Proteolytic activity in vivo and encapsidation of recombinant human immunodeficiency virus type 1 proteinase expressed in baculovirus-infected cells

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The activity in vivo of HIV-1 proteinase (PR) was analysed in the baculovirus expression system, using eight different constructs of the prt gene under the control of polyhedrin (PH) promoters of various strengths. None of the active PRs was expressed in substantial quantities, and only PH-fused and/or non-functional PR mutants accumulated in high amounts in insect cells. However, enough PR activity was generated from a lengthened PR construct in insect cells to process Gag polyprotein substrate co-expressed in the same cells in trans. Fusion of the first 58 residues from the PH sequence to the PR N terminus did not significantly change its activity and specificity of cleavage of the Gag substrate. When analysed under mild denaturing conditions, PH-fused or unfused full-length PR point mutants, as well as PH-fused or unfused C-terminal deletion mutants, showed a propensity to multimerize, with a predominant occurrence of dimers. The incorporation of PR into Gag particles was studied using eight Gag–PR fusion constructs, all containing a non-functional PR mutant. The PR domain was fused to the C-terminal p6 domain of Gag (p6geo), or translated in frame with NCp7 (as in frameshifted Gag–Pol polyprotein) and followed by downstream sequences of increasing lengths from the Pol domain or the bacterial β-galactosidase. The results suggested that the presence of the p6geo domain was detrimental to the encapsidation of polyprotein-embedded PR.

Introduction

The structural proteins of human immunodeficiency virus type 1 (HIV-1) – the viral matrix (MAp17), the capsid (CAp24/p25) and the internal nucleoprotein NCp15, as well as the enzymes reverse transcriptase (RT), integrase (IN) and proteinase (PR) – originate from a large (160 kDa) gag–pol polyprotein precursor as a result of autoprocessing events due to specific cleavage by the pol-encoded PR (reviewed in Cann & Karn, 1989; Wills & Craven, 1991). This proteolytic processing thus depends upon PR, whose translation, in turn, depends upon the efficiency of the ribosomal frameshifting occurring at the 3′ end of the gag gene, near the NCp7–p6geo junction. Ribosomal frameshifting has been found to be a general mechanism by which retroviruses translate overlapping out-of-phase sequences into an in-phase fusion gene product (Jacks et al., 1988). This strategy is thus used by HIV-1, which expresses its fused gag–pol genes by frameshifting, a phenomenon which occurs at a frequency of 1:10 to 1:20. HIV frameshifting has been found to proceed in vitro (Jacks et al., 1988) and in yeast (Wilson et al., 1988) with the same apparent efficiency as in mammalian cells in vivo. Insect cells infected with recombinant baculoviruses expressing Gag–PR constructs also contain processed Gag polyprotein, suggesting that HIV PR had been produced by authentic ribosomal frameshifting (Hughes et al., 1993; Madsen et al., 1987; Overton et al., 1989).

The mature HIV-1 PR, 99 amino acids in length, belongs to the aspartyl protease family (reviewed in Kraüßlich & Wimmer, 1988). It is active as a dimer (Katoh et al., 1989; Kraüßlich, 1991; Kraüßlich et al., 1992; Miller et al., 1989), and its active site contains the consensus D-T-G sequence (Fontenot et al., 1992; Loeb et al., 1989). Since PR activity and the resulting proteolytic processing of Gag and Gag–Pol are essential for the generation of mature, infectious HIV-1 virions (Kaplan et al., 1993; Kohl et al., 1988; Kraüßlich, 1992; Konvalinka et al., 1995; Park & Morrow, 1993; Peng et al., 1989; Rosé et al., 1995), PR represents one of the major targets for enzyme-directed anti-AIDS therapy (Arrigo & Huffman, 1995; for reviews, Debock, 1995; Wlodaver & Erickson, 1993), although the reported emergence of multiple resistant variants raises serious issues (Borman et al., 1996).
Fig. 1. Proteinase constructs and their cloning in intermediate baculovirus vectors pGmAc115T, pGm532 and pGmAc03. The solid bars represent the PR sequence, and the diagonally light hatched boxes represent the upstream octapeptide MGTVSFNF contained in the transframe region (TF) resulting from the frameshift. Full-length PR precursor of 107 amino acids (PR107), the active site mutant (asterisk) PRD33G and the C-terminal deletion mutant PR77 were expressed as (a) unfused recombinant PR, or (b) fused to the polyhedrin (PH) N-terminal sequence of 58 amino acids (represented by thick hatched boxes). In (c), the PH promoter (symbolized by vertically hatched boxes) was partially deleted (∆), giving rise to the two low-expressor clones ∆PR107 and ∆PR77. fs, Frameshift signal.

In previous studies, we have used the recombinant baculovirus expression system to analyse the mechanisms of assembly of pseudo-viral Gag particles and their release by budding from the plasma membrane of infected insect cells (reviewed in Boulanger & Jones, 1996). The aim of the present study was to express various constructs of recombinant HIV-1 PR and to analyse their proteolytic activity with Gag precursor, the natural substrate of PR, co-expressed in trans in the same baculovirus-infected cells. We found that both active and inactive forms of PR were expressed at low levels in insect cells, whatever the strength of the promoter upstream of the cloned gene. However, enough active PR was synthesized to specifically cleave the Gag substrate provided in the same cells in trans. The PR activity was not significantly altered when a foreign sequence, from the baculoviral polyhedrin, was fused to its N terminus. In addition, we studied the sequence requirements for encapsidation of a PR point mutant embedded in Gag–Pol or Gag–β-galactosidase polyprotein. The occurrence of the p6gag domain at the Gag–PR junction appeared to be detrimental to this process, suggesting that another level of regulation for HIV-1 PR activation and virion maturation could occur at the stage of Gag and Gag–Pol co-assembly.

Methods

Cells, baculoviruses and intermediate transfer vectors. Spodoptera frugiperda Sf9 cells were maintained as monolayers at 28 °C in Grace’s antheraea medium supplemented with 10% foetal calf serum, Yeastolate and lactalbumin hydrolysate (Luckow & Summers, 1989). Recombinant baculoviruses were obtained by in vivo recombination in Sf9 cells between the transfer vector carrying the foreign gene and Bsu36I-digested DNA from the AcMNPV-derived BacPAK6 virus (Clontech), and recombinant clones were selected by their β-galactosidase(-) phenotype. Recombinants expressing HIV-1 Gag–PR–β-galactosidase fusion proteins were recovered from recombination in vivo with genomic DNA from wild-type (WT) AcMNPV, and recombinants selected by their occlusion body(-) and β-galactosidase(+)- phenotype. The four types of baculovirus transfer vectors used in this study to express genes of interest under the control of the polyhedrin (PH) promoter, pGmAc03, pGmAc532, pGmAc34T and pGmAc115T, have been described in previous studies (Carrière et al., 1995; Chazal et al., 1994, 1995; Hong & Boulanger, 1993; Royer et al., 1991, 1992). They led
to various levels of expression of recombinant proteins: low, high and high in fusion with the PH N-terminal sequence, respectively.

**Gene constructs and nomenclature.** All the genetic constructs used in this study have been described elsewhere (Royer, 1993), and the details of these constructs are available upon request. The AcMNPV baculoviruses expressing HIV-1 PR were identified by the letters PR followed by the number of amino acids in the recombinant PR sequence. The synthetic gene encoding the HIV-1 PR used in the present study corresponded to a PR precursor of 107 residues which contained the N-terminal self-processing site with the phenylalanine–proline bond of the RT–PR junction, MGTVSFNF-P (Valverde et al., 1992). Thus, recombinant PR107 expressed the (WT) PR precursor. The C-terminally-truncated form of PR, deleted of the last 30 amino acids, was referred to as PR77. The PR precursor mutant of 107 residues carrying the aspartic acid to glycine substitution in the active site was referred to as PRD33G, as the aspartic acid residue 25 in the maturated PR occupied position 33 in our PR107 precursor. The corresponding PH-fused proteases were designated PR58–107, PR58–77 and PR58–D33G, respectively. Two recombinant PRs, produced by the low expresser pGmAc03 with a partially deleted PH promoter, were referred to as APR107 and APR77, as they corresponded to the precursor of 107 residues and its C-terminal deletion mutant, respectively. The different PR constructs are depicted in Fig. 1.

Pr55Gag refers to the WT Gag precursor of 55 kDa expressed by Gag12myr(+). Pr47Gag refers to the non-N-myristylated, intracytoplasmic Gag precursor of 438 residues lacking the p6gag domain and expressed by clone Gag170 (Royer et al., 1992). In-phase Pr55Gag–PR fusion proteins are indicated by their apparent molecular masses in kDa: GagPR67, GagPR84, GagPR112, GagPR126 and GagPR160 all contain a prt gene mutant (D to G mutation in the D-T-G triad of the active site) fused to the C-terminus of the p6 domain of Pr55Gag. GagPR67 contains no extra downstream sequence, whereas GagPR84 contains the N-terminal third of the RT up to residue Tyr-144 (EcoRV site at position 2556), GagPR112 contains the RT sequence up to residue Trp-426 (KpnI site at nucleotide 3405), GagPR126 contains almost the entire RT sequence up to Trp-535 (KpnI site at nucleotide 3733) and GagPR160 contains the entire RT and IN domains. The frameshifted equivalent of GagPR160, lacking p6gag and referred to as AcH7fs (a gift from I. Jones, NRC IVEM, Oxford, UK) was a double mutant expressing a constitutively frameshifted gag–pol message and an in-phase Gag–PR–Pol polyprotein (Hughes et al., 1993). The in-phase Pr55Gag–PR–β-galactosidase polyprotein was referred to as GagPRBG182, and Pr47Gag–PR–β-galactosidase, a derived polyprotein deleted of the p6gag domain and part of the PR, was referred to as GagPRBG168. The different Gag–PR–Pol and Gag–PR–β-gal fusion constructs are shown in Fig. 2.

Recombinant baculoviruses expressing the 46 kDa Gag precursor from human T-lymphotropic virus 1 (HTLV-1 Pr46Gag) and HTLV-1 PR were both obtained from B. Guillemaud (INSERM, U-328 Bordeaux, France) (Ménard et al., 1993). Recombinant baculovirus expressing the

**Fig. 2.** Gag–PR fusion constructs. All the constructs were made in the pGmAc115T intermediate vector and included a non-functional PR carrying a D-to-G substitution in its active site (symbolized by an asterisk). The solid bars represent the PR domain, the stippled boxes represent the Pol domains, reverse transcriptase (RT) and integrase (IN), and the thick hatched boxes represent the bacterial β-galactosidase sequence. In (a), all the fusion polyproteins had the p6gag domain, connected to PR via the junction heptapeptide STVSFNF (jp; light hatched boxes). In (b), the genetic construct in AcH7fs maintained the gag–pol frameshift signal (fs) and the p6gag domain was not translated. In (c), the PR domain was embedded in the β-galactosidase sequence and fused to p6gag via the jp, as in GagPRBG182, or directly to Ncp7, as in p6-deleted GagPRBG168.
Fig. 3. SDS–PAGE and immunoblot analyses of recombinant PR expressed in SF9 cells at different times post-infection (p.i.) using different recombinant baculoviruses. (a) Coomassie blue-stained gel. Cells were harvested at 8 h (lane 1), 24 h (lane 2) and 36 h (lane 3) p.i. Only two clones, PR58–D33G and PR58–77, showed a detectable signal of recombinant protein, at 18
72 kDa Gag precursor from human spumavirus (HSRV Pr72Gag) was constructed in our laboratory (Carrière et al., 1995) and baculovirus expressing cauliflower mosaic virus (CaMV) gene IV-encoded virion coat precursor protein P57 was obtained from G. Devauchelle (INRA, St Christol-les-Alès, France).

**In vivo proteinase assays.** HIV-1 PR activity in trans on its Gag precursor substrate was assayed in Sf9 cells co-infected with two recombinants, one expressing the PR, the other expressing the Gag substrate. Since N-myristylated Pr55Gag was budding massively at the plasma membrane and its non-N-myristylated counterpart was mainly accumulated in the nucleus (Royer et al., 1991, 1992), our standard assays were performed using non-N-myristylated Pr47Gag expressed by clone Gag170. In addition, since a cytotoxic effect due to baculovirus expression could occur at a high m.o.i. and result in non-specific proteolysis of Gag, the total m.o.i. was maintained constant, and relative m.o.i. varied between PR- and Gag-substrate-expressing viruses. For example, in a typical experiment Sf9 cells were simultaneously infected by a mixed inoculum of Gag170:PR:107 at 50:0 (control, no PR), 45:5, 40:10, 35:15, 30:20, 25:25, 15:35, 10:40, 5:45 and 0:50 (control, no Gag) p.f.u. per cell. Cell lysates taken 40 h after infection were immunologically analysed by SDS–PAGE and immunoblotting with Gag monoclonal antibody (MAb) for the occurrence of the specific p24–p25 doublet band of CA protein.

**Biochemical and immunological analyses.** SDS–PAGE analysis of proteins, electric transfer, immunoblotting and ultracentrifugation of Gag particles in sucrose gradient have been described in detail in previous studies (Carrière et al., 1995; Chazal et al., 1994, 1995; Royer et al., 1991, 1992). Northern blot analysis was performed on RNA extracted from Sf9 cells using the guanidinium technique (RNAzol B, Bioprobe Systems). Probes were labelled with [32P]dCTP using a nick translation method (Sanger et al., 1977) and Sequenase kit, version 2.0 (USB Sequenase, Amersham).

**Antibodies.** Mouse MAb anti-Pr55Gag-p24CA (Epiclone-5001) was obtained from Epitope Inc. Anti-Pr55Gag and anti-HIV-1 PR polyclonal antibodies (laboratory-made) were both prepared in rabbit by immunization with Pr55Gag or bacterially expressed PR purified by preparative SDS–PAGE. Rabbit polyclonal antibodies against HTLV-1 Gag and CaMV Gag were obligingly supplied by B. Guillemain and G. Devauchelle, respectively. Rabbit serum against HSRV Gag was laboratory-made (Carrière et al., 1995). All antibodies were used at working dilutions ranging from 1:1000 to 1:2000.

**Electron microscopy (EM) and immunoelectron microscopy (IEM).** Cell specimens were fixed, embedded and processed as previously described (Chazal et al., 1994; 1995; Carrière et al., 1995) and sections examined under the Hitachi HU-7000 electron microscope.

**Results**

### Level of expression of recombinant PR in Sf9 cells

HIV-1 PR has been found to produce a cytotoxic effect upon induction in bacterial cells, an effect which was apparently independent of its proteolytic activity since it also occurred with the non-functional mutant D33G (Valverde et al., 1992). To obviate a possible deletion effect of recombinant PR on baculovirus-infected insect cells, the genes coding for PR107 and PR77 were cloned under the control of a partially deleted PH promoter lacking a few nucleotides upstream of the PH ATG codon (Fig. 1c). This construct has been shown to result in low levels of expression of cloned gene products (Luckow & Summers, 1989; Royer et al., 1992). This generated the two low-expressor clones ΔPR107 and ΔPR77. For comparison, WT and mutant PR were cloned under the intact PH promoter as fused (PR107, PR77 and PRD33G; Fig. 1a) or PH-fused recombinant proteins (PR58–107, PR58–D33G and PR58–77; Fig. 1b). In the two latter cases, the vectors were high expressers (Royer et al., 1992).

Although low expression of PR was expected to have a lesser cytotoxic effect than a high level of expression, an early cytopathic effect (CPE), different from the late CPE observed in the course of AcMNPV infection, was observed in Sf9 cells expressing all unfused PR constructs, even the inactive PR mutants PR77 and PRD33G. This CPE was not observed with their PH-fused counterparts PR58–77 and PR58–D33G (not shown). None of the active forms of PR yielded by the two high expressers PR107 or PR58–107 was found to be synthetized at detectable levels in whole cell extracts analysed by Coomassie blue-stained SDS–PAGE (Fig. 3a). As expected, no PR signal was detected for the two low expressers ΔPR107 and ΔPR77. Rather unexpectedly, however, the PR mutants synthesized by the high expressers PR77 and PRD33G were not visible over the background (Fig. 3a). The only two clones yielding visible recombinant PR bands in stained gels were PR58–D33G (18 kDa) and PR58–77 (16 kDa). Both were non-functional PR mutants (cf. below), and both contained the N-terminal 58 amino acids of baculoviral PH fused to the N-terminal self-processing site of the PR (refer to Fig. 1b). Scanning the gel showed that the PR58–77 band represented 8–10% of the total protein content of the cell, whereas the band of PR58–D33G did not exceed 1–2%.

In immunoblotting with PR antibodies (Fig. 3b), a strong signal was observed with the two PH-fusion forms PR58–D33G and PR58–77, and the two unfused mutants PRD33G (12 kDa) and PR77 (8 kDa) were also visible. No band was detected for PR107 and its PH-fusion equivalent PR58–107. Fusion of the full-length PR mutant PRD33G with the PH N-terminal 58 amino acids, as in PR58–D33G, therefore restored the yields of the recombinant PR to detectable levels, although inferior to the range usually obtained with non-toxic recombinant proteins expressed in the baculovirus system under the
natural PH promoter (Luckow & Summers, 1989; Royer et al., 1992). C-terminal deletion, in addition to the fusion to the PH N-terminal sequence, significantly increased the recombinant PR expression to high-type levels, as in PR58–77. The fact that both inactive PRD33G and PR77 were still synthesized in low amounts confirmed that the cytotoxicity of PR in insect cells was not entirely due to its protease nature, as already observed in bacterial cells (Valverde et al., 1992), and suggested that PR-mediated CPE was confined to within residues 1 to 70 in the mature PR sequence.

Northern blot analysis showed no significant difference between the two high-expresser clones PR107 and PR77 with respect to the PR-specifying mRNA levels (data not shown). Likewise, the mRNA levels of the low-expresser clones APR107 and APR77 were found to be similar, both of them being 4–5 times lower than for the high-expresser clones PR107 and PR77 (not shown). This has already been observed for a recombinant Gag clone expressed under the control of the same partially deleted PH promoter [Gag159myr(+); Royer et al., 1992]. This confirmed that the difference in the level of expression between inactive and active PR forms PR107 and PR77 resided at the post-transcriptional level.

**Oligomerization of recombinant PR**

Since HIV-1 PR is active as a dimer, and since PR dimerization could constitute a regulatory step in Gag proteolysis and assembly (Vogt, 1996), it was important to examine the dimer and/or multimer status of our recombinant PRs. Extracts of Sf9 cells infected with the recombinant baculoviruses expressing the detectable forms of PR were analysed by SDS–PAGE and immunoblotting after mild SDS denaturation at room temperature. In addition, DTT and urea were omitted from the sample buffer. The unfused protease PRD33G occurred mainly as a band migrating with an apparent molecular mass of about 24 kDa, compatible with that of a dimeric form (Fig. 3c). For the C-truncated form PR77, a 16 kDa protein was the prominent species. For both PH-fused full-length and C-truncated PR (PR58–D33G and PR58–77), the monomer species of 18 and 16 kDa, respectively, appeared to co-exist with slower components which could represent dimer species (36 and 32 kDa, respectively) and higher order oligomers.

**In vivo proteolytic activity on HIV-1 Gag substrates provided in trans**

(i) **Unfused PRs.** Sf9 cells were simultaneously infected with a baculovirus expressing Pr47Gag (a non-N-myristylated p6-deleted Gag precursor which accumulates within the cytoplasm) (Royer et al., 1991) and a second baculovirus expressing PR. With the low-expresser APR107, cleavage of the Gag precursor occurred, generating the specific p24–p25 doublet band characteristic of the CA protein (Henderson et al., 1992). The cleavage was evident after 24 and 36 h of co-expression. By contrast, Pr47Gag substrate remained intact in cells co-infected with the C-terminal deletion mutant APR77 (Fig. 4). This indicated that, even though no PR band was detectable by SDS–PAGE and immunoblotting of whole cell lysates (Fig. 3b), there was enough PR synthesized in the infected cells to

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**Fig. 4. In vivo PR activity assay on intracytoplasmic Gag precursor substrate.** Sf9 cells were co-infected with a recombinant expressing Pr47Gag, a p6myr-deleted non-N-myristylated Gag precursor, and low expresser recombinants ΔPR107 (expressing active PR) or ΔPR77 (expressing a C-terminal deletion mutant). The total m.o.i. remained constant in all cell samples (50 p.f.u. per cell). Lanes 1–11: co-infections with Gag170:ΔPR107 at p.f.u./cell ratios of 50:0 (lane 1: control sample, single infection with Gag170), 45:5 (lane 2), 40:10 (lane 3), 35:15 (lane 4), 30:20 (lane 5), 25:25 (lane 6), 20:30 (lane 7), 15:35 (lane 8), 10:40 (lane 9), 5:45 (lane 10) and 0:50 (lane 11: control, single infection with ΔPR107), respectively. Lanes 12–19: co-infections with Gag170:ΔPR77 at 45:5 (lane 12), 40:10 (lane 13), 35:15 (lane 14), 30:20 (lane 15), 25:25 (lane 16), 20:30 (lane 17), 15:35 (lane 18) and 5:45 (lane 19) p.f.u. per cell, respectively. Whole cell extracts (36 h p.i.) were analysed by SDS–PAGE and immunoblotting with anti-Pr55-p24 MAb (Epiclone-5001). m, Pre-stained molecular mass markers (kDa).
achieve the specific processing in trans of its natural substrate, Gag. A similar Gag cleavage at equivalent p.f.u. ratios of co-infecting recombinant baculoviruses was observed with the high-expresser PR107, as shown below.

(ii) Influence of foreign sequence fused to PR. The pattern of digestion in vivo of Pr47Gag by PH-fused PR58–107 seemed slightly less efficient than by unfused PR107, a difference which only appeared at a low m.o.i. ratio of PR- to Gag-expressing recombinant and became non-significant at a higher m.o.i. (not shown). This suggested that an additional polypeptide sequence located on the N-terminal side of the PR self-processing site did not significantly impair the recognition of its Gag substrate and its proteolytic activity.

The N-terminal tail of both PR precursors PR107 and PH-fused PR58–107 contained the natural upstream self-processing site of the PR, as the heptapeptide (M)GTVSFNF-P. It was therefore of interest to determine whether the F–P peptide bond was cleaved, and whether the PR was active as the mature 99 residue enzyme or as the precursor of 107 residues. However, since there was no visible protein band of active PR107 or PR58–107 (refer to Fig. 3 b), possible self-processing in cis could not be assayed. Thus, PR processing at the N terminus was investigated in trans using the two detectable and proteolytically inactive clones PRD33G (107 residues) or PR58–D33G (165 residues) as PR substrates in co-expression with active PR107 within the same cells. No change was observed in the electrophoretic mobility of the PR bands in immunoblots, even after 72 h of co-infection and with a high m.o.i. of PR107 (not shown). This would suggest that cleavage of the N-terminal tail was not required for PR activity, and that the two proteolytically active forms PR107 and PR58–107 could function as uncleaved miniprecursor and fusion protein, respectively. This was reminiscent of previous results obtained with various forms of PR translated in rabbit reticulocyte lysate, which have shown that autoprocessing is very inefficient when PR is expressed as part of a precursor that lacks Gag sequences (Zybarth & Carter, 1995). This was different from the bacterial expression of PR107 precursor, which has been found to be processed and accumulates as a mature form of 99 residues (Valverde et al., 1992). However, it could not be excluded that the enzyme substrates, as mutant PR subunits, (i) might interact with the active PR subunits to produce inactive dimers, or (ii) that the cleavage sites in the mutant PRs might be conformationally inaccessible as a result of the alterations. In both cases, the result would be the absence of apparent trans-processing.

(iii) Influence of N-myristylation on HIV-1 Gag processing in vivo. HIV-1 full-length Gag precursor in two configurations, Pr55Gagmyr(+)(–) and Pr55Gagmyr(-) expressed by clones Gag12myr(+)(–) and GagG2A mutant, respectively (Chazal et al., 1994; 1995), was expressed in Sf9 cells in a double infection with PR107. There were slightly more cleavage products with non-N-myristylated substrate GagG2A than with Pr55Gagmyr(+), mainly detected as the MAp17 band (not shown). This pattern could result from different half-lives for the two forms of MA, rather than from differences in Gag processing. Alternatively, the cleavage pattern could reflect a discrete variation in the conformational structure between the two Gag precursor forms, resulting in a change in the accessibility of the PR site at the MA–CA junction, as previously suggested for some Gag insertion mutants (Chazal et al., 1994). The difference could also be due to the cellular compartmentalization of the Gag substrates: whilst non-N-myristylated Gag accumulates within the cytosol (Royer et al., 1991, 1992), the molecules of N-myristylated Gag, which are efficiently transported to the plasma membrane where Gag particles assemble and bud into the extracellular medium, would escape PR processing. Indeed, significant amounts of unprocessed Pr55Gagmyr(+)(–) were found in the medium of co-infected cells (not shown).
Incorporation of recombinant PR into Gag particles

Retroviral Gag particles with a high efficiency of assembly have been proposed as packaging systems (termed retro-secretion of recombinant proteins) to isolate, in a particulate form, proteins of interest fused in-phase with the Gag precursor sequence (Wills, 1989). Considering that HIV-1 PR is encapsidated as an internal domain embedded in a Gag–Pol polyprotein, the capacity of PR to be packaged with Gag precursor when fused to its C terminus (cis-incorporation), or to be co-packaged with WT Pr55Gag precursor provided in trans (trans-incorporation) was investigated in our baculovirus expression system. Various Gag–PR fusion proteins were thus constructed. Gag–PR–Pol polyproteins contained, or lacked, the pol′60′ domain upstream of PR embedded in downstream pol-encoded sequences of increasing lengths. In two chimeric constructs, GagPRBG182 and GagPRBG168, Gag and PR were in-phase with a downstream foreign sequence belonging to the bacterial β-galactosidase (Fig. 2). Since it has been shown that there is no Gag particle assembly in the baculovirus system in the presence of a functional PR expressed in cis or trans (Hughes et al., 1993; Overton et al., 1989), all our constructs contained a PR mutant inactivated by substitution at its active site (D33G). All the recombinant polyproteins were produced in high yields, and all except one, GagPR160, accumulated as stable primary gene products (Fig. 5). For GagPR160, which corresponded to Gag precursor fused to full-length pol-polyprotein PR–RT–IN, the expected product of 160 kDa was replaced by a 115 kDa band. DNA sequencing ruled out a stop codon accidentally introduced into the pol gene. Similarly, recombinant AcH7fs, expressing a permanently frameshifted gag–pol message and an in-phase Gag–Pol polyprotein including a non-functional PR mutant, as in our GagPR160 clone, has been reported to be unstable in baculovirus-infected cells, and to accumulate as a 95–90 kDa Gag–Pol cleavage product (Hughes et al., 1993).

(i) Single infection and cis-incorporation of PR into Gag particles. The efficiency of assembly and extracellular release of membrane-enveloped Gag particles by Sf9 cells expressing our different Gag–PR fusion proteins was immunologically assayed in cell culture fluid samples (Chazal et al., 1994, 1995; Carrie et al., 1995). The results were compared to WT Pr55Gag particles released by Gag12myr(-)-infected cells. Only GagPR67 yielded detectable amounts of Gag particles, although at levels 5 to 10 times lower than WT Pr55Gag. GagPR67 particles sedimented at a lower apparent density (1±15 g/l), and appeared smaller in diameter (50–70 nm) and irregular in shape when compared to the regular WT Pr55Gag particles of 120–130 nm (Fig. 6a,b). The other constructs GagPR84, GagPR112, GagPR126, GagPR160 and GagPRBG182 failed to produce Gag particles in significant amounts. EM analysis of Sf9 cells expressing these different clones showed dense material accumulating at the plasma membrane (Fig. 6c) and reacting with both Gag and PR antibodies in IEM (not shown).

(ii) Effects in trans on WT Gag particle assembly and budding. Sf9 cells were co-infected at an equal m.o.i. with Gag12myr(+), expressing WT Pr55Gag, and a second baculovirus expressing a Gag–PR fusion construct. The amounts of WT Gag particles released into the culture medium were immunologically
assayed in sucrose gradient fractions. As shown in Fig. 7(a), a significant negative effect on WT Gag particle assembly and release was observed in co-expression with five fusion proteins, GagPR84, GagPR112, GagPR126, GagPR160 and GagPRBG182. The decrease was 2- to 4-fold, compared to cells solely expressing WT Pr55Gag. No negative effect was shown by GagPR67, AcH7fs or GagPRBG168. If one compares pairwise GagPR160 with AcH7fs on one hand and GagPRBG182 with GagPRBG168 on the other hand the only difference, and common feature with all the trans-dominant negative clones, resided in the presence of the p6<sup>gag</sup> domain upstream of the PR domain embedded in RT (GagPR160) or β-galactosidase sequence (GagPRBG182). By contrast, both AcH7fs and GagPRBG168, which showed no detectable negative effect, lacked p6<sup>gag</sup> and contained all, or part of, the PR domain (refer to Fig. 2).

(ii) Co-expression and trans-encapsulation of PR. As a result of the frameshift events, PR is physiologically co-encapsidated with Gag precursor Pr55Gag in the form of a polyprotein Gag–PR–Pol lacking the p6<sup>gag</sup> domain. It was therefore of interest to test whether the presence of p6<sup>gag</sup> in our Gag–PR fusion proteins was also detrimental to their rescue in trans by WT Pr55Gag and co-incorporation into Gag particles. The six p6<sup>gag</sup>-containing fusion constructs GagPR67, GagPR84, GagPR112, GagPR126, GagPR160 and GagPRBG182, and the two p6<sup>gag</sup>-lacking AcH7fs and GagPRBG168 were thus co-expressed with WT Pr55Gag and the amount of co-encapsidated Gag–PR fusion protein was assayed by SDS–PAGE and immunoblot analysis of extracellular Gag particles. There was no apparent correlation between the efficiency of Gag–PR co-encapsulation and the length of RT domain in which the PR was embedded: the fusion protein represented about 5% of the total Gag proteins for GagPR67, 2.5% for GagPR84, 17% for GagPR112 and 0.5% for GagPR126 (Fig. 7b). Thus, the domain downstream of the PR seemed to influence the efficiency of Gag–PR co-encapsulation in a sequence-dependent rather than in a precursor length-dependent manner. Gag–PR co-encapsulation also seemed to greatly depend upon the nature of the sequence upstream of the PR: the fraction of GagPR polyprotein co-encapsidated was about 15% for GagPR160, but more than 30% for its equivalent AcH7fs. Likewise, for the two Gag–PR–β-galactosidase fusion proteins, GagPRBG182 was co-encapsidated at barely detectable levels (about 1%), whereas GagPRBG168 was found to represent more than 17% of the total particulate Gag. The β-galactosidase assays performed on Gag particles confirmed the Gag antigen content analysis, showing a 30-fold higher co-encapsulation level for GagPRBG168 compared to GagPRBG182 (Fig. 7b). Again, for both polyprotein pairs, GagPR160 and AcH7fs on one hand and GagPRBG182 and GagPRBG168 on the other, the common difference resided in the p6<sup>gag</sup> domain inserted, or not, between NCp7 and PR.

Discussion

In contrast with bacterial expression, none of the active PRs expressed in insect cells, PR107 (the miniprecursor of 107 residues with a short N-terminal tail from the transframe region) or PR58–107 (the PR107 precursor fused to the N-terminal 58 amino acids from the polyhedrin sequence) was
expressed in substantial amounts (Fig. 3a, b). The difference in the levels of PR expression was found to reside at a post-transcriptional step. Cytotoxicity of HIV-1 PR has already been observed in bacterial (Valverde et al., 1992) and mammalian cells (Shoeman et al., 1990). From our data, however, it seemed that the PR-mediated CPE in SF9 cells was not only related to its proteolytic functions and to the cleavage of cytoskeletal filament proteins or transcription machinery elements (Rivière et al., 1991; Shoeman et al., 1990). The observation that our mutants PRD33G and PR77 were synthesized in low quantities suggested that PR-mediated CPE could also result from some cytotoxicity inherent to the protein itself, and carried by its N-terminal 70 amino acids. This toxic effect was efficiently neutralized by fusion of the PH (M)GTVSFNF from the transframe domain embedded in Gag–Pol polyproteins of increasing lengths (Gag–PR–Pol polyproteins), or in Gag–β-galactosidase fusion proteins (Gag–PR–β-gal chimeras GagPRBG182 and GagPRBG168). The results suggested that fusion of PR to the C-terminal β domain of Gag, whatever the length of the downstream RT sequence in which PR was embedded, had a detrimental effect in cis on Gag particle assembly and release from the plasma membrane, and on the final level of PR cis-encapsulation (Fig. 6). Likewise, the co-incorporation of different Gag–PR–Pol polyproteins and Gag–PR–β-gal chimeras into WT GagPr55 particles was found to occur at low levels for all peαγ-containing constructs. More specifically, the pairwise comparison of polyproteins differing by the peαγ region, GagPR160 with AcH7fs on one hand and GagPRBG182 with GagPRBG168 on the other hand, revealed that the co-encapsulation efficiency of PR-containing Gag polyproteins was significantly lower for the peαγ-bearing than for the peαγ-lacking constructs (Fig. 7). This suggested that the presence of the peαγ region was detrimental to the trans-rescue and co-encapsulation of PR domain embedded in retroviral or foreign sequences.

The functional importance of the structure of the Gag–Pol junction and the domain upstream of PR for PR activation and polyprotein processing in relation to Gag particle assembly has recently been evidenced in avian leukemia virus (Stewart & Vogt, 1994) and HIV-1. In HIV-1, PR polyproteins containing
the upstream transframe region and entire NC domain showed efficient dimerization and self-processing activity, whereas PR precursors containing only the transframe region were defective for both properties (Zybarth & Carter, 1995). Also, it has been reported that the p6\textsuperscript{trans} domain is not required for HIV-1 particle assembly and release in the absence of active PR, suggesting a functional linkage between p6\textsuperscript{trans} and PR-mediated Gag processing (Huang et al., 1995).

The results of the present study confirm the functional relationship between the two genetically overlapping domains p6\textsuperscript{trans} and PR. Ribosomal frameshifting is generally considered as a mechanism for limiting the synthesis of active HIV-1 PR, as the frameshift events occur at a frequency of 1 to 20 (Jacks et al., 1988). If, for some reason, the frameshift signal was bypassed by the ribosomes and a constitutive in-phase translation of the gag–pol message occurred at the frameshift site, the subsequent excessive level of PR would provoke the premature cleavage of Gag precursors which would thus fail to assemble into immature particles. Therefore, there is a positive pressure, via PR-mediated Gag processing, to maintain the ribosomal frameshifting, and hence maintain PR synthesis to limited amounts. Our data would suggest an additional mechanism of positive selection of the frameshift event, which takes place at the Gag protein structure and Gag assembly level. A one base pair insertion occurring near the 3' end of the gag gene could result in the readthrough of the gag stop codon and an in-frame translation of the gag–pol junction. In this case, the resulting mis-sense polyprotein containing the full-length Gag precursor (including its p6\textsuperscript{trans} domain), PR and RT domains would be excluded from the encapsidation process, and would exert a trans-dominant negative effect on Gag particle assembly. Further studies on the p6\textsuperscript{trans} domain and the overlapping transframe region (abbreviated p6\textsuperscript{trans}) connecting the NCp7 to the PR domain would be required to confirm this novel indirect mechanism of PR down-regulation.

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