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Interaction of human immunodeficiency virus type 1 Vif with Gag and Gag–Pol precursors: co-encapsulation and interference with viral protease-mediated Gag processing

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Interactions of human immunodeficiency virus type 1 (HIV-1) Vif protein with various forms of Gag and Gag–Pol precursors expressed in insect cells were investigated *in vivo* and *in vitro* by co-encapsulation, co-precipitation and viral protease (PR)-mediated Gag processing assays. Addressing of Gag to the plasma membrane, its budding as extracellular virus-like particles (VLP) and the presence of the p6 domain were apparently not required for Vif encapsidation, as non-N-myristoylated Δ p6-Gag and Vif proteins were co-encapsidated into intracellular VLP. Encapsidation of Vif occurred at significantly higher copy numbers in extracellular VLP formed from N-myristoylated, budding-competent Gag–Pol precursors harbouring an inactive PR domain or in chimaeric VLP composed of Gag and Gag–Pol precursors compared with the Vif content of Pr55Gag VLP. Vif encapsidation efficiency did not seem to correlate directly with VLP morphology, since these chimaeric VLP were comparable in size and shape to Pr55Gag VLP. Vif apparently inhibited PR-mediated Pr55Gag processing *in vitro*, with preferential protection of cleavage sites at the MA–CA and CA–NC junctions. Vif was resistant to PR action *in vitro* under conditions that allowed full Gag processing, and no direct interaction between Vif and PR was detected *in vivo* or *in vitro*. This suggested that inhibition by Vif of PR-mediated Gag processing resulted from interaction of Vif with the Gag substrate and not with the enzyme. Likewise, the higher efficiency of Vif encapsidation by Gag–Pol precursor compared with Pr55Gag was probably not mediated by direct binding of Vif to the Gag–Pol-embedded PR domain, but more likely resulted from a particular conformation of the Gag structural domains of the Gag–Pol precursor.

Introduction

Of the six auxiliary gene products of human immunodeficiency virus type 1 (HIV-1), the highly conserved virion infectivity factor, Vif (Oberste & Gonda, 1992; Sova *et al.*, 1995; Wieland *et al.*, 1994), is probably the most elusive in terms of structure and function (Camaur & Trono, 1996; Cohen *et al.*, 1996; Trono, 1995). Vif protein is essential for virus growth in primary blood lymphocytes, macrophages and

certain T cell lines (Bouyac *et al.*, 1997a; Courcoul *et al.*, 1995; Gabuzda *et al.*, 1992, 1994; Goncalves *et al.*, 1996; von Schwedler *et al.*, 1993; Sova & Volsky, 1993). It was hypothesized at first that the *vif*-defective phenotype resulted from a defect in entry and uncoating of the infecting virions (Borman *et al.*, 1995; von Schwedler *et al.*, 1993) or/and from improper core protein maturation and packing, based on EM observation (Borman *et al.*, 1995; Bouyac *et al.*, 1997b; Höglund *et al.*, 1994; Simm *et al.*, 1995). However, other EM and biochemical analyses revealed no detectable differences in the ultrastructure (Ochsenbauer *et al.*, 1997) and protein patterns (Fouchier *et al.*, 1996; Ochsenbauer *et al.*, 1997) of *vif*⁺ and *vif*⁻ virions.

Likewise, the cell-species restriction for *vif*-defective virus had originally led to the conclusion that cellular factor(s) could complement *in trans* the absence of *vif* functions. However,

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recent data suggest that Vif can counteract an inhibitory function present in non-permissive cell lines such as human T cells that interferes at late stages in the virus life cycle (Madani & Kabat, 1998; Simon *et al.*, 1998*a, b*). Thus, Hck, a tyrosine kinase of the Src family, has been shown to be able to inhibit the production and infectivity of *vif*-deleted virus, but not that of wild-type (WT) virus. The negative effect of Hck on HIV-1 replication is overcome by Vif (Hassaine *et al.*, 2001). Vif has also been shown to interact with cytoskeleton elements (Karczewski & Strebel, 1996) and to inhibit the proteolytic activity of HIV-1 protease (PR) *in vitro* and in bacteria, a function assigned to its N-terminal domain (Baraz *et al.*, 1998; Friedler *et al.*, 1999; Kotler *et al.*, 1997; Potash *et al.*, 1998).

Vif has been found to co-localize with Gag protein in human T cells (Simon *et al.*, 1997, 1999) and to be associated with HIV-1 particles (Borman *et al.*, 1995; Goncalves *et al.*, 1995) as a genuine constitutive element of the virus core (Liu *et al.*, 1995). In good agreement with these data, co-precipitation experiments performed with HIV-infected H9 cell lysates suggested that a specific Gag–Vif interaction occurred in human cells *in vivo* and that the NC domain was an essential determinant for the binding of Gag to Vif (Bouyac *et al.*, 1997*b*). Likewise, in insect cells that expressed both HIV-1 Gag precursor (Pr55Gag) and Vif protein, significant amounts of Vif were found to be co-encapsidated with Pr55Gag into membrane-enveloped Gag particles budding at the plasma membrane (Huvent *et al.*, 1998). Major Vif-interacting sites in Gag were mapped to two regions: (i) the C-terminal domain of Pr55Gag, spanning the second zinc finger of the NC domain, the spacer peptide (sp) 1 and the sp1–p6 junction as far as the N-terminal proline-rich motif of the p6 domain (Huvent *et al.*, 1998); and (ii) the MA–CA junction (Bouyac *et al.*, 1997*b*). In Vif, the basic C-terminal domain and a discrete central region within residues 68–100 were identified as major Gag-binding sites (Huvent *et al.*, 1998). Interestingly, mutations affecting the C-terminal domain of Vif and the central Gag-binding sites resulted in a *vif*[−] phenotype of HIV-1 virions produced by human cells (Bouyac *et al.*, 1997*b*).

Conflicting reports have concluded that Vif is associated with (Liu *et al.*, 1995; Y. Sun, J. Van Velkinburg and C. Aiken, personal communication) or is absent from (Dettenhofer & Yu, 1999) highly purified HIV-1 virions and that the presence of Vif within the virion correlates with the level of the protein within the infected cells, rather than relating to specific viral incorporation of Vif (Simon *et al.*, 1998*c*). However, Vif has been shown to increase the stability of the virus core (Öhagen & Gabuzda, 2000), suggesting that some core component could be a target for Vif function. Consistent with this, Vif has been characterized as an RNA-binding protein (Dettenhofer *et al.*, 2000; Zhang *et al.*, 2000) that might be involved in intracellular trafficking and packaging of HIV-1 genomic RNA (Zhang *et al.*, 2000).

The aim of the present study was to investigate further the

mechanism of interaction of Vif with Gag and Gag–Pol precursors and their co-encapsidation into retrovirus-like particles (VLP). We found that Vif, expressed as a recombinant protein in insect cells, was efficiently co-encapsidated with non-*N*-myristoylated, budding-defective, p6-deleted Gag precursor into intracytoplasmic VLP, suggesting that the presence of the p6 domain, the addressing of Gag to the plasma membrane and VLP budding were not required for Vif encapsidation. We also found that Vif was encapsidated with significantly higher efficiency into extracellular VLP formed from *N*-myristoylated Gag–Pol precursor harbouring an inactive PR domain compared with Pr55Gag or into chimaeric VLP composed of two different precursor species, Gag and Gag–Pol. Vif exerted an inhibitory effect on Gag proteolytic processing, mainly shown by protection of the cleavage sites at the MA–CA and CA–NC junctions. However, no direct interaction between Vif and PR could be detected *in vivo* by electron microscopy or *in vitro* in co-precipitation assays, and Vif showed complete resistance to PR action *in vitro*. Although a transient interaction of Vif with PR could not be excluded, this suggests that the Vif-mediated inhibition of Gag processing resulted from direct binding of Vif to the Gag substrate. Moreover, our data suggest that the enhancement of Vif encapsidation efficiency by Gag–Pol precursor was not mediated by p6* or the Gag–Pol-embedded PR domain, but resulted from a more favourable conformation of the Gag domain when expressed as a Gag–Pol precursor.

Methods

■ Insect cells, recombinant baculoviruses and virus infections. *Spodoptera frugiperda* cells (Sf9 subclone) were maintained as monolayers in Grace's insect medium supplemented with L-amino acids (Gibco BRL) and 10% foetal calf serum. The construction and phenotypes of recombinant baculoviruses used in the present study have been described in detail elsewhere (Carrière *et al.*, 1995; Chazal *et al.*, 1994, 1995; Gay *et al.*, 1998; Hong & Boulanger, 1993; Huvent *et al.*, 1998; Royer *et al.*, 1991, 1992, 1997). Sf9 cells were infected at an m.o.i. ranging from 2 to 10 p.f.u. per cell for single infections. In double- or triple-infection experiments, cells were infected simultaneously with 10 p.f.u. per cell of each recombinant baculovirus. Expression of recombinant proteins was verified by SDS–PAGE and immunoblotting analysis of cell lysates and immunofluorescence (IF) analysis of fixed cells, performed on cultures infected in parallel, using the appropriate antibodies.

Gag and Gag–Pol clones. The Gag precursors used consisted of full-length Pr55Gag (WT precursor of 55 kDa), C-terminal deletion mutants (amber; amb) Gag-amb462 (p6 C-terminal moiety deleted), Gag-amb438 (p6 deleted), Gag-amb426 (p6 + sp1 deleted) and the L-to-P substitution mutant GagL268P (Fig. 1). They were expressed as *N*-myristoylated (myr⁺), budding-competent, or non-*N*-myristoylated (myr[−]), budding-defective recombinant proteins. *N*-myristoylated Gag–Pol precursors consisted of Gag(FS)p6*Pol and Gagp6–Pol. Gag(FS)p6*Pol (originally named Ach7fs; Hughes *et al.*, 1993) is a frameshift mutant with constitutively frameshifted translation of the *gag-pol* mRNA. In Gagp6–Pol, the PR domain was fused to the C-terminal p6 domain (Royer *et al.*, 1997). Both Gag–Pol precursors were PR-defec-

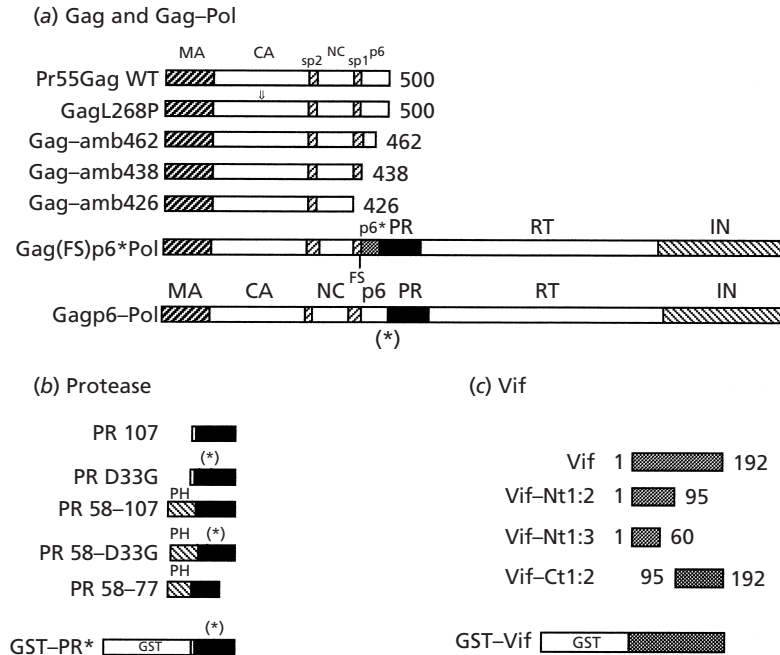


Fig. 1. Schematic drawing of the different recombinant proteins used in this study, with their structural domains. (a) Gag and Gag–Pol precursors; (b) viral proteases (PR); (c) Vif and Vif-derived proteins. The structural domains in proteins are indicated according to the accepted nomenclature. Numerals correspond to amino acid residues that initiate and terminate the protein sequences. PH, Viral polyhedrin; (*), active site mutation (D → G substitution) in PR.

tive, by the double substitution GR⁸⁶ → EF in the PR domain of Gag(FS)p6*Pol (Göttlinger *et al.*, 1989; Hughes *et al.*, 1993), or by the PR active-site mutation D²⁵ → G in Gagp6–Pol (Royer *et al.*, 1997).

Vif. The recombinant HIV-1 Vif-expressing baculovirus, derived from the pNDK molecular clone (Spire *et al.*, 1989), has been described previously (Huvent *et al.*, 1998).

Protease (PR). PR was expressed in baculovirus-infected cells in three polyhedrin-tagged versions, active PR58-107 (full-length), inactive PR58-D33G (full-length, catalytic-site mutant) and inactive PR58-77 (C-terminally truncated mutant) (Fig. 1). They all carried the N-terminal 58 amino acid residues from the baculovirus polyhedrin sequence at their N termini (Royer *et al.*, 1997).

Control vector. MR15 was an empty baculovirus vector used as a negative control in single or double infections. MR15 harboured an out-of-phase PR sequence in the locus of the deleted polyhedrin gene.

Isotopic labelling and immunoprecipitation. Sf9 cells were infected simultaneously with equal m.o.i. of two recombinant baculoviruses (10 p.f.u. per cell), one expressing Vif, the other expressing the PR58-D33G or PR58-77 mutant protease. Cells were labelled (Redivue PRO-MIX, Amersham Pharmacia Biotech; 37 TBq/mmol; 5.3 MBq/ml of methionine-free medium) at 16 h after infection (p.i.). At 48 h p.i., cells were harvested and lysed and cell lysates were clarified and used for co-immunoprecipitation using anti-polyhedrin, anti-PR or anti-Vif rabbit antibodies. Immune precipitates were collected by selection on Protein A–Sepharose CL-4B affinity gel (Sigma) as described previously (Karayan *et al.*, 1994). Proteins retained on the affinity gel were analysed by SDS–PAGE and autoradiography, or blotted and analysed with anti-Vif (diluted 1:3000) and anti-PR (1:2000) antibodies. To avoid the masking of the Vif band (23 kDa) on blots by the undesirable reaction of the secondary anti-rabbit IgG with the IgG light chain (22 kDa) present in the immunoprecipitate, the alkaline phosphatase (or peroxidase) conjugate used was a monoclonal anti-rabbit IgG (γ-chain specific, clone RG-96, Sigma; diluted 1:5000) and the portion of the

membranes where the IgG heavy chains had blotted was excised prior to antibody reaction.

■ Bacterial clones and bacterially expressed recombinant proteins

Recombinant protease. Recombinant HIV-1 PR107 was an active protease form of 107 residues containing 7 residues from the p6* domain at its N terminus plus the methionine initiator (Valverde *et al.*, 1992). Both PR107 and its inactive version PRD33G (mutated at the catalytic site) were expressed in bacterial cells (*E. coli* strain MC1061) using the inducible *araB* promoter (Valverde *et al.*, 1992).

Nucleocapsid (NC) protein. The cDNA of NCp15 (NCp7 + p6 domain) was obtained by PCR amplification of the corresponding HIV-1_{LAI} gag sequence, and cloned into the pKK233-3 vector (Amersham Pharmacia Biotech) according to conventional methods. Recombinant NCp15, starting at Met-378, was overproduced in *E. coli* strain JM105 by induction with IPTG, as described below.

Recombinant Vif. The pD10Vif bacterial expression plasmid, carrying the *vif* gene from the HIV-1 proviral clone pHXB2, was obtained from D. Gabuzda (Yang *et al.*, 1996). In plasmid pD10Vif, a 6 × His tag was fused to the Vif N terminus lacking the initiation methionine codon (MRGSHHHHHHGS–Vif). Vif protein was purified essentially according to the method of Yang *et al.* (1996). The plasmid was transformed into *E. coli* MC1061 and expression of Vif was induced by addition of 0.5 mM IPTG to exponential-phase bacterial cultures (OD₆₀₀ = 0.6–0.8). After induction for 4 h at 37 °C, the bacterial cells were lysed in 6 M guanidine–HCl, 0.1 M sodium phosphate, pH 8.0, at room temperature with overnight stirring. Insoluble cell debris was removed by centrifugation at 15 000 r.p.m. in an SS-34 rotor for 30 min and the supernatant was loaded onto a Ni–NTA–agarose column (Qiagen). The column was washed extensively with lysis buffer and eluted sequentially with the same solution at decreasing pH (pH 6.5, 6.0, 5.8, 5.5 and 5.0). The Vif-containing fractions that eluted at pH 5.5 were pooled, diluted to 200 μg/ml and dialysed successively

against 50 mM MOPS–NaOH, pH 6.5, containing 150 mM NaCl and 3.0, 1.5, 0.75, 0.42, 0.21 and 0 M guanidine–HCl. The protein was then concentrated with a Centriprep-10 concentrator (Amicon) and insoluble aggregates were removed by centrifugation at 100 000 *g* for 30 min at 4 °C. The soluble fraction was adjusted to 10% glycerol and stored in aliquots at –70 °C.

Glutathione-S-transferase (GST)-fused Vif. The construction of GST–Vif from the NL4.3 strain of HIV-1 has been described elsewhere (Bouyac *et al.*, 1997b).

GST-fused protease. The protease was inactivated (PR*) by mutagenesis (D25G substitution) by a two-step recombinant PCR method with the following primers: PR-7-GAV-*EcoRI*-(s), PR-PQI-*EcoRI*-(s), PR-D25G-(s/as), PR-Stop-LNF-*NotI*-(as) (see below). GST–PR*–7, containing 7 residues from p6* at its N terminus, as in PR107, and GST–PR* (99 residues) were obtained by digestion of the PCR products with *EcoRI/NotI* and cloning into the pGEX-5X2 expression vector. The cloning oligonucleotides used were: PR-7-GAV-*EcoRI*-(s), 5' AAAG-GAATTCCTTGGAACTGTATCCTTTAACTTC 3'; PR-PQI-*EcoRI*-(s), 5' AAAGGAATTCCTCAGATCACTCTTTGGCAACG 3'; PR-D25G-(s/as), 5' GCTCTATTAGGTACAGGAGCAG 3' and 5' CTG-CTCCTGTACCTAATAGAGC 3'; and PR-Stop-LNF-*NotI*-(as), 5' TC-ACGATGCGGCCGCTAAAAATTTAAAGTGCAACCAATC 3'.

Expression and purification of GST-fusion proteins. *E. coli* Top10 cells (Invitrogen), transformed with fusion protein-expression plasmids, were grown at 30 °C and protein expression was induced by IPTG, as described above. The bacteria were lysed by sonication (3 × 30 s) on ice and the lysate was incubated for 30 min at 4 °C in the presence of 1% Triton X-100 with shaking. Insoluble material was pelleted for 30 min at 14 000 *g* and the supernatant was incubated overnight at 4 °C with 20 µl 50% (v/v) glutathione (GSH)–agarose beads (Sigma) per ml of lysate. After three successive washes with 1 M NaCl and PBS, the GST-fusion proteins immobilized on GSH–agarose beads were quantified by SDS–PAGE. The beads were stored at 4 °C in the presence of a protease inhibitor cocktail (1 mg/ml aprotinin, 1 mg/ml leupeptin, 2 mg/ml pepstatin and 1 mg/ml antipain) for further analysis.

■ **In vitro transcription–translation.** For *in vitro* protein synthesis, appropriate genes were amplified by PCR with 5'-oligonucleotides that contained the T3 RNA polymerase promoter upstream of the initiation position and 3'-oligonucleotides that contained a stop codon. Amplified DNAs were subjected to *in vitro* transcription–translation using the TNT coupled wheat germ extract system (Promega) as recommended by the manufacturer. Proteins were translated in the presence of [³⁵S]methionine (37 TBq/mmol; Amersham Pharmacia Biotech), analysed by SDS–PAGE and quantified by autoradiography and phosphorimager analysis. The oligonucleotides used for protein *in vitro* translation were 5' GTTAT-TAACCTCACTAAAGGGAAGATTATGGAAAACAGATGGCA-GGTGATG 3', referred to as T3 Vif-1-(s), and 5' ATTCTGCTAT-GTTGACAC 3', referred to as Vif-stop-192-(as).

■ **GST-pull down assays.** Binding reactions were performed overnight at 4 °C in TBST binding buffer containing 50 mM Tris–HCl, pH 7.0, 0.2% Tween 20 and appropriate concentrations of NaCl (150–350 mM) in the presence of BSA (200 µg/ml) in a total volume of 300 µl. GSH–agarose beads bound to GST-fusion protein were incubated overnight with either 8 µl *in vitro*-translated ³⁵S-labelled proteins or 200 µl cytoplasmic extract and then washed extensively in TBST buffer. Samples were resuspended in 25 µl SDS sample buffer and bound proteins were analysed by SDS–PAGE and autoradiography or Western blotting (Huvent *et al.*, 1998).

■ **VLP analysis.** Membrane-enveloped VLP formed from *N*-myristoylated Gag or Gag–Pol polyproteins and released by extracellular budding were analysed by ultracentrifugation in sucrose–D₂O gradients (Liu *et al.*, 1995) after proteolytic digestion with subtilisin, aimed at hydrolysing soluble or membrane-bound Vif protein, which would not be truly Gag-encapsidated and could contaminate the VLP fractions (Goncalves *et al.*, 1995; Huvent *et al.*, 1998; Simon *et al.*, 1997). Linear gradients (10 ml total volume, 30–50% w/v) were centrifuged for 18 h at 28 000 r.p.m. in a Beckman SW41 rotor. The 50% sucrose solution was made in D₂O buffered to pH 7.2 with NaOH and the 30% sucrose solution was made in 10 mM Tris–HCl, pH 7.2, 150 mM NaCl, 5.7 mM disodium EDTA. Aliquots of 0.4 ml were collected from the bottom and proteins were analysed by SDS–PAGE and immunoblotting.

■ **EM and immunoelectron microscopy (IEM).** For conventional EM, baculovirus-infected Sf9 cells were harvested at 40 h p.i., pelleted, fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.5, post-fixed with osmium tetroxide (2% in H₂O) and treated with 0.5% tannic acid solution in H₂O. The specimens were dehydrated and embedded in Epon (Epon-812). Sections were stained with 2.6% alkaline lead citrate and 0.5% uranyl acetate in 50% ethanol and post-stained with 0.5% uranyl acetate solution in H₂O. For IEM and antibody–immunogold labelling (Gay *et al.*, 1998; Huvent *et al.*, 1998), cell specimens were embedded in hydrophilic metacrylic resin (Lowicryl K4M; Chemische Werke Lowi) and sections were reacted with anti-Cap24 and anti-MAP17 mouse MABs and anti-Vif rabbit antibody, both diluted 1:100 in Tris-buffered saline (TBS) overnight at 4 °C. For double labelling, the reaction with gold-labelled secondary antibody was carried out at room temperature for 1 h, using anti-mouse IgG labelled with 5 nm colloidal gold particles and 10 nm gold-tagged anti-rabbit IgG (EM-GAM5 and EM-GAM10, respectively; BioCell Research Lab) diluted 1:100 in TBS. For single labelling, 5 nm gold-labelled secondary antibody was used. Sections were post-stained with 0.5% uranyl acetate. Specimens were examined under a Hitachi-H7100 electron microscope.

■ PR activity *in vitro* on Pr55Gag and Vif substrates

Recombinant Gag substrate. Proteolytic processing of Gag was assayed using the soluble, assembly-defective, non-*N*-myristoylated full-length polyprotein mutant of 55 kDa, Gag-G2A (Chazal *et al.*, 1995), as the protein substrate of viral PR. Sf9 cells were infected with Gag-G2A-expressing baculovirus and infection was allowed to proceed for 48 h. Cells were pelleted, resuspended in hypotonic buffer (10 mM Tris–HCl, pH 7.0, 1 mM disodium EDTA) containing a cocktail of protease inhibitors (Complete protease inhibitor cocktail tablets; Boehringer) at 1 tablet for 50 ml and lysed in a tight-pestle Dounce homogenizer. The cell lysate was clarified by centrifugation at 12 000 *g* for 20 min. Soluble Gag-G2A polyprotein was recovered in the cytosolic fraction and stored frozen at a total protein concentration of 0.4–0.5 mg/ml. As Gag-G2A represented 5–10% of the cytosolic protein (Royer *et al.*, 1992), the cytosolic fraction was used as protease substrate without further purification (Valverde *et al.*, 1992).

Recombinant PR. Bacterially expressed protease PR107 and its mutant PRD33G were isolated as described previously (Valverde *et al.*, 1992) with minor modifications. Briefly, bacterial cells (*E. coli* MC1061, 5 ml aliquots) taken at an OD₆₀₀ of 0.5 were treated with L-arabinose at 20% for 4 h to induce PR expression and lysed in 0.1 ml 50 mM Tris–HCl, pH 7.0, containing 1 mM DTT, 0.01% lysozyme and the complete protease inhibitor cocktail described above. After standing on ice for 10 min, the samples were treated with 0.1% NP40 and DNase I at a final concentration of 1 mg/ml in the presence of 10 mM MgCl₂. The lysates were clarified by centrifugation at 12 000 *g* for 20 min and

the supernatant was used as the source of PR. The amount of PR was estimated by Coomassie blue staining of SDS–polyacrylamide gels by comparison with a range of titrated lysozyme samples. In some experiments, recombinant, affinity-purified PR (expressed in *E. coli*; Bachem AG) was also used.

Standard PR assays. In standard reactions, 5 μ l aliquots of Sf9 cytosolic fraction containing Gag-G2A substrate (0.5–1.0 pmol) were mixed with 10 μ l aliquots of PR sample (0.05–0.01 pmol PR107) and 5 μ l buffer containing 50 mM MOPS–NaOH, pH 6.5, 150 mM NaCl and increasing amounts of recombinant Vif protein, ranging from 4 to 40 pmol (0.1–1.0 μ g) per sample. Digestion was conducted for 1 h at 37 °C and the enzymatic reaction was stopped by addition of 20 μ l SDS–PAGE sample buffer. In negative-control samples, the inactive PR mutant PRD33G was used in place of PR107 or PR107 was used in the presence of the inhibitor Saquinavir (Bragman, 1996). Saquinavir was kindly supplied by F. Mammano (INSERM–Hôpital Bichat, Paris) and used at concentrations ranging from 0 to 2.0 mM.

PR assays using nascent Gag and Vif substrates. In typical reactions, 2.5 μ l [³⁵S]methionine-labelled Pr55Gag or Vif protein, obtained by *in vitro* translation as described above, was brought to a final volume of 35 μ l with 50 mM Tris–HCl, pH 6.8, 150 mM NaCl, 1 mM disodium EDTA, 10% glycerol. An aliquot of 1 μ l of bacterially expressed, purified HIV-1 PR (H 1256; Bachem) at a concentration of 540 ng/ μ l was added to the mixture and digestion was allowed to proceed at 37 °C for 4 h. Digestion was stopped by heating in SDS–PAGE loading buffer and samples were analysed by SDS–PAGE and autoradiography.

■ **Immunoblot analysis and antibodies.** Proteins were analysed by SDS–PAGE and immunoblotted, as described previously (Huvent *et al.*, 1998). Anti-HIV-1 MA protein rabbit polyclonal antibody (laboratory-made; Huvent *et al.*, 1998) was raised in a rabbit by injection of GST-fused, bacterially expressed and affinity chromatography-purified MAb17. Mouse MAbs against MAb17 (Epiclone 5003) and against CAp24 (Epiclone 5001) were obtained from Cylex Inc. and a MAb against HIV-1 reverse transcriptase was obtained from IntraCell. Mouse MAb HH3 directed against NCp7 was obtained from R. Benarous (Tanchou *et al.*, 1994). Anti-p6 rat MAb M35/2F8 was kindly provided by M. G. Sarngadharan (Veronese *et al.*, 1987; Carrière *et al.*, 1995) and the rabbit anti-Vif serum was provided by D. Gabuzda. Anti-HIV-1 PR and anti-polyhedrin rabbit sera were both laboratory-made (Royer *et al.*, 1997). Phosphatase-labelled anti-rabbit, -mouse and -rat IgG conjugates were purchased from Sigma and horseradish peroxidase-labelled conjugates were purchased from Boehringer. For luminograms, chemiluminescent peroxidase substrate Supersignal (Pierce) was used. For immunological quantification of Gag and Vif proteins, blots were reacted with ¹²⁵I-labelled anti-rabbit or anti-mouse whole IgG antibody (Amersham Pharmacia Biotech; 6.9 mCi/mg; 3 μ Ci per blot) and exposed to radiographic film (Hyperfilm- β max, Amersham Pharmacia Biotech). Autoradiographs were scanned at 610 nm using an automatic densitometer (REP-EDC, Helena Laboratories) or protein bands were excised from blots and radioactivity was measured in a scintillation counter (Beckman LS-6500) as described previously (Huvent *et al.*, 1998).

Results

Co-encapsidation of Vif and Gag proteins in intracellular VLP in Sf9 cells

We have shown previously that Gag particles formed from Gag-amb438myr⁺, a Gag precursor lacking the p6 domain,

were more homogeneous in size and shape than VLP composed of full-length Pr55Gag (Gay *et al.*, 1998; Wilk *et al.*, 2001). Its non-N-myristoylated counterpart, Gag-amb438myr⁻, assembled intracytoplasmic VLP efficiently in insect cells (Royer *et al.*, 1991, 1992; Carrière *et al.*, 1995). Gag-amb438myr⁻ was therefore co-expressed with Vif in Sf9 cells and cell sections were examined under the EM after single immunogold labelling of Vif protein or differential immunogold labelling of both Vif and Gag proteins (Huvent *et al.*, 1998). Rabbit anti-Vif antibody was detected with 10 nm gold-tagged anti-rabbit IgG antibody and mouse anti-CAp24 and anti-MAb17 MAbs were detected with 5 nm gold-tagged anti-mouse IgG antibody (Fig. 2).

In single Vif-labelling experiments, gold grains were mostly found to be associated with intracytoplasmic VLP and frequently delineated the VLP contours (Fig. 2*a*). In double immunogold labelling, gold grains of 5 nm (Gag) and 10 nm (Vif) diameter accumulated mostly in section areas that showed VLP, whereas areas devoid of VLP were poorly labelled (Fig. 2*b*). Gold grains of both diameters were observed in close association with intracytoplasmic VLP and the EM pattern suggested a co-assembly of Gag and Vif proteins into VLP (Fig. 2*b–d*). However, it is noteworthy that patches with double Vif and Gag labelling showed more intense Vif labelling at the periphery than in the centre, where VLP with clear contours were most abundant (Fig. 2*b*). In the peripheral zones, Vif labelling was associated with electron-dense, amorphous material with less Gag labelling. Although some degree of mutual exclusion between Vif and Gag antibodies could not be ruled out, the EM pattern would be compatible with Gag particles seen at different stages of assembly, and would suggest that the affinity of Vif for the Gag molecules varied during the course of the Gag assembly process and decreased in properly assembled particles, as suggested recently (Sova *et al.*, 2001).

These EM data thus confirmed that the Vif–Gag interaction could occur intracellularly, as suggested previously (Bouyac *et al.*, 1997*a*; Huvent *et al.*, 1998). The observation that co-encapsidation of Vif and the myristoylation-defective, C-terminal deletion mutant Gag-amb438myr⁻ could take place within the cytoplasm implied that the interaction between Gag and Vif did not depend upon the presence of the p6 domain in the Gag precursor. It also implied that Vif and Gag co-packaging did not result simply from the addressing of Gag and Vif to the cell surface, their co-localization at the plasma membrane and the extracellular budding of Gag particles.

Co-encapsidation of Vif with Gag or Gag–Pol precursors into extracellular VLP

Before maturation, HIV-1 particles are composed transiently of two Gag precursor species, the Gag polyprotein of 55 kDa (Pr55Gag) and the Gag–Pol polyprotein of 160 kDa (Pr160Gag–Pol), the latter precursor containing the p6* and

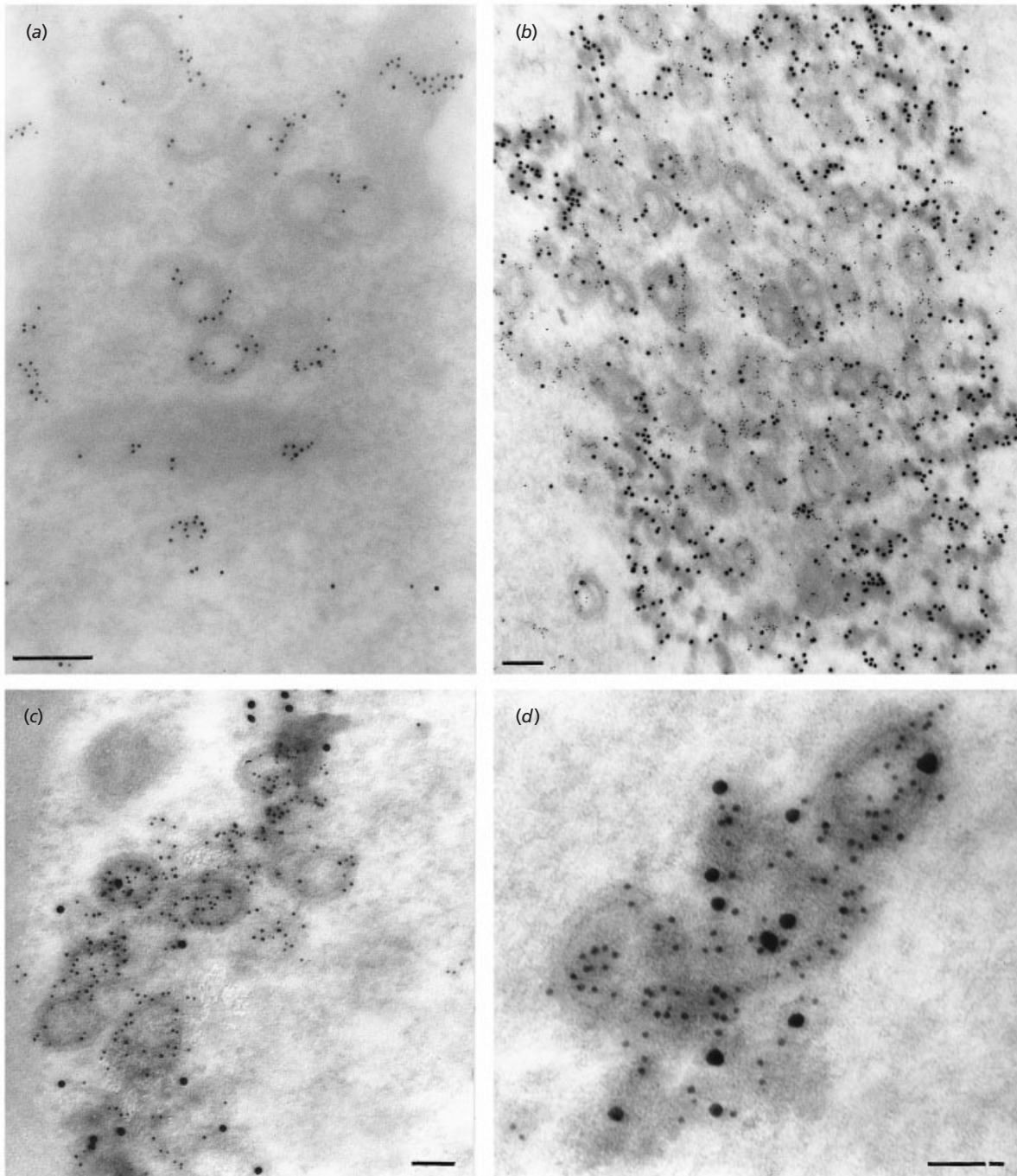


Fig. 2. IEM analysis of Sf9 cells co-expressing Vif and non-*N*-myristoylated, $\Delta p6$ -mutant Gag precursor Gag-amb438myr⁻. (a) Single immunogold labelling of cell sections with anti-Vif rabbit polyclonal antibody and 5 nm colloidal gold-conjugated anti-rabbit IgG antibody. Note that gold grains often delineate the contours of intracytoplasmic Gag particles (VLP). (b)–(d) Double immunogold labelling of sections examined at low (b) and high (c, d) magnification. Sections were reacted simultaneously with anti-Vif rabbit antibody and anti-MA and anti-CA mouse MAbs followed by 10 nm gold-conjugated anti-rabbit IgG antibody and 5 nm gold-conjugated anti-mouse IgG antibody. Bars, 125 (a, b) and 66 (c, d) nm.

PR domains upstream of the RT-IN domain (Fig. 1). Since direct interactions between Vif-derived peptides and viral PR (Friedler *et al.*, 1999) have been reported, we investigated the possible influence of PR on Vif encapsidation efficiency in an

assay in which PR was provided as an inactivated, constitutive domain of the Gag-Pol precursor. Vif was co-expressed with a single Gag (WT Pr55Gag) or Gag-Pol precursor species and their VLP Vif contents were compared by quantitative

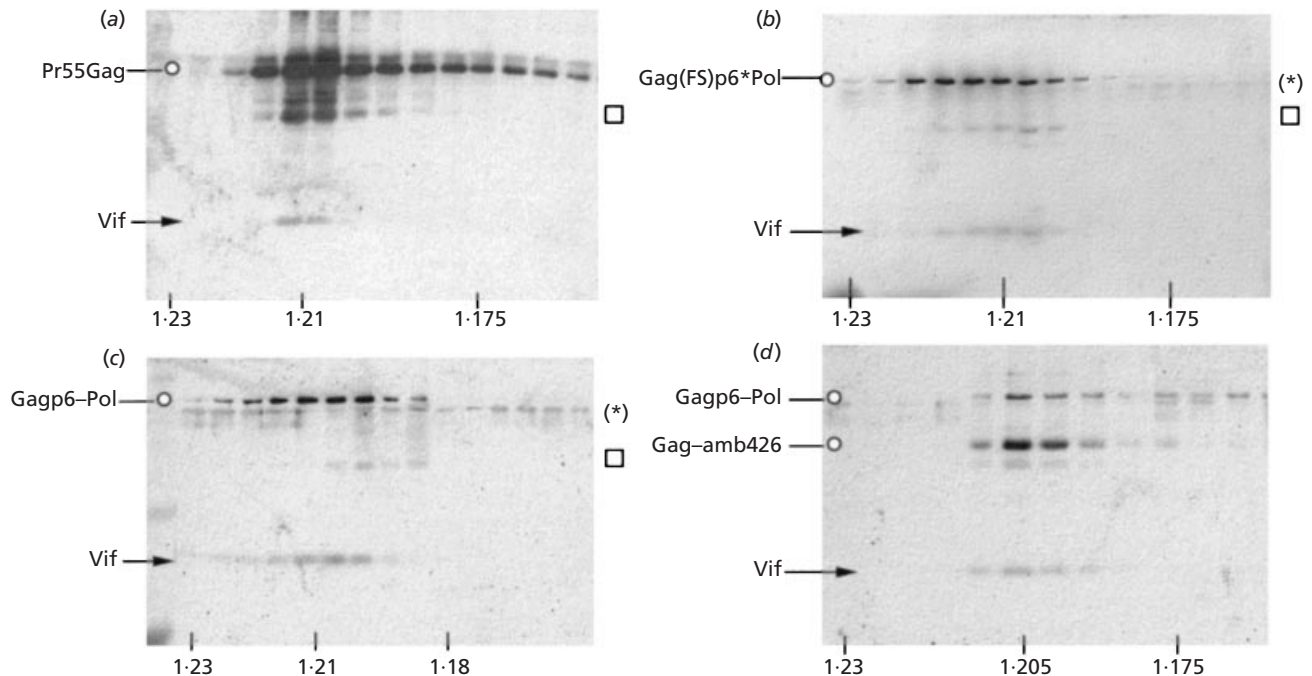


Fig. 3. Sedimentation analysis of Vif encapsidation into various extracellular VLP budding from Sf9 cells. Homogeneous VLP were formed from Pr55Gag (a), Gag(FS)p6*Pol (b) or Gagp6–Pol (c) precursors and chimaeric VLP were constituted from two Gag precursors, Gagp6–Pol and Gag-amb426myr⁺ (d). VLP were isolated in a sucrose–D₂O gradient as described in Methods. Gradient fractions were analysed by SDS–PAGE and immunoblotting using anti-MA and anti-Vif rabbit antibodies and ¹²⁵I-labelled anti-rabbit IgG secondary antibody. Autoradiographs of the gradient fractions spanning density 1.228 (bottom) to 1.119 (top) are shown and the densities of significant fractions are indicated below each panel. The apparent densities found for Gag and Gag–Pol VLP ranged from 1.22 to 1.18. Positions of Gag and Gag–Pol precursors (○), Vif protein (→) and Gag major cleavage products (□) are indicated. (*), Background band seen occasionally on blots. Note that chimaeric VLP formed from Gag–Pol and Gag-amb426myr⁺ precursors (d) sedimented as a sharper peak than Gag–Pol VLP (b, c), confirming EM observations (Fig. 4; refer to Hughes *et al.*, 1993; Royer *et al.*, 1997).

immunoblot analysis of VLP isolated by velocity gradient ultracentrifugation (Huvent *et al.*, 1998) and estimated semi-quantitatively by IEM analysis of budding VLP in EM sections (Carrière *et al.*, 1995). As shown in Fig. 3(a, b) and Table 1, Gag(FS)p6*Pol, a budding-competent, *N*-myristoylated Gag–Pol precursor containing a mutant PR, encapsidated 6-fold more copies of Vif per Gag molecule than did Pr55Gag. This was confirmed by IEM analysis of VLP (Fig. 4). Sections of VLP composed of Gag(FS)p6*Pol were labelled with Vif antibody at significantly higher levels than VLP formed from Pr55Gag: the mean number of Vif-associated gold grains per VLP was found to be 17.52 (SEM = 2.69; SD = 13.26; *n* = 93) for Gag(FS)p6*Pol (Fig. 4b) versus 2.62 (SEM = 0.60; SD = 2.60; *n* = 72) for Pr55Gag (Fig. 4a). The background anti-Vif labelling of Pr55Gag VLP assembled in the absence of Vif expression was 0.13 grains per VLP (SEM = 0.08; SD = 0.56; *n* = 176). The difference was therefore significant at the *P* = 0.05 confidence level.

In order to evaluate the possible role of the p6* domain in the enhancement of Vif packaging efficiency, encapsidation was next studied by using another Gag–Pol precursor, Gagp6–Pol. In the Gagp6–Pol fusion construct, the mutant PR domain has been artificially fused to the C terminus of the p6

domain (Royer *et al.*, 1997) whereas, in the permanently frameshifted Gag(FS)p6*Pol, the PR domain was located at the C terminus of the natural p6* domain (Hughes *et al.*, 1993). Thus, the major difference between Gagp6–Pol and Gag(FS)p6*Pol resided in their p6 and p6* domains, respectively (Fig. 1). The molar ratio of Vif to Gag copies found in VLP formed from Gagp6–Pol precursor was 4-fold higher than in Pr55Gag VLP (Fig. 3c; Table 1), a value not significantly different from that obtained with Gag(FS)p6*Pol at the *P* = 0.05 confidence level. This would imply that the occurrence of p6* in place of p6 in the Gag–Pol precursor did not affect the Vif–Gag co-encapsidation process significantly, confirming our EM observation of the dispensability of p6 for Vif packaging into intracellular VLP (Fig. 2). This would also suggest that the sequences responsible for the higher Vif encapsidation level were located downstream of the Gag domains and acted either directly, via extra Vif-binding sites in the Pol domain, or indirectly, via Pol-mediated conformational modifications of the Gag domains.

Morphology of VLP and Vif encapsidation

It has been reported recently that Vif was packaged at

Table 1. Co-encapsidation of Vif protein with various Gag and Gag–Pol precursors

The *N*-myristoylated Gag-amb426myr⁺ is a budding-competent, C-terminal deletion mutant (p6 + sp1-deleted), whereas its non-*N*-myristoylated equivalent Gag-amb426myr⁻ is a budding-defective mutant (Carrière *et al.*, 1995). GagL268P⁺ is a CA substitution mutant with an assembly-defective phenotype, releasing membrane-enveloped Gag particles in small numbers and with aberrant morphology (Hong & Boulanger, 1993). Both *N*-myristoylated frameshift mutant Gag(FS)p6*Pol and the Gagp6–Pol fusion construct harbour an inactivated PR domain (Hughes *et al.*, 1993; Royer *et al.*, 1997).

Baculovirus vectors	VLP Vif content*	No. of copies of Vif (per 2000 copies of Gag)†
Double infections		
Vif + empty vector‡	0§	0§
Vif + Gag-amb426myr ⁻	0§	0§
Vif + WT Pr55Gagmyr ⁺	5.9 ± 1.6	73 ± 10 (1.0)
Vif + Gag-amb426myr ⁺	1.3 ± 0.4	16 ± 3 (0.2)
Vif + GagL268Pmyr ⁺	72.0 ± 2.5	775 ± 28 (10.6)
Vif + Gag(FS)p6*Pol	38.0 ± 15.6	470 ± 64 (6.4)
Vif + Gagp6–Pol	25.3 ± 14.5	312 ± 43 (4.3)
Triple infections		
Vif + Gag(FS)p6*Pol + Gag-amb426myr ⁺	31.5 ± 13.0	389 ± 53 (5.3)
Vif + Gagp6–Pol + Gag-amb426myr ⁺	17.5 ± 8.8	216 ± 30 (2.9)

* The amount of Vif co-encapsidated with Gag or/and Gag–Pol was assayed as described in Methods. Results are expressed as the percentage of Gag and Gag–Pol content (Huvent *et al.*, 1998). Values represent means ± SD of four separate isolations of VLP. In the triple-infection experiments, the total content of Gag–Pol and Gag-amb426 precursor molecules in chimaeric VLP was taken into account for the calculation of the Vif:Gag molar ratio.

† The number of 2000 copies of Pr55Gag precursor per VLP has been estimated by Layne *et al.* (1992).

Values in parentheses are the fold increase over the number of Vif copies per VLP composed of WT Pr55Gag.

‡ Empty vector MR15 has an out-of-phase PR sequence within the deleted polyhedrin gene.

§ No Gag VLP were released by Sf9 cells infected with empty vector or non-*N*-myristoylated Gag-amb426myr⁻. The background levels of radioactivity at the positions of Gag, Gag–Pol and Vif in the immunoblots of the corresponding gradient fractions were subtracted from the values obtained for the other samples.

significantly higher levels in HIV-1 particles composed of Gag CA deletion mutants with aberrant morphology (Sova *et al.*, 2001). This was confirmed by our own observation that GagL268Pmyr⁺ encapsidated 10-fold more Vif per Gag molecule than did Pr55Gag (Table 1). GagL268Pmyr⁺ is a CA substitution mutant that self-assembles with low efficiency and releases large, membrane-enveloped Gag particles (Hong & Boulanger, 1993). We then analysed the influence of VLP morphology on Vif encapsidation using chimaeric VLP composed of two Gag precursor species, as occurs in immature virus particles. Co-expression of *N*-myristoylated Gag(FS)p6*Pol (or Gagp6–Pol) and p6-deleted GagPr47 (or Gag-amb438myr⁺, our Δp6 equivalent of Pr47) in insect cells has been found to result in chimaeric VLP that appear to be more homogeneous in size and shape under the EM than VLP formed from single Gag(FS)p6*Pol molecules (Hughes *et al.*, 1993) or Gagp6–Pol (Royer *et al.*, 1997). The same effect was observed in co-expression of Gag(FS)p6*Pol with Gag-amb426myr⁺ (Fig. 4; Table 2). Gag(FS)p6*Pol molecules assembled into VLP with a diameter 2-fold larger (mean

242 nm) than that of WT Pr55Gag VLP (125 nm). Moreover, their heterogeneity and lack of sphericity, as estimated respectively by the value of the SD of the mean diameter and the ratio of their minimum to maximum diameters (d:D), were significantly greater than those of WT VLP (Table 2). By contrast, the morphology of chimaeric VLP composed of Gag-amb426myr⁺ + Gagp6–Pol or of Gag-amb426myr⁺ + Gag(FS)p6*Pol (with or without Vif) was similar to that of WT Pr55Gag VLP (Fig. 4c, d; Table 2).

Gag-amb426myr⁺, which lacks p6 and the sp1 spacer peptide between the NC and p6 domains, has been shown to be defective in Vif packaging, with a 5- to 10-fold reduction of the Vif content of VLP formed from Gag-amb426myr⁺ compared with WT Pr55Gag VLP (Huvent *et al.*, 1998; Table 1). Thus, when assayed in triple-infection experiments involving Gag–Pol and Gag-amb426myr⁺, packaging of Vif into chimaeric VLP would essentially depend upon the Gag–Pol precursor species. Sf9 cells were thus infected simultaneously with three recombinant baculoviruses expressing Vif, Gag-amb426myr⁺ and Gag(FS)p6*Pol (or Gagp6–Pol) and the

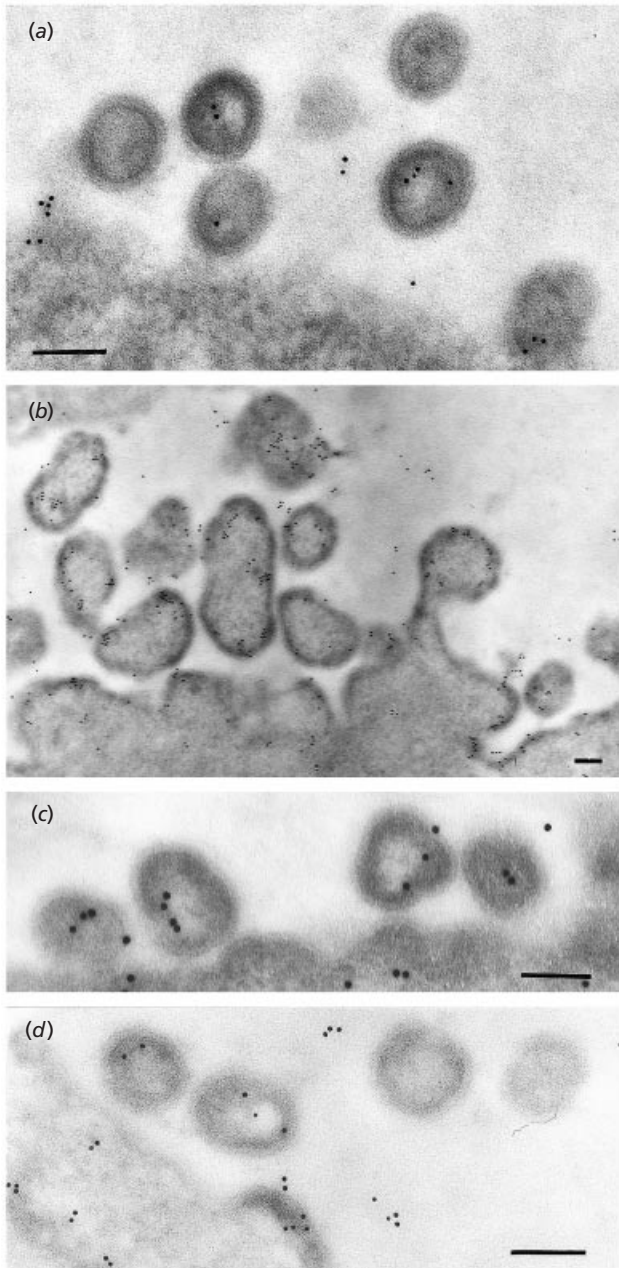


Fig. 4. IEM analysis of plasma membrane of Sf9 cells co-expressing Vif and various Gag or/and Gag–Pol precursors. VLP formed from single Gag species WT Pr55Gag (a) or Gag(FS)p6*Pol (b) and chimaeric VLP formed from Gag-amb426myr⁺ and Gag(FS)p6*Pol (c, d) are shown. Cell sections were immunolabelled with rabbit anti-Vif antibody and primary antibody molecules were detected by 5 nm gold-conjugated anti-rabbit IgG. The parameters of VLP are given in Table 2. Bars, 100 nm.

expression of the three recombinant proteins was verified *in situ* by IF microscopy and by SDS–PAGE analysis and immunoblotting of Sf9 cell lysates (not shown). The VLP released from the co-infected cells were isolated in velocity gradients and assayed for Vif and Gag content (Table 1). The results showed that Vif was encapsidated at significantly

higher levels in chimaeric VLP than in VLP constituted of the single Pr55Gag species (Table 1; Fig. 3, compare a and d), although the VLP showed similar size and morphology under the EM (Table 2; Fig. 4, compare a with c and d). Interestingly, the number of copies of Vif was larger in mixed VLP containing the natural Gag(FS)p6*Pol precursor than in mixed VLP containing the Gagp6–Pol fusion construct, 5- and 3-fold, respectively, over the Pr55Gag VLP content (Table 1). These data suggested that Vif encapsidation into Gag particles was not a simple consequence of an aberrant morphology of these particles, but would favour the hypothesis of a conformational role of the Gag–Pol precursor in Vif–Gag interaction and co-encapsidation, e.g. dimerization induced or/and stabilized by the PR–Pol domains. However, since a direct interaction between Vif and PR could not be excluded at this stage, the next experiments were designed to address this point.

Absence of detectable Vif–PR interaction *in vivo* and *in vitro*

The possible direct interaction between Vif and PR was examined *in vivo* under the EM on sections of Sf9 cells co-infected by a baculovirus expressing Vif and a high-expresser of polyhedrin-tagged, inactive PR* (PR58-D33G or PR58-77; Fig. 1). No pattern of co-localization of the PR* and Vif protein inclusions was visible in the cytoplasm and nucleus of Sf9 cells in EM and IEM (not shown). In co-immunoprecipitation assays of Sf9 cell lysates, neither of the individual proteins, Vif or PR*, was found to co-precipitate with the other with any of the specific antibodies used, anti-PR, anti-polyhedrin or anti-Vif (not shown). Likewise *in vitro*, in GST-pull down assays, no significant amount of Vif protein was found to bind GST–PR* (inactivated by a D → G substitution at its catalytic site) using full-length Vif or its isolated N-terminal or C-terminal moieties. In reverse experiments using GST–Vif, no detectable PR* was pelleted from PR58-D33G- or PR58-77-expressing Sf9 lysates (not shown). Moreover, pull down of active recombinant PR (commercially available from Bachem) by GST–Vif was also unsuccessful (not shown).

Altogether, these data apparently argued against a stable interaction between Vif and the viral PR, although a transient interaction between Vif and PR, which would account for the Vif-mediated inhibition of PR activity (Kotler *et al.*, 1997; Potash *et al.*, 1998), could not be excluded. Similarly, PR-mediated proteolytic degradation of Vif within maturing virus particles could explain its absence or ‘quasi-absence’ from mature virions, as reported previously (Dettenhofer & Yu, 1999). To test this hypothesis, *in vitro*-translated nascent Vif and Gag proteins were incubated with chromatographically purified, recombinant HIV-1 PR (Bachem) and cleavage products were analysed by SDS–PAGE and autoradiography. As shown in Fig. 5(a), PR was capable of processing Pr55Gag precursor, as shown by the occurrence of a major p24 protein species. In contrast, Vif appeared to be totally resistant to viral

Table 2. Morphological parameters of VLP released by Sf9 cells expressing Gag or/and Gag–Pol precursors

Samples of recombinant baculovirus-infected Sf9 cells were harvested 48 h after infection, fixed and processed for EM. Membrane-enveloped VLP released by budding from the plasma membrane were examined under the EM and their maximum (D) and minimum (d) diameters were measured (Gay *et al.*, 1998).

Gag precursor species in VLP	VLP diameter* (nm) (mean \pm SD)	Heterogeneity in shape†	Sphericity index‡ (mean \pm SD)	Sample size (n)
Single-Gag VLP				
WT Pr55Gagmyr ⁺	125 \pm 12	12	0.89 \pm 0.01	51
Gag-amb426myr ⁺	127 \pm 11	11	0.91 \pm 0.08	24
Gag(FS)p6*Pol	242 \pm 62	62	0.76 \pm 0.15	128
Gagp6–Pol	111 \pm 34	34	0.78 \pm 0.09	25
Chimaeric VLP				
Gag(FS)p6*Pol + Gag-amb426myr ⁺	144 \pm 13	13	0.82 \pm 0.13	42
Gagp6–Pol + Gag-amb426myr ⁺	138 \pm 19	19	0.80 \pm 0.11	88
Gag(FS)p6*Pol + Gag-amb426myr ⁺ + Vif§	139 \pm 24	24	0.83 \pm 0.12	77

* The mean diameter of each particle was obtained from the formula (D + d)/2.

† Estimated as the SD of the mean diameter (Campbell & Vogt, 1997; Gay *et al.*, 1998).

‡ Evaluated from the ratio of the minimum to maximum diameter (d:D) (Campbell & Vogt, 1997; Gay *et al.*, 1998).

§ VLP released from triple-infected cells, as shown in Fig. 4(c, d).

PR *in vitro*, at least under conditions that allowed efficient cleavage of Pr55Gag.

Influence of Vif protein on PR-mediated Gag processing *in vitro*

To determine whether Vif could affect the proteolytic activity of PR, we developed an *in vitro*-processing assay using a soluble, non-*N*-myristoylated, budding-defective Gag precursor mutant (Gag-G2A) as the viral substrate and bacterially expressed recombinant PR (PR107; Valverde *et al.*, 1992) as the enzyme under conditions defined previously (Valverde *et al.*, 1992). Negative-control samples contained active-site mutant protease PRD33G (Valverde *et al.*, 1992) or PR107 with the inhibitor Saquinavir. With Gag and PR alone, discrete bands corresponding to the expected cleavage products migrating as MAp17, the doublet band of CAp24–CAp25 (CAp24 \pm sp2) and NCp15 were observed on Western blots (not shown; see Royer *et al.*, 1997). However, special attention was aimed at three major intermediate Gag-cleavage products, migrating with apparent molecular masses of 40–41 (P41), 39 (P39) and 17.5 (P17.5) kDa. These proteolytic products were identified by using three parameters: (i) their molecular mass in SDS–PAGE, (ii) their reactivity to antibodies directed towards specific domains of Gag (Fig. 5*b, d*) and (iii) the proteolytic pattern of Gag amber mutants (Fig. 6). P41, which reacted with both anti-MA and anti-CA antibodies but not with anti-p6 antibody, corresponded to the N-terminal moiety of the Gag precursor, MAp17–CAp24 or MAp17–CAp25 (Mervis *et al.*, 1988) (Fig. 5*b*). As P39 reacted with both anti-CA and anti-p6 antibodies but not with anti-MA antibody, it corresponded to the Gag C-terminal moiety. P39 resulted from the hydrolysis

of the major processing site at the MA–CA junction and was composed of the CAp24, sp2, NCp7, sp1 and p6 domains (Pettit *et al.*, 1994) (Fig. 5*d*). The Gag fragment P17.5, which reacted with both anti-p6 and anti-NC antibodies, spans sp2, NC and p6 (Fig. 5*d*). Both P41 and P17.5 resulted from the cleavage of Gag at the CA–NC junction on either side of the sp2 spacing peptide (Henderson *et al.*, 1992; Pettit *et al.*, 1994) (Fig. 5*b, d*).

A significant change was observed in the proteolytic pattern of Gag precursor in the presence of Vif and more particularly in the occurrence of the intermediate cleavage product P41 detected by anti-MA antibody, which decreased progressively in a Vif-dependent manner (Fig. 5*b*). A similar effect was obtained with the PR-inhibitor Saquinavir, over the range 1.0 to 1.7 mM (Fig. 5*c*). As the decrease in the P41 signal was not accompanied by the occurrence of smaller products, the data implied inhibition of Gag processing, with selective protection of the cleavage site at the CA–sp2 junction. The effect on the Gag proteolytic pattern was only visible over 8–16 pmol Vif for 1 pmol Gag substrate and 0.1 pmol PR, and this apparent threshold suggested that oligomeric forms of Vif might be involved. This is consistent with recent data showing that Vif oligomerization is required for its function (Yang *et al.*, 2001).

Non-*N*-myristoylated Gag precursors of various lengths (Gag-amb462, Gag-amb438 and Gag-amb426), carrying amber stop codons at different positions in the C-terminal p6 or sp1 domain (Fig. 1), were incubated with PR in the presence or absence of Vif. The same anti-MA-reacting P41 band appeared to be attenuated in the presence of Vif in all Gag mutant patterns (Fig. 6), indicating that P41 corresponded to the N-

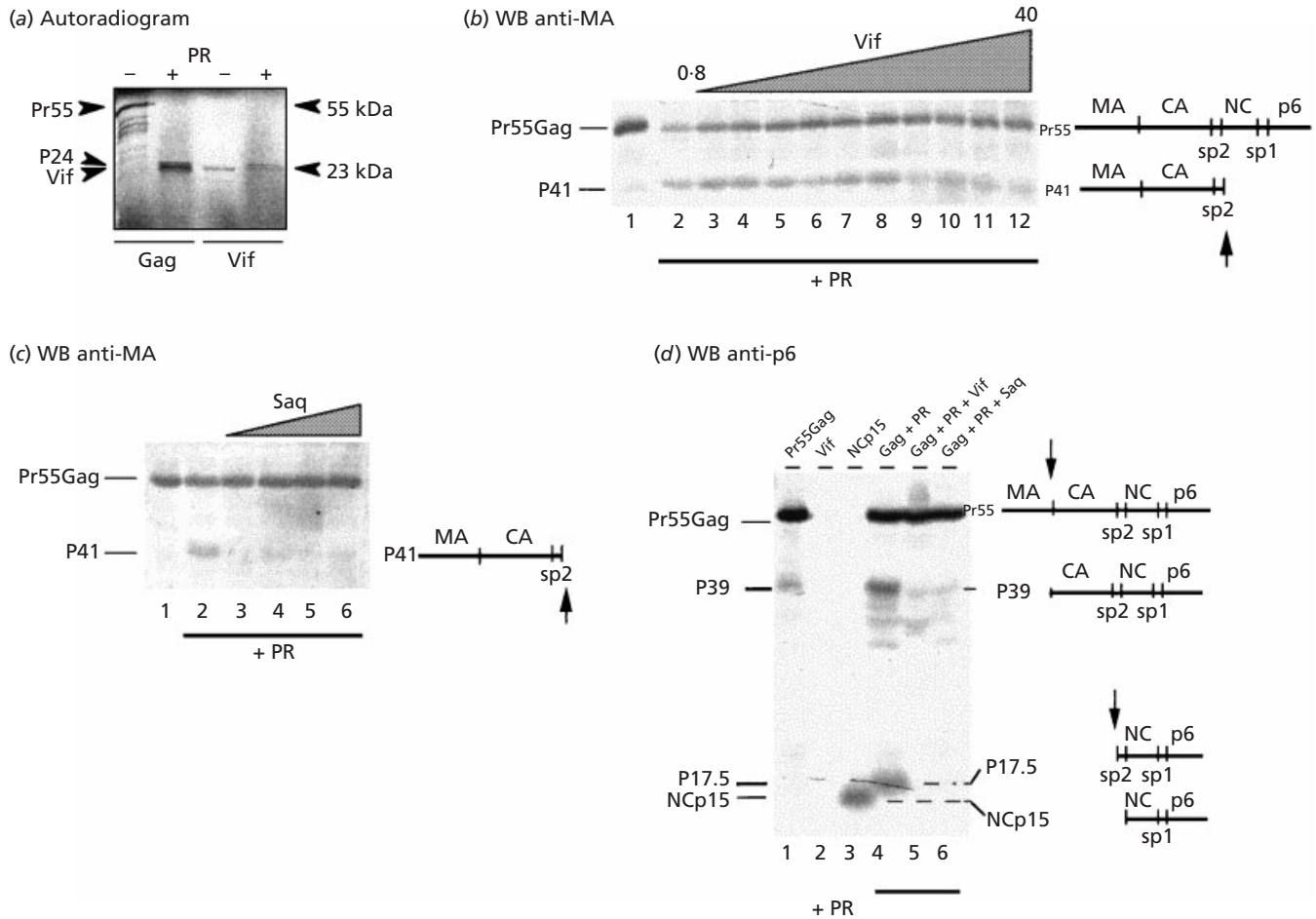


Fig. 5. (a) Comparative sensitivities of Gag and Vif, used as separate substrates, to PR digestion *in vitro*. [³⁵S]Methionine-labelled full-length Gag precursor (Pr55) and Vif protein (23 kDa) were incubated without (–) or with (+) purified, recombinant PR at 37 °C for 4 h and cleavage products were analysed by SDS–PAGE and autoradiography or phosphorimaging. (b) Influence of Vif on PR-mediated Gag processing: analysis of the N-terminal moiety. Full-length, non-*N*-myristoylated precursor of 55 kDa (Gag-G2A substrate; 1 pmol) was incubated with 0.1 pmol aliquots of recombinant viral protease (PR107) and increasing amounts of recombinant Vif protein, ranging from 0.8 to 40 pmol. Control samples were: lane 1, negative control, Gag alone; 2, positive control, Gag incubated with PR107, without Vif. Lanes 3–12: Gag incubated with PR107 in the presence of 0.8, 1.6, 2.4, 3.2, 4, 8, 16, 24, 32 and 40 pmol Vif. (c) Influence of Saquinavir on PR-mediated Gag processing. Gag substrate (1 pmol) was incubated with 0.1 pmol aliquots of recombinant viral protease (PR107) and increasing amounts of inhibitor Saquinavir at 0 (lane 2), 0.6 (3), 1.0 (4), 1.3 (5) and 1.7 (6) mM. Lane 1: negative control, Gag alone. In (b) and (c), samples were analysed by SDS–PAGE and immunoblotting with anti-MA antibody (WB, Western blot). Linear representations of the initial 55 kDa Gag substrate and the cleavage product P41 with their different domains are depicted on the right. Arrows indicate the putative PR cleavage site responsible for the occurrence of the P41 cleavage product. (d) Influence of Vif on PR-mediated Gag processing: analysis of the C-terminal moiety. Gag-G2A substrate (1 pmol) was incubated alone (lane 1), with 0.1 pmol PR (4), with 0.1 pmol PR and 40 pmol Vif (5) or with 0.1 pmol PR and 1.7 mM Saquinavir (6). Lane 2, Vif protein alone; 3, recombinant NCp15 protein used as a marker. Samples were analysed by SDS–PAGE and immunoblotting with anti-p6 antibody. A linear representation of the initial 55 kDa Gag substrate and the two major cleavage products, P39 and P17.5, with their different domains is depicted on the right. The arrows indicate the putative PR cleavage sites that generate the P41 and P17.5 cleavage products visible on the blot.

terminal moiety of the Gag precursor. This also suggested that the absence of p6 and p6 + sp1 domains apparently did not modify the negative influence of Vif on PR-mediated cleavage of the upstream sites bounding the sp2 domain.

The proteolytic pattern of Gag in immunoblots using anti-p6 antibody confirmed the negative effect of Vif on PR-mediated Gag processing: almost complete extinction of the

P17.5 signal was observed in the presence of Vif, suggesting inhibition of cleavage at the CA–sp2 junction (Fig. 5*d*; compare lanes 4 and 5). It also showed a simultaneous decrease in the intensity of the cleavage product P39, which implied protection of the cleavage site at the MA–CA junction. The negative effect on Gag processing of 40 pmol Vif mimicked the anti-PR activity of Saquinavir at 1.7 mM (Fig. 5*d*, lanes 5 and 6).

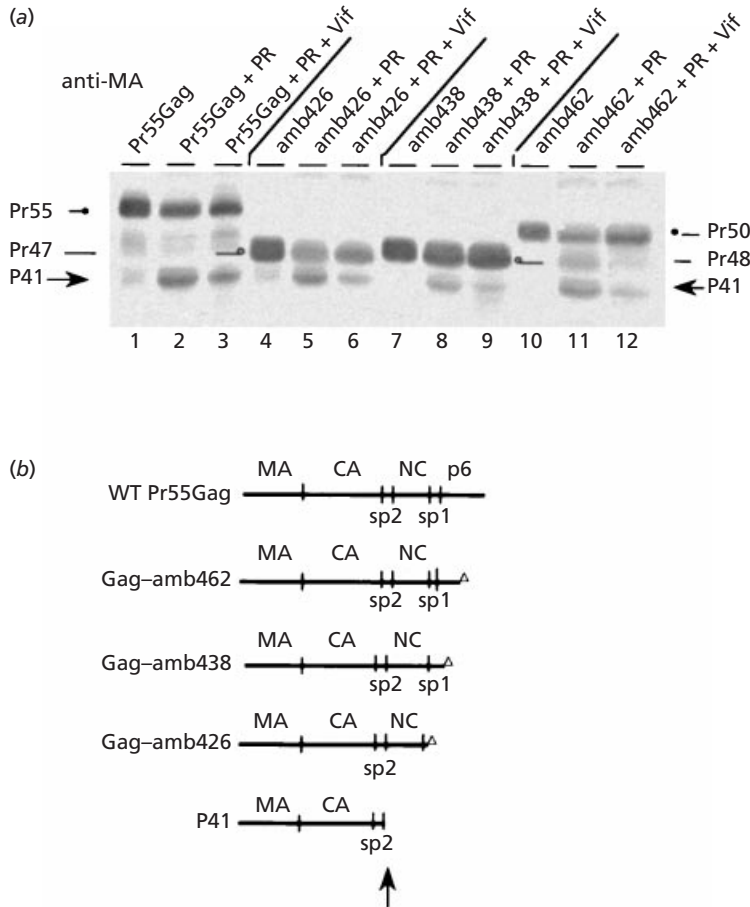


Fig. 6. Comparative PR-mediated processing of full-length Gag and C-terminal Gag deletion mutants (amber). (a) Gag substrate (1 pmol) was incubated alone (lanes 1, 4, 7 and 10), with 0.1 pmol PR (2, 5, 8 and 11) or with 0.1 pmol PR and 40 pmol Vif (3, 6, 9 and 12). Gag substrates were the non-*N*-myristoylated full-length (55kDa Gag) Gag-G2A (lanes 1–3), Gag-amb426 (4–6), Gag-amb438 (7–9) and Gag-amb462 (10–12). Samples were analysed by SDS-PAGE and immunoblotting with anti-MA antibody. (b) Schematic drawing of the Gag amber mutants with their different structural domains. The arrow indicates the putative PR cleavage site that generates the P41 cleavage product visible in (a).

Discussion

The molecular mechanisms of the regulatory functions of Vif in the HIV-1 life cycle and the biochemical basis for the possible association of Vif protein with the virus core remain matters of controversy. In this study, we investigated by *in vivo* and *in vitro* assays the interactions between Vif and Gag precursor and between Vif and PR, which represents a structural domain of the Gag–Pol precursor as well as an enzyme essential for virion maturation and infectivity. We observed efficient co-encapsulation of Vif with Gag precursor into intracellular VLP assembled by the non-*N*-myristoylated, budding-defective, Δp6 mutant Gag-amb438myr⁻, confirming previous results on direct Gag–Vif interaction (Bouyac *et al.*, 1997*b*; Huvent *et al.*, 1998). Our results implied that Vif encapsidation did not depend on Gag addressing to the plasma membrane and could also occur independently of Gag *N*-myristoylation and VLP budding and did not require the presence of the p6 domain in the Gag precursor.

Gag–Vif co-encapsulation into budding VLP released by Gag + Vif-co-expressing cells was also assayed using budding-competent, *N*-myristoylated Gag and Gag–Pol precursors. Vif was found to be encapsidated into extracellular VLP with significantly higher efficiency when co-expressed with Gag–

Pol precursors harbouring an inactive PR domain compared with Pr55Gag. Furthermore, Vif was encapsidated at higher levels into chimaeric VLP composed of Gag–Pol precursor and p6 + sp1-deleted Gag-amb426myr⁺ compared with single Pr55Gag VLP (Fig. 3; Table 1). The chimaeric VLP showed mean size, sphericity and homogeneity in shape similar to those of Pr55Gag VLP (Fig. 4; Table 2), which seemed to exclude the possibility that Vif encapsidation efficiency was simply related to the morphology of VLP, as hypothesized recently (Sova *et al.*, 2001), or to the amount of cytoplasmic material or plasma membrane enclosed in VLP. This rather suggested that Vif had some affinity for a downstream domain(s) of the Gag–Pol precursor and/or that the presence of the PR–Pol domains at the Gag C terminus positively affected the binding of Vif to the upstream regions of Pr55Gag identified previously (Bouyac *et al.*, 1997*b*; Huvent *et al.*, 1998). Co-encapsulation of Vif with a naturally frameshifted Gag–Pol precursor containing the p6* domain, compared with a Gag–Pol fusion construct containing the p6 domain in place of p6*, suggested that the effect on Vif encapsidation was not due directly to p6* but to further downstream sequences. Since we were unable to detect any significant interaction *in vivo* or *in vitro* between Vif and PR, direct participation of the PR domain of Gag–Pol precursor in Vif packaging was unlikely.

Although some binding of Vif to regions of the Pol domain could not be totally excluded, the results of our encapsidation assays suggested that the conformation of the Gag domain was more favourable to Gag–Vif interaction in the Gag–Pol precursor than in Pr55Gag.

Vif was also analysed with respect to its possible influence on the proteolytic activity of PR *in vitro*, using soluble Pr55Gag precursor as the substrate. We found that Vif was totally resistant to PR action *in vitro* (Fig. 5*a*), but exerted an inhibitory effect on PR-mediated Gag processing (Figs 5*b–d* and 6). Although some subtle mechanism of PR inactivation by Vif could not be excluded, the data suggested that Vif-mediated inhibition of Gag processing resulted from binding of Vif to the Gag substrate rather than to the PR enzyme. The proteolytic pattern suggested preferential protection by Vif of two major cleavage sites, at the MA–CA and CA–NC junctions (Figs 5 and 6). Interestingly, these two junction regions were revealed previously as the preferential binding sites of Vif (Bouyac *et al.*, 1997*b*; Huvent *et al.*, 1998). The peptide bond at the sp2–NC junction is cleaved at the highest rate by the viral PR (Pettit *et al.*, 1994). In addition, sp2 is not only essential for sequential processing of Pr55Gag (Pettit *et al.*, 1994), but is also a major determinant of Gag particle assembly (Campbell & Vogt, 1997; Gay *et al.*, 1998; Gross *et al.*, 2000). This suggested that Vif preferentially inhibited the cleavage of peptide bonds integrity of which is crucial for temporal regulation and correct assembly of HIV-1 virions.

We therefore hypothesize that Vif could regulate negatively the proteolytic activity of PR by interacting with the Gag/Gag–Pol substrate in the cytoplasm of infected cells, as suggested previously (Bouyac *et al.*, 1997*b*; Huvent *et al.*, 1998). This interaction would be transient and would maintain the integrity of the Gag and Gag–Pol molecules during their addressing to the plasma membrane and their assembly into immature particles. At later stages of assembly, a majority of Vif molecules would be excluded from the particles, as suggested recently (Sova *et al.*, 2001). The molecular mechanism of Vif exclusion is still unknown, but it could occur via a conformational change of Gag, probably resulting from Gag proteolysis, that lowered its affinity for Vif, or/and by exchange of Vif with a viral or cellular ligand(s) acting as a competitor(s) for Gag binding. Two lines of evidence support this Vif ligand-switching hypothesis. (i) Vif interacts with the cytoplasmic side of the plasma membrane, an association mediated by intrinsic membrane components and the C-terminal domain of Vif (Goncalves *et al.*, 1994, 1995; Simon *et al.*, 1997). (ii) The affinity of Vif for RNA decreased in the presence of Gag precursors, while the latter still bound RNA, suggesting a displacement and exchange of RNA-bound proteins during genome packaging (Zhang *et al.*, 2000).

It must be considered that our results might apply only to insect cells, which could lack some crucial mammalian cell factors. It is noteworthy that, in our system of baculovirus infection, we probably favoured incorporation of Vif into VLP

for at least two reasons: (i) the Gag and Gag–Pol precursors were not cleaved, since the Gag–Pol constructs were defective in PR activity, and (ii) insect cells lack certain cellular factors such as some tyrosine kinases of the Src family that may retain Vif at the cell membrane (Hassaine *et al.*, 2001). Further analyses are needed to reunite the multiple functions assigned to Vif. Several of them, e.g. the role of Vif in RNA folding and packaging and its role in virus core morphology and stability (Höglund *et al.*, 1994; Öhagen & Gabuzda, 2000) and in regulation of Gag/Gag–Pol processing during capsid assembly, would be compatible with a function as a viral chaperone in virus morphogenesis and genome encapsidation.

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