig. 5. Western blot analysis showing recombinant proteins expressed in Sf9 cells (6×10^4) infected with recombinant baculovirus (5 pfu/cell). Recombinant proteins were detected by the p24 specific monoclonal antibody [41]. *1* Supernatant (10 µl) of b-p55 infected Sf9 cells 5 days p.i., *2–4* extracts of Sf9 cells harvested 5 days after infection with b-p55 (*2*), b-p55PR (*3*) and with AcMNPV wild type baculovirus (*4*). Molecular weight markers are indicated (kDa)

B-p55 infected cells showed multiple lentivirus-like budding of particles with 100–160 nm in diameter strongly resembling those budding from v-p55 and v-p55Pol infected cells at the cell membrane surface (Fig. 4). These structures were not detected in Sf9 cells infected by b-p55PR and with wild type AcMNPV (data not shown). Assembly of the budding structures occurs at the cytoplasmic site of the plasma membrane. No particles or particulate structures were detected intracellularly. Production of the budding particles was abundant, leading to the release of single particles (Fig. 6A) and to oligomeric aggregation (n = 2–4; Fig. 6B) caused by incompletely dissociated complexes of budding structures or by monomeric particles fused immediately following the budding event. The structures dissociating from the cell membrane differed slightly in size and shape.

Immature particles, produced in Sf9 cells showed an electron translucent center, which was covered by a dark, electron-dense ring and an outer 14–17 nm medium grey regular shell. The b-p55 derived particulate structures were surrounded by a 6–7 nm plasma membrane derived from the infected cell. Within the lumen of p55 *gag* particles formed by the baculovirus system, electron-dense granules with diameters of about 15–20 nm were observed (Fig. 6). These extracellular p55 *gag* particles were similar to structures previously described as immature HIV-1 virions [5, 16]. Ultrathin section electron microscopy performed with b-p55PR infected cells revealed a dramatic decrease in p55 particle formation, possibly due to the observed complete intracellular processing of the *gag*-precursor (Fig. 5), thus confirming that complete intracellular processing prevents core particle formation.

In order to confirm p55 particle formation and shedding of the particulate structures into the culture medium, we performed sucrose gradient sedimentation analyzing the culture supernatant of Sf9 cells harvested 1–5 days after infection with b-p55. Fractions collected from the gradient were analyzed by immunoblotting followed by densitometric scanning. Highest yields of budding

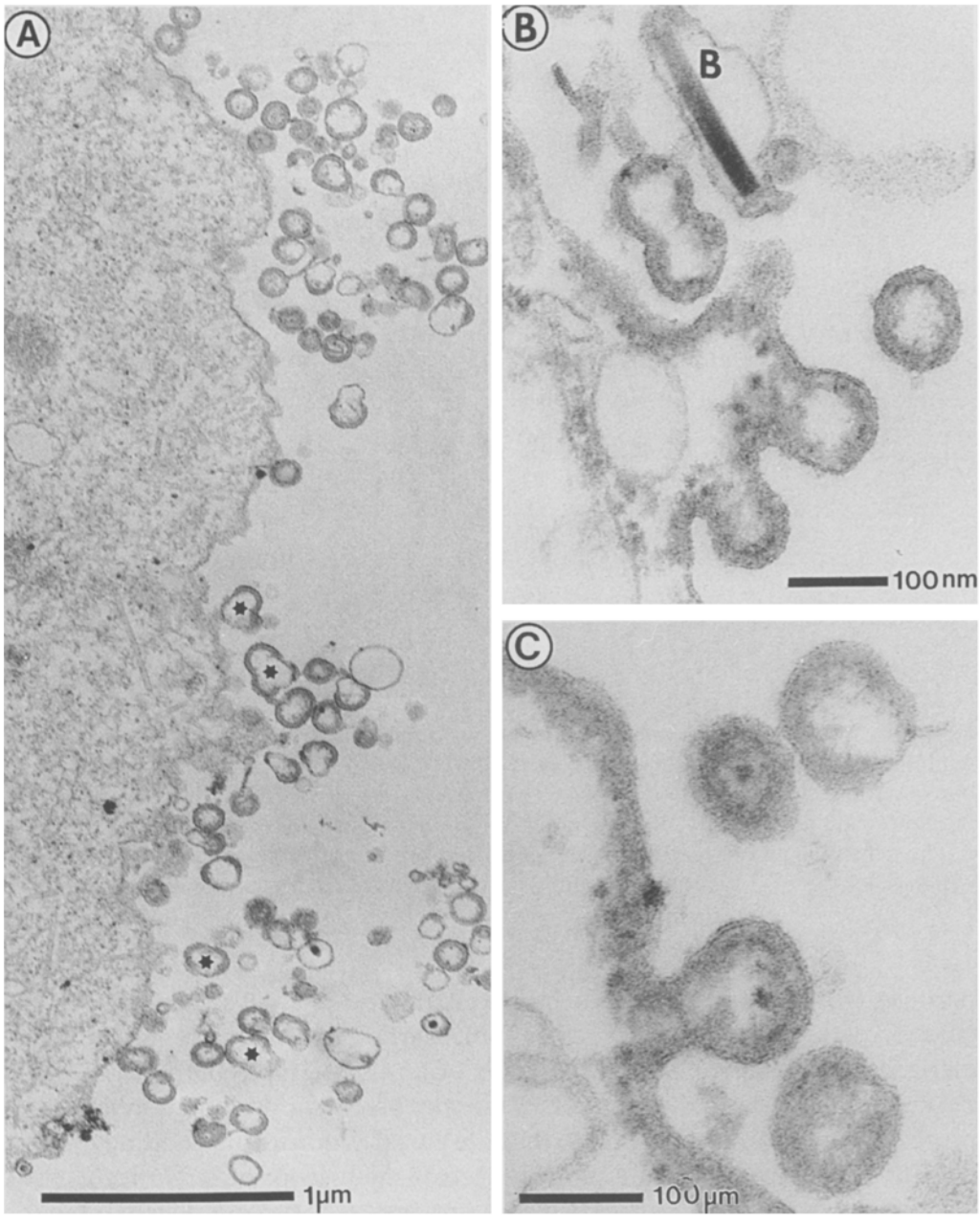


Fig. 6. Ultrathin section of Sf 9 cells infected with a b-p55 recombinant baculovirus showing multiple budding of immature lentivirus like particles (**A**). The proposed mechanism of budding (**B** and **C**) is analogous to that observed at the plasma membrane of SW 480 cells infected by the corresponding recombinant vaccinia virus (v-p55, Fig. 4). **A** Dramatic budding in close vicinity obviously results in the formation of oligomeric and giant particles (asterisks). **B** Due to their rod-like shape, recombinant baculoviruses (*B*) can be easily distinguished from the budding p 55 core particles

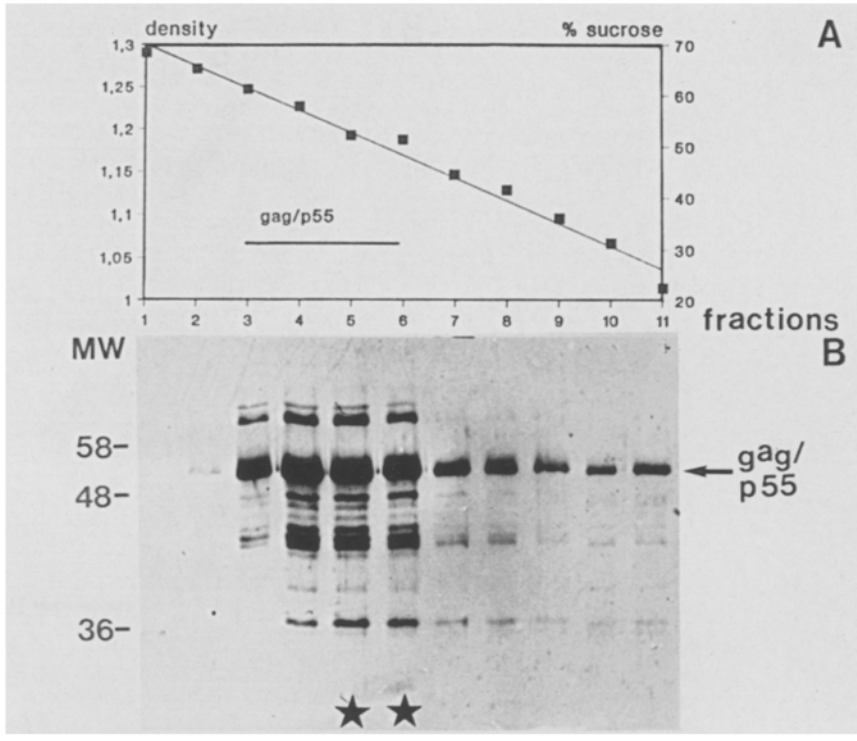


Fig. 7. Sucrose sedimentation analysis. Cells were infected with 10 pfu/cell b-p55. Supernatant was harvested 5 days p.i., precleared (3000 × **g**) in a Kontron centrifuge and layered onto a sucrose gradient (10–50%). After 2 h of centrifugation in a TST 41.14 rotor, sequential fractions were analyzed by Western blot analysis using the p 24 monoclonal antibody (**B**). **A** The density of the serial fractions is indicated (■); antigenic peak fractions are labeled with a black bar. **B** Fractions containing particulate p 55 core structures are indicated (★)

particles were obtained from supernatants harvested 5 days p.i. (Fig. 7). Specifically detected bands of a higher molecular weight might represent oligomerized p 55 precursor proteins. Signals of a reduced molecular weight might be due to a not specific degradation of the core precursor protein by cytoplasmic proteases randomly packaged into the core particles during the budding process. Negative staining and examination by electron microscope of the antigenic peak fractions demonstrated particulate core antigens at a sucrose density of 1.16–1.19 kg/l. Particulate structures were budding into the culture supernatant to an estimated concentration of 20–30 mg/l. Peak fractions corresponded to a major 55 kDa protein species which was specifically detected in Western blots by monoclonal antibodies to p 24 (Fig. 7). In gradients performed with b-p55 PR derived culture supernatants, this peak fraction, containing particulate structures was missing (data not shown). These data clearly confirmed that (*i*) p55 *gag* particles are budding from b-p55 infected Sf 9 cells into the culture medium and that (*ii*) particle formation is considerably reduced by HIV-1 protease mediated processing as demonstrated in b-p55 PR infected Sf 9 cells.

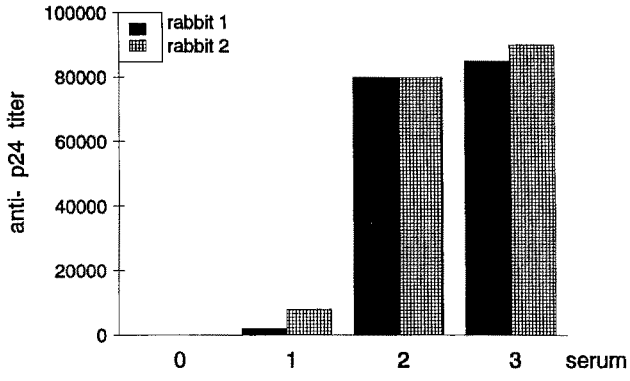


Fig. 8. p 24 specific IgG antibody response induced in 2 rabbits. Sera were taken 10 days following the injection of purified p55 *gag* particles; *0* preimmune sera; *1–3* antisera

Immunogenicity of immature HIV virus-like particles

To demonstrate the immunogenicity of HIV-1 virus like particles, supernatants of b-p55 infected Sf9 cells were harvested and the immature particles were partially purified as described in Material and methods. Primary immunisation of two rabbits was performed using 67 µg of HIV-core antigen in complete Freund's adjuvant, followed by two booster injections with the same antigen dose in incomplete Freund's adjuvant after 2 and 6 weeks. Ten days after each immunisation serum samples were tested for anti p24 IgG production by a commercial antigen specific ELISA (Behringwerke AG, Marburg, Federal Republic of Germany). Antibody titers following the primary and booster injections are shown in Fig. 8. The induced antibody titers were similar in both animals. The animals developed a strong anamnestic response following the first booster injection reaching titers in excess of $1/10^5$. A further booster injection did not markedly increase the response.

Discussion

Intrinsic adjuvant properties of particulate antigens are indicated by successful and routine application of recombinant 22 nm hepatitis B virus (HBV) surface antigen (HBsAG) particles as a protective vaccine in humans [40]. Particle formation was also reported for the production of HBV core antigen in *E. coli* and for the capsid precursors of several retroviruses and retrovirus-like particles expressed in eucaryotic expression systems [1, 7, 17, 18]. Autologous and chimeric Ty-particles were producted in yeast cells and purified for the application in immunization experiments and clinical studies [27]. Vaccine mediated induction of an extensive humoral and cellular immune response to the HIV-1 core proteins might prove important in HIV vaccine development. The HIV-1 group specific antigen is highly conserved amongst different HIV-1 and HIV-2 isolates and was characterized in the past as an important target of cell mediated [28, 42] and humoral immunity [30, 44]. We suggest that HIV-1 core particles themselves or products derived by adding additional sequence information are well suited as a safe and effective candidate vaccine. This study

aimed at investigating the parameters (e.g., HIV protease mediated processing or amino-terminal myristylation of the *gag* precursor) involved in budding of HIV-1 core particles after expression of artificial *gag*-gene constructs in various expression systems. In order to optimize HIV-1 core particle formation, different procaryotic and eucaryotic expression systems were compared. For optimal conditions of comparison, identical basic constructs were used for the production of different *gag* derived recombinant proteins.

Core particle formation was not found in *E. coli*. One explanation could be the lack of posttranslational modifications which occur in authentic HIV infections [8, 41], but not in *E. coli*. As confirmed by our experiments analysing v-p55 and v-p55 M^- infected SW 480 cells, glycine dependent NH_2-terminal myristylation is essential for the budding of 100–160 nm immature p 55 particles (Fig. 1). In contrast to the nuclear localization of unmyristylated p 55 produced in the baculovirus system [6], we could not detected an accumulation of particulate structures in the nucleus of v-p55 M^- infected SW 480 cells. Interestingly, amino-terminal myristylation of the *gagpol* precursor is not an absolute prerequisite for consecutive processing. This is shown by the processing of the core precursor in v-p55 M^- Pol infected CV-1 cells thus underlining major differences in the capsid morphogenesis between lentiviruses and type D retroviruses [8] (Fig. 2 A).

Comparing both of the tested eucaryotic expression systems with respect to their p 55 particle forming capacity, we conclude that budding of immature *gag* particles is available from v-p55 infected mammalian cells as well as from Sf 9 insect cells infected with a p 55 recombinant baculovirus. Detailed morphological analysis of p55*gag* particles budding from infected SW 480 and Sf 9 cells by ultrathin section electron microscopy demonstrated a fine structure very similar to the immature *gag* particles detected in authentic HIV infections [5]. This proposes a common mechanism for assembly of immature particles at the cytoplasmic side of the plasma membrane (Figs. 4 and 6). However, we found dramatic differences in the amounts of budding of immature core particles between the expression systems tested. Corresponding to the fulminant expression of p 55 in b-p55 infected cells, we found cluster-like accumulations of *gag* molecules at the inner cell membrane. A cluster of membrane bound *gag* precursor molecules results in hotspots of budding particles (Fig. 6). Due to the abundant budding, a considerable part of the detected free particles produced from b-p55 infected insect cells is specified by oligomeric aggregates. In contrast, we find only small amounts of single, not oligomerized p 55 particles budding from the plasma membrane of v-p55 infected SW 480 cells, possibly due to the significantly weaker expression of the *gag* precursor in the vaccinia system. Based on densitometric analysis of immunoblots we found a 50 fold excess of *gag*-protein synthesis in the baculovirus system (b-p55) as compared to the production of the core precursor by recombinant vaccinia virus in mammalian cells (data not shown). According to the described existence of cellular myristyl moiety receptor molecules [32, 33], a reduced number of these receptors in the

tested mammalian cells might be responsible for the comparably low yield of p55 *gag* particles in the vaccinia virus system. Moreover, differences in growth conditions between Sf9 cells (28 °C) and SW 480 cells (37 °C) might influence the membrane fluidity and consequently the formation of *gag*-particles.

Addition of PR- or *pol*-coding sequences to the *gag* gene did not provide for the synthesis of particles that resemble naturally occurring mature capsid structures including the typical conical p 24 core [5, 16]. Interestingly, addition of the exact PR coding sequence to the *gag* gene in its original reading frame (v-p55 PR) was not sufficient to induce significant processing of the core precursor in the vaccinia virus system (Fig. 2 A). This unexpected result is in agreement with findings of Shioda et al. [36]), but is in clear contrast to the observed complete processing of the *gag*-precursor in extracts of b-p55 PR infected Sf9 cells (Fig. 5). Addition of the entire *pol* coding sequence to the *gag* gene and expression by vaccinia viruses (v-p55 Pol, v-p55 M^- Pol) resulted in a significant, but not complete processing of the polyprotein precursors, that resembled the authentic situation in HIV-infected H 9 cells. This strongly indicates the involvement of regions COOH-terminal from the actual PR-domain in efficient formation of enzymatically active protease dimers (Fig. 2 A). In contrast, complete processing of the *gag* precursor shown by b-p55 PR infected Sf9 cells (Fig. 5, lane 3) and by v-p55 PR-FS infected mammalian cells (Fig. 2 B) drastically reduced core particle formation (Fig. 1). We propose, that dimerization of monomeric PR-domains, suggested to be necessary for complete processing of precursor molecules, which is not found in HIV infected cells, requires high intracellular concentrations of the *gag* PR-readthrough proteins within the cell. Such concentrations possibly are only reached in artificial expression systems like, e.g., b-p55 PR infected Sf9 cells or in CV-1 cells infected with the v-p55 PR-FS vaccinia virus.

However, even partial processing did not finish the core maturation process. As demonstrated in a number of ultrathin sections, we could only find core particles budding from the plasma membrane described as immature virus particles by Gelderblom et al. [5]. These results clearly indicate, that the synthesis of mature capsids in artificial expression systems can not be achieved by simple addition of the protease or polymerase gene to the *gag* open reading frame. Recent publications described the synthesis of *gag* particles containing an electron dense core and HIV-RNA using a vaccinia driven expression system [11] and in stable transfection experiments [12]. A common feature of both experiments is the use of DNA constructs including, in addition to the PR coding sequence, an RNA packaging signal, located only few basepairs upstream of the p 55 coding sequence [22]. In contrast, using *gag* sequences lacking the packaging signal sequence, we could not find RNA packaged in our *gag* particle preparations in Nothern blot analysis (data not shown). Taken together, these observations suggest, that viral HIV RNA containing the packaging signal is at least partially involved in the capsid maturation process.

Immunological evaluation of immature *gag* particles purified from b-p55

infected Sf 9 cells showed a high immunogenicity in rabbits. Preliminary results, using p 55 recombinant vaccinia viruses (v-p55) for immunisation of rabbits gave significantly lower titers of specific antibodies suggesting that recombinant core particles were more immunogenic than the vaccinia constructs (unpubl. data). Taken together, we conclude that empty immature p55 *gag* particles lacking viral RNA and produced by recombinant baculovirus are well suited as a noninfectious vaccine component. Parallel studies try to identify regions in the *gag* precursor that can be exchanged by immunologically relevant epitopes encoded by other HIV-1 genes without disturbing the p 55 particle forming capacity. This approach aimed at extending the immunological spectrum of the p55 *gag* derived particles by inserting cytotoxic and helper T-cell determinants as well as neutralizing epitopes such as the gp 120 principal neutralizing determinant V 3 (PND). Adverse side effects such as gp 120 dependent, antibody mediated enhancement of infection or the induction of immune suppresive effects would be excluded. The safety of engineered particulate vaccines would be guaranteed by the lack of viral LTR elements and primer binding sites. Moreover, deletion of the RNA packaging signal, the lack of enzymes (PR, RT, IN) and essential regulatory proteins (Tat, Rev, Vif) important for a possible recombination to infectious particles would enhance the safety of such a candidate vaccine.

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