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Phenotypic Characterization of Insertion Mutants of the Human Immunodeficiency Virus Type 1 Gag Precursor Expressed in Recombinant Baculovirus-Infected Cells

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A panel of 28 insertion mutants of the human immunodeficiency virus type 1 (HIV-1) Gag precursor (Pr55^{Gag}) was constructed by linker-insertion mutagenesis and expressed in recombinant baculovirus-infected insect cells. One set of 14 mutants carried the normal N-myristylation signal; the other set constituted their non-N-myristylated counterparts. The mutants were characterized with respect to (i) assembly and extracellular release of membrane-enveloped budding Gag particles, (ii) intracellular assembly and nuclear transport of Gag cores, (iii) specific processing of Pr55^{Gag} by HIV-1 protease *in vivo*, and (iv) binding of Pr55^{Gag} to an HIV-1 genomic RNA probe in Northwestern blotting. Insertions within the region between amino acid residues 209 and 334 in the CA domain appeared to be the most detrimental to Gag particle assembly and release of Gag into the external medium, whereas a narrower window, between residues 209 and 241, was found to be critical for secretion of soluble Pr55^{Gag}. Differences in Pr55^{Gag} processing *in vivo* and RNA binding *in vitro* between N-myristylated and non-N-myristylated Gag mutants suggested a major conformational role for the myristylated N terminus of Gag precursor. In coinfection experiments using wild-type Gag- and mutant Gag-expressing recombinants, a transdominant negative effect on Gag particle assembly and release was observed for insertions located in two separate domains, the matrix and nucleocapsid.

Three major structural proteins of the human immunodeficiency virus type 1 (HIV-1) virion originate from the specific cleavage of a 55,000-Da polyprotein precursor (Pr55^{Gag}) by the virus-coded protease: the p17 matrix (MA), p24 capsid (CA), and p15 nucleocapsid (NC) proteins (for reviews, see references 6, 12, and 44). In previous studies, it has been shown that recombinant Pr55^{Gag} synthesized in baculovirus-infected insect cells is capable of self-assembly. N-myristylated Pr55^{Gag} forms membrane-enveloped virus-like particles which are released into the external medium by budding from the plasma membrane, while assembly of non-N-myristylated Pr55^{Gag} occurs intracellularly (8, 13, 19, 30, 34). The carboxy-terminal domain p6 of the NC protein has been found to be dispensable for intracellular assembly of non-N-myristylated Gag core-like particles and for budding and extracellular release of N-myristylated Gag particles by insect cells (34, 35), in contrast to mammalian cells (14).

Deletion of the p15 domain from non-N-myristylated Gag precursor (Pr41^{Gag}) abolished its intracellular assembly (34). For N-myristylated Pr41^{Gag}, the downstream boundary of the Gag precursor sequence required for particle budding has been mapped to the p24-p25 junction (21). Two substitution mutations in the carboxy-terminal moiety of the p24 domain, L268P and L322S, have been found to be deleterious for the intracellular assembly function of non-N-myristylated Gag precursor. However, only the L268P mutation abolished the budding of N-myristylated Gag particles and the secretion of soluble Gag precursor (18). This finding suggested that discrete subdomains of the HIV-1 Gag precursor were involved at

various degrees in the self-assembly and budding processes of virus-like particles.

To further characterize the polypeptide domains of HIV-1 Gag precursor involved in the mechanisms of assembly of virus capsids and release of membrane-enveloped, budding Gag particles by the infected cells, we used a suppressor-linker insertional method (25) to introduce small in-frame 12-bp insertions into HIV-1 *gag* DNA. Two sets of 14 insertion mutants each were generated; one set of mutants carried the normal N-myristylation signal, and the other set constituted their non-N-myristylated counterparts. Three insertion mutants were thus obtained in the p17 domain (*in40*, *in100*, and *in120*), seven were obtained in the p24 domain (*in143*, *in184*, *in209*, *in241*, *in334*, *in341*, and *in357*), one was obtained in the p25 additional sequence (*in374*), two were obtained in the p7 domain (*in426* and *in438*), and one was obtained in the p6 domain (*in462*). The resulting phenotype of the Pr55^{Gag} mutants expressed in the baculovirus system was characterized with respect to the following criteria: (i) extracellular release of membrane-enveloped Gag particles and of soluble Gag, (ii) intracellular assembly and subcellular location of Gag cores, (iii) processing *in vivo* by the coexpressed HIV-1 protease, and (iv) binding to a viral genomic RNA probe.

An essential sequence for Gag particle assembly and budding, as well as for soluble Gag secretion, was mapped to the C-terminal two-thirds of the capsid domain. Furthermore, a novel conformational function was found for the myristyl group at the N terminus of the Gag precursor, in addition to its role in membrane targeting (15). Insertion mutants of Pr55^{Gag} which were assembly defective when expressed in the non-N-myristylated form were restored to wild-type (WT) particle budding phenotype when their insertions were placed in the N-myristylated Gag polypeptide backbone. This observation confirmed previous results obtained with deletion (21) and substitution (18) mutants. Conversely, RNA binding activity of

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TABLE 1. Sequences of HIV-1 Gag insertion mutants

Mutant ^a	Site of insertion	Nucleotide position ^b	gag codon ^c	Domain affected ^d	aa sequence ^e	
					From	To
<i>in40</i>	<i>AluI</i>	452	40	p17	EL	ELEFQL
<i>in100</i>	<i>AluI</i>	630	100	p17	AL	AGIPAL
<i>in120</i>	<i>PvuII</i>	690	120	p17	AD	AGIPAD
<i>in143</i>	<i>RsaI</i>	760	143	p24	VH	VWNSRH
<i>in184</i>	<i>DraI</i>	883	184	p24	LN	FWNSRN
<i>in209</i>	<i>AluI</i>	957	209	p24	AA	AGIPAA
<i>in241</i>	<i>RsaI</i>	1055	241	p24	ST	SLEFQT
<i>in334</i>	<i>DraI</i>	1333	334	p24	LK	FWNSRK
<i>in341</i>	<i>AluI</i>	1353	341	p24	AT	AGIPAT
<i>in357</i>	<i>HaeIII</i>	1402	357	p24	GH	GWNSSH
<i>in374</i>	<i>AluI</i>	1452	374	p25	AT	AGIPAT
<i>in426</i>	<i>RsaI</i>	1610	426	p7	CT	CLEFQT
<i>in438</i>	<i>HaeIII</i>	1646	438	p7	WP	WLEFQP
<i>in462</i>	<i>AluI</i>	1753	462	p6	SF	SWNSSF

^a Each was constructed in its N-myristylated and non-N-myristylated versions.

^b The base pair preceding the inserted sequence is numbered from the cap site of the HIV-1_{Bru} genome sequence (42).

^c The position of the mutation is indicated by the codon in the gag gene sequence immediately preceding or modified by the insertion.

^d The p17 matrix, p24 and p25 capsid, p15 and p7 nucleocapsid, and p6 domains were defined by HIV-1 protease cleavage at the following peptide bonds: Y-132-P-133, L-363-A-364, M-377-M-378, and F-448-L-449 (28).

^e The amino acids (aa) involved by the insertion are indicated by the single-letter code, and the inserted tetrapeptides are indicated in boldface.

Gag mutants was restored to WT levels when the N-terminal myristylation signal was deleted. Hence, the N-myristyl group could likely function as a global second-site suppressor of mutations (11) in gag which affect the conformation of the Gag polyprotein molecule as a whole.

MATERIALS AND METHODS

Virus and cells. Two parental recombinants of *Autographa californica* nuclear polyhedrosis virus (AcNPV), Gag12myr⁺ and G2A, were used as parental clones for insertion mutagenesis. Gag12myr⁺ (34, 35) expresses the WT form of N-myristylated full-length Gag precursor (GagPr55myr⁺). In G2A, a G-to-C substitution was introduced by oligonucleotide-directed mutagenesis at the second nucleotide of the second codon in the gag sequence, resulting in a GGT (glycine)-to-GCT (alanine) mutation. As a consequence of the loss of the N-myristylation signal, G2A expressed an N-myristylated-minus mutant form of Pr55^{Gag} (GagPr55myr⁻). Gag mutant Och184 was obtained by inserting a TAA stop codon at position 184 in the Gag sequence, resulting in a C-truncated Gag product consisting of the entire matrix domain and the downstream 50 amino acids belonging to the capsid domain. The gag gene product expressed by recombinant Och184 was a protein of 183 residues and 22 kDa in apparent molecular mass. WT and mutant Pr55^{Gag} were produced as recombinant proteins in *Spodoptera frugiperda* Sf9 cells maintained in Grace's medium supplemented with 10% fetal calf serum. Recombinants AcNPV-PR107 and AcNPV-PR77 contain a synthetic gene for an active form and a carboxy-terminally deleted, inactive form of the HIV-1 protease (18, 39), respectively. Both are expressed under the control of the polyhedrin promoter. In coinfection experiments, Sf9 cells were first infected with the recombinant expressing the Gag substrate at a multiplicity of infection (MOI) of 20 PFU per cell for 1 h at room temperature, after which the virus inoculum was removed and the cells were further incubated for 1 h with the second recombinant expressing the HIV-1 protease, at an MOI of 10 PFU per cell. Cells were harvested at 24 h postinfection (p.i.), and cell lysates were analyzed for Gag precursor processing.

Suppressor-linker insertional mutagenesis. Mutagenesis was conducted on a pUC18 plasmid containing the coding sequence for the gag gene (p18^{gag}). The insertion mutations were generated by the strategy of Lobel and Goff (25), which consists of introducing small in-frame 12-bp insertions into genes. The p18^{gag} plasmid was subjected to partial restriction nuclease digestion in separate reactions, with the following blunt cutters: *AluI*, *DraI*, *HaeIII*, *PvuII*, and *RsaI* (Boehringer, Mannheim, Germany). The linearized molecules were purified by agarose gel electrophoresis, blunt-end ligated to the *PvuII* suppressor-linker DNA fragment, and transfected into *Escherichia coli* CC114. Transformants were selected on MacConkey lactose agar (Difco Laboratories). Plasmids containing insertions were digested with *EcoRI* and recircularized. The sites of insertion were mapped by restriction enzyme digestion. Each insertion in the gag gene was isolated by digestion at unique restriction sites and reinserted into the corresponding sites of an intermediate baculovirus plasmid containing the coding sequence for the N-myristylated (WT) or non-N-myristylated (G2A) form of Pr55^{Gag}, cloned downstream of the polyhedrin promoter. Each mutant gag-containing plasmid was recombined with DNA from AcNPV as previously described (34, 35). Recombination with the first type of plasmid vector generated a set of insertion mutants of N-myristylated Pr55^{Gag}, whereas recombination with the second type of plasmid gave rise to their non-N-myristylated equivalents.

Nomenclature. Each mutant name indicates the location of its insertion, numbered according to the codon in the gag sequence which was immediately preceding or modified by the insertion; e.g., *in40myr*⁺ indicates that codons 40 and 41 of Pr55^{Gag}, which code for glutamic and leucine residues, respectively, were separated by an inserted tetrapeptide sequence in the mutant (E)-40-LEFQ-L41; Table 1). The non-N-myristylated counterpart of each GagPr55myr⁺ insertion mutant is indicated by the suffix "myr⁻" following its number, e.g., *in40myr*⁻.

EM. Sf9 cells infected at an MOI of 10 PFU per cell were harvested for electron microscopic (EM) processing at 48 h p.i. The cell pellets were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.5), postfix with osmium tetroxide (2% in H₂O), and treated with tannic acid (0.5% in H₂O).

After dehydration, the specimens were embedded in Epon-812 (Fulham, Latham, N.Y.). Sections were stained with 2.6% alkaline lead citrate and 0.5% uranyl acetate in 50% ethanol. Specimens were poststained with 0.5% uranyl acetate and examined under a Hitachi-H7100 EM.

Electrophoretic and immunological analyses. Protein samples were electrophoresed in sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gels (acrylamide/bisacrylamide ratio of 50:0.8), using a discontinuous buffer system (22). The proteins were electrically transferred from the SDS-gel onto nitrocellulose membranes (Hybond-ECL [enhanced chemiluminescence]; Amersham) at 180 mA for 90 min, using a semidry system (Cambridge Electrophoresis, Ltd., Cambridge, England). After saturation in 5% skimmed milk in 20 mM Tris-HCl (pH 7.5)–150 mM NaCl–0.05% Tween 20 (TBS-T buffer) for 1 h at room temperature, Pr55^{Gag} was detected on blots by successive incubations with anti-Gag antibody, alkaline phosphatase-labeled anti-immunoglobulin G conjugate (1:1,000; Sigma), and nitroblue tetrazolium–5-bromo-4-chloro-3-indolyl phosphate toluidinium cosubstrate-substrate (Boehringer) for color development (18). The primary anti-Gag antibodies were mouse monoclonal antibody (MAb) directed against Pr55-p24 (Epiclone 5001; Epitope Inc., Beaverton, Oreg.), mouse MAb anti-Pr55-p17 (Epiclone 5003), rat MAb anti-p6 (40), and homemade rabbit serum against Pr55^{Gag}. They were used at a dilution of 1:1,000 to 1:1,500 in TBS-T for 16 h at room temperature.

Gag secretion. Pr55^{Gag} has been shown to be released in the extracellular medium in two different forms, membrane-enveloped virus-like particles and nonassembled, soluble Gag polyproteins (35). Total secretion of Pr55^{Gag} (soluble plus particulate) was immunologically assayed on unfractionated cell culture medium collected at 48 h p.i. and clarified by centrifugation at $8,000 \times g$ for 5 min, using a protein blotting technique. The amount of Pr55^{Gag} released as budding particles was estimated by the same technique, performed on pelletable fraction of Pr55^{Gag}, obtained by a two-step sucrose gradient centrifugation method (37). Aliquots of infected cell culture supernatants were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), and proteins were transferred onto Hybond-ECL membranes as described above. Two of our insertion mutations were found to disrupt the Pr55^{Gag} epitope recognized by Epiclone 5001, whereas none modified its reactivity toward Epiclone 5003, which is specific for the p17 domain. Hybond-ECL membranes were therefore stepwise reacted with MAb Epiclone 5003 (1:1,000, overnight at room temperature), horseradish peroxidase-labeled anti-mouse immunoglobulin G conjugate (1:1,000; Amersham), and chemiluminescent substrate (Luminol; Amersham). Immunoblots were exposed to radiographic films (Hyperfilm-ECL; Amersham) for different lengths of time to prevent misevaluations of overexposed bands, and the luminograms were scanned at 610 nm, using an automatic scanner (REP-EDC densitometer system; Helena Laboratories, Beaumont, Tex.). The values obtained for secreted mutant Pr55^{Gag} were calculated from the intensity of their signals relative to that of WT GagPr55myr⁺. ECL blots were calibrated with samples of Gag12myr-infected cell supernatant, the total GagPr55myr⁺ concentration of which had been previously determined by antigen capture enzyme-linked immunosorbent assay as previously described (35). WT Pr55^{Gag} concentration, expressed as micrograms per milliliter of culture medium (1 ml corresponding to the supernatant of 10^6 cells), usually ranged between 20 and 30 $\mu\text{g}/\text{ml}$ per 10^6 cells in Gag12myr-infected cell supernatants.

DNA sequence analysis. The fragment of recombinant baculovirus DNA carrying the gag insertion was amplified by PCR,

using oligonucleotide primers hybridizing to sequences upstream and downstream of the mutation. Oligonucleotides were synthesized by Eurogentec (Seraing, Belgium). PCR fragments were sequenced by a dideoxynucleotide method (Sequenase sequencing kit; United States Biochemical Corp., Cleveland, Ohio) under the conditions recommended by the supplier.

Viral RNA binding. Each insertion mutant of Pr55^{Gag} was assayed for RNA binding capability by the Northwestern blotting technique developed by Luban and Goff (26). The *SacI*–*XmnI* fragment from the gag gene DNA (nucleotides 221 to 815 from the cap site in the HIV-1_{BRU} sequence [42]), which contained the encapsidation signal (7, 17, 24), was cloned between the *SacI* and *HincII* sites of pBluescript II KS(–). ³²P-labeled RNA probe was obtained by in vitro transcription of the resulting plasmid linearized at the internal *XmnI* site at nucleotide 381, using an RNA transcription kit (Stratagene). The genomic RNA probe, transcribed from the T7 promoter, corresponded to the 5'-terminal region (*SacI*–*XmnI*, nucleotides 221 to 381), spanning the splice donor site and the start of the gag gene, as used by Jowett et al. (21). Nonspecific RNA probe, transcribed from the T3 promoter on the complementary strand, corresponded to the *XmnI*–*XmnI* fragment (nucleotides 815 to 381). After SDS-PAGE and electrical transfer to ECL membranes, proteins of whole cell lysates were renatured by immersing the membrane in 30 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-KOH buffer (pH 7.5)–200 mM KCl–10 μM ZnCl₂–2 mM dithiothreitol containing 500 μg of heparin per ml (NW buffer). Blots were reacted with 5×10^5 cpm per blot (20 to 25 ng of RNA) for 1 h at room temperature in NW buffer and autoradiographed.

RESULTS

Construction of linker-insertion mutations in GagPr55. In-frame insertions of 12 bp were introduced into the DNA sequence of the HIV-1 gag gene, at sites generated by the blunt cutters *AluI*, *DraI*, *HaeIII*, *PvuII*, and *RsaI* (25). Fourteen insertion mutants in the N-myristylated recombinant GagPr55 were thus generated, and the equivalent insertions were similarly constructed in the non-N-myristylated form of recombinant Pr55^{Gag}. Table 1 shows the nature of the inserted amino acids and the locations of the mutation sites in the gag gene sequence. Insertion mutants were isolated as follows: three in the p17 matrix domain (*in40*, *in100*, and *in120*), seven in the p24 capsid (*in143*, *in184*, *in209*, *in241*, *in334*, *in341*, and *in357*), one in the p1.5 extra peptide between the p25 and p24 cleavage sites (*in374*), two in the p7 nucleocapsid (*in426* and *in438*), and one in the p6 domain (*in462*).

Mutant *in120* had its insertion within a region of high homology between membrane-associated proteins of many RNA viruses other than retroviruses (4), corresponding to residues 119 to 128 in the Gag_{BRU} sequence (42). Insertions *in40* and *in120* were located in regions of the MA domain with a high probability of helical conformation, according to structure predictions (2). Insertions at positions *in184*, *in209*, *in241*, *in334*, and *in341* in the CA domain involved residues which have been shown to be highly conserved in all retrovirus and several picornavirus capsid proteins (3). Two of them, *in241* and *in334*, were located on each side of the major homology region of retrovirus capsid proteins spanning residues 285 to 304 in HIV-1 Pr55^{Gag}, which constitutes part of the postulated puff (3, 23) and also corresponds to the assembly domain AD3 of Gag (44). In the p7 domain, the *in426* tetrapeptide was inserted at the carboxylic side of the last cysteine residue implicated in the second zinc finger, and the *in438* insertion lay

downstream of the second zinc finger, on the carboxylic side of the second tryptophan residue of p7. The insertion in the p6 domain (*in462*) was located immediately downstream of the conserved PTAPP sequence at residues 455 to 459 (29).

Expression and proteolytic processing of mutant Gag precursors. Pr55^{Gag} was found to be expressed at similar levels in all but one insertion mutant-infected cell cultures. With both N-myristylated and non-N-myristylated forms of *in40* mutant, a gag gene product of about 39 kDa was detected as the major anti-Gag antibody-reacting signal. Only trace amounts of Pr55^{Gag} were visible at all stages of infection, and pulse-chase experiments suggested a rapid cleavage of newly synthesized or nascent Pr55^{Gag} by host cell proteases (data not shown). Epitope analysis with MAbs directed against the p17 and p6 domains showed that the 39-kDa species contained the carboxy-terminal region p6, not p17 (see Fig. 4). This finding implied that the proteolytic cleavage occurred preferentially at or near the p17-p24 junction. None of the insertion mutants except *in209* showed any particular cytopathic effect other than the late cytopathic effect induced by the baculovirus itself. Two insertions, *in334* and *in341*, completely destroyed the reactivity of Pr55^{Gag} with MAb Epiclone 5001 (not shown), which allowed us to map its epitope to a sequence spanning residues 334 to 341 (LKALGPAA). All mutant Pr55^{Gag} but one, *in334*, showed an electrophoretic mobility similar to that of WT Pr55^{Gag} expressed by Gag12myr⁺; Pr55^{Gag} expressed by *in334* migrated slightly more slowly than WT Pr55^{Gag} (Fig. 1).

Gag polyprotein is the natural substrate for HIV-1 protease, which specifically cleaves it into p17, p24-p25 doublet, and p15 by hydrolysis of the Tyr-Pro (132-133), Leu-Ala (363-364), and Met-Met (377-378) bonds (28). Possible conformational alterations of the Pr55^{Gag} insertion mutants were investigated by their susceptibility to HIV-1 protease coexpressed in vivo. Sf9 cells were coinfecting with separate recombinants, one expressing gag and the other expressing a synthetic gene for HIV-1 protease (39), both under the control of the polyhedrin promoter (AcNPV-PR107 [18]). In parallel, as a control for nonspecific Gag processing by host cell proteases, cell cultures were doubly infected with each Gag recombinant and AcNPV-PR77, a recombinant expressing an inactive, carboxy-truncated form of HIV-1 protease (33a). Pr55^{Gag} cleavage was assayed by SDS-PAGE and immunoblotting analysis. A certain degree of spontaneous cleavage of WT and mutant Pr55^{Gag} by host cell enzymes occurred at late times of coinfection with AcNPV-PR77, and anti-Gag antibody-reacting products migrating at 47 and 41 kDa were always visible (not shown) (18, 35). This proteolytic effect was observed at similar levels with both N-myristylated and non-N-myristylated forms of Pr55^{Gag}.

In coinfection with the recombinant expressing the active protease, the expected specific p17 and p24-p25 Gag products were visible in most patterns, and their electrophoretic mobility was consistent with proteins carrying a four-amino-acid insertion (Fig. 1). Four mutants, *in40myr*⁺, *in209myr*⁺ (Fig. 1a), *in40myr*⁻, and *in241myr*⁻ (Fig. 1b), showed a cleavage pattern significantly different from the parental one, with almost no detectable p25-p24 doublet. In *in40myr*⁺, *in40myr*⁻, and *in241myr*⁺, a major band of about 39 kDa appeared to resist further protease cleavage. The absence of the specific p25-p24 doublet in these mutant patterns suggested that Gag-p39 was not a preferred substrate for HIV-1 protease in vivo and that only Pr41^{Gag}, resulting from an initial cleavage of Pr55^{Gag} at the capsid-nucleocapsid junction, was able to generate the p24-p25 capsid proteins. Comparisons of the cleavage patterns of N-myristylated and non-N-myristylated forms of Pr55^{Gag} seemed to indicate that all of the N-myristylated forms of recombinant Pr55^{Gag} were processed

FIG. 1. Immunoblotting patterns of Sf9 cell extracts coinfecting with HIV-1 Gag-AcNPV recombinant (20 PFU per cell) and protease-AcNPV recombinant (10 PFU per cell). In panel a, the cells expressed the N-myristylated versions of WT (Gag12myr⁺) or insertion mutant Pr55^{Gag}; in panel b, cells expressed the non-N-myristylated versions. Cells were harvested at 24 h p.i., and cell lysates were analyzed by SDS-PAGE and immunoblotting with rabbit polyclonal anti-Gag antibody. Note the difference in the cleavage patterns between *in209myr*⁺ and *in209myr*⁻ and between *in241myr*⁺ and *in241myr*⁻. Sizes are indicated in kilodaltons.

with a higher efficiency by HIV-1 protease than the non-N-myristylated forms were (compare Fig. 1a and b). This might be due to conformational differences between GagPr55myr⁺ and GagPr55myr⁻, resulting in a preferential cleavage of the GagPr55myr⁺ substrate at the MA-CA and CA-NC junction sites, to a colocalization of protease and GagPr55myr⁺ within the cell, or both. The first hypothesis was supported by the observation that *in209* and *in241* showed significantly different cleavage patterns in their N-myristylated and non-N-myristylated configurations (Fig. 1).

Assembly and budding of N-myristylated Pr55^{Gag} mutants. Release of Gag virus-like particles by budding from the plasma membrane was qualitatively analyzed by EM of cell samples harvested at 48 h p.i. The EM patterns of N-myristylated mutants of Pr55^{Gag} could be arranged into four groups. Group I corresponded to mutants with a WT budding pattern, as shown by parental recombinant Gag12myr⁺, and comprised *in100myr*⁺, *in120myr*⁺, *in143myr*⁺, *in184myr*⁺, *in341myr*⁺, *in374myr*⁺, *in426myr*⁺, *in438myr*⁺, and *in462myr*⁺. Four budding-defective mutants, *in40myr*⁺, *in209myr*⁺, *in241myr*⁺, and

in334myr⁺, constituted group II. Group III consisted of mutants, represented by *in438myr*⁺, which differed from the parental type in the cellular sublocation of Gag particles. One particular mutant, *in357myr*⁺, which showed budding particles with abnormal size and shape, was assigned to a single group, group IV. Figure 2 illustrates these patterns for some members of the four groups.

Mutant *in374myr*⁺ (group I) showed budding Gag particles indistinguishable from the WT (Fig. 2a), whereas *in357myr*⁺ (group IV) released budding particles heterogeneous in size and shape, with many particles having a diameter smaller than that released by WT Gag12myr⁺, 70 nm versus 120 to 130 nm (Fig. 2c). Among the group II mutants, which were completely defective in budding, *in209myr*⁺ provoked profound alterations of the cell surface, with indentations and local disruptions of the plasma membrane, suggesting an increased rigidity of the membranal bilayer compared with parental Gag-expressing cells (Fig. 2d and e). This was reminiscent of the EM pattern shown by a frameshift mutant of Gag-Pol expressed in insect cells and accumulating a C-truncated 90-kDa fusion protein (20). Three mutants, *in40myr*⁺, *in357myr*⁺, and *in438myr*⁺, insertions of which were localized in different areas of the Pr55^{Gag} sequence, shared properties with members of different groups, suggesting that the same insertion could have pleiotropic effects. For example, membrane-enveloped Gag particles assembled by *in438myr*⁺ and by *in357myr*⁺ were rarely found at the cell surface (as for the group I members) but preferentially accumulated within intracytoplasmic vesicles (Fig. 2b). On the other hand, *in40myr*⁺, which failed to release budding particles from the plasma membrane (as for the group II members), assembled some Gag particles of 65 to 70 nm in diameter within the cytoplasm (not shown). These particles did not react with anti-p17 antibody in immunoelectron microscopy but reacted with anti-p6 antibody (not shown). This observation, and the rapid cleavage of *in40*-GagPr55 by cellular proteases at the p17-p24 junction, suggested that the 39-kDa Gag product, containing the p24 and p15 domains, could assemble intracellularly into small core-like particles.

Intracellular assembly and nuclear localization of non-N-myristylated Pr55^{Gag} mutants. In insect cells infected by recombinant baculovirus expressing non-N-myristylated full-length Gag precursor, we have shown that Gag particles were massively accumulated in the nucleus, in contrast to p6-deleted mutant of Pr55^{Gag}, which mainly assembled Gag particles in the cytoplasm (34). This finding suggested that some nuclear targeting function was directly or indirectly associated with the p6 domain. EM observation of cells infected by non-N-myristylated parental G2A clone and by each of our 14 insertion mutants showed that in general, the non-N-myristylated insertion mutants formed significantly less core-like structures than did parental G2A Gag within the nucleus (34) and that most of the intranuclear Gag material accumulated as amorphous inclusions. The phenotypic groups previously defined for the N-myristylated Gag family were tentatively applied to the non-N-myristylated versions of mutant Pr55^{Gag}. However, no mutant showed any particles resembling the intranuclear Gag cores assembled by parental G2A and could thus be individualized as group I. Assembly-defective mutants (group II) comprised two mutants, *in100myr*⁻ and *in334myr*⁻. Group III was a heterogeneous group, characterized by a variety of Gag topological patterns differing from that of the parental recombinant G2A. Four of them, *in374myr*⁻, *in426myr*⁻, *in438myr*⁻, and *in462myr*⁻, exhibited rare particles within the cytoplasm and none in the nucleus (Fig. 2h). One mutant, *in209myr*⁻, assembled particles which budded inside the perinuclear cisterna, but none were detected within the nucleoplasm (Fig. 2f).

Group IV consisted of mutants showing amorphous intranuclear inclusions of Gag material, with rare or no individualized core particles. This was the case for *in40myr*⁻, *in120myr*⁻, *in143myr*⁻, *in184myr*⁻, *in241myr*⁻, *in341myr*⁻, and *in357myr*⁻ (Fig. 2g). Taken together, the EM observations suggested that most of our insertions were more detrimental to Gag assembly when expressed in the non-N-myristylated context than in the N-myristylated context of Pr55^{Gag}.

Extracellular release of mutant Gag precursors. N-myristylated Gag precursor has been shown to be released into the culture medium of recombinant-infected insect cells as soluble Gag polyproteins and membrane-enveloped budding particles, whereas non-N-myristylated Gag precursor is released only as unassembled, soluble material (35). The amount of extracellular Gag precursor secreted in particulate form was quantitatively estimated by SDS-PAGE and chemiluminescent protein blotting analysis of aliquots of Gag particle pellets isolated from cell culture media. The same assay was used to quantitate the total Gag secretion in aliquots of unfractionated cell culture media. Particulate Gag represented 70 to 85% of the total WT Pr55^{Gag} secreted by Gag12myr⁺-infected cells. Extracellular release of both particulate and soluble Gag was almost totally abolished in *in40myr*⁺, a result consistent with the loss of the N-myristylated p17 domain of Gag precursor. This result suggested that the p39 Gag product (p24-p15) was not secreted at detectable levels by Sf9 cells. With the exception of three mutants, *in426myr*⁺, *in438myr*⁺, and *in462myr*⁺, which were slightly reduced in Gag particle release compared with the WT, all insertions were detrimental to some extent to the secretion of particulate Gag (Fig. 3a). The histogram of Gag particle concentration in the media at 48 h p.i. showed that the maximum effect occurred for insertion mutants within positions 143 to 374, i.e., a region overlapping virtually the entire CA domain (Fig. 3a). This result confirmed previous observations that mutations in the CA domain are particularly critical for virion assembly (10, 16, 36, 44).

When the culture supernatants were assayed for total Gag (particulate plus soluble), the region between residues 209 and 334 appeared to be critical for secretion of N-myristylated Gag precursor (Fig. 3a). These results confirmed the EM analyses, in which no particles were found budding from the plasma membrane of cells infected by *in209myr*⁺, *in241myr*⁺, and *in334myr*⁺ at 48 h p.i. (Fig. 2). However, for five mutants, *in143myr*⁺, *in184myr*⁺, *in341myr*⁺, *in357myr*⁺, and *in374myr*⁺, the amounts of total Gag secreted represented 5 to 10 times the amounts of particulate Gag, suggesting a higher efficiency of soluble Pr55^{Gag} secretion compared with WT (Fig. 3a).

Total Pr55^{Gag} assayed in the culture supernatants of non-N-myristylated recombinant-infected cells represented nonenveloped, soluble Gag polyproteins (35). The critical domain for secretion of soluble non-N-myristylated Pr55^{Gag} coincided with the region responsible for the secretion-defective phenotype of N-myristylated Gag, although spanning a narrower sequence, between residues 209 and 241 (Fig. 3b). On each boundary of this region, some insertion mutants secreted soluble non-N-myristylated Gag with a higher efficiency than the parental recombinant G2A. This was the case for *in184myr*⁻, *in334myr*⁻, *in341myr*⁻, and *in357myr*⁻ (Fig. 3b).

Viral RNA binding. The capacity of WT and mutant Pr55^{Gag} to bind to viral RNA was investigated by Northwestern blotting assay (26) of WT- and mutant-infected cell extracts analyzed by SDS-PAGE. The viral RNA probe used contained the encapsidation signal psi of the RNA genome (7, 17, 24). In both N-myristylated and non-N-myristylated Gag autoradio-

FIG. 2. EM analysis of Sf9 cells expressing N-myristylated and non-N-myristylated insertion mutants of recombinant Pr55^{Gag}. Arrows in panel d show local disruptions of the plasma membrane. Arrowheads in panel e point to plasma membrane indentations. Open arrowheads in panel f point to Gag core-like particles budding in the perinuclear cisterna. Note the accumulation of Gag particles (120 to 130 nm in diameter) in vesicles of *in438myr*⁺-infected cells (b) and the heterogeneity in shape and diameter (65 to 70 nm for the boxed particle) of intravesicular particles in *in357myr*⁺-infected cells (c). V, virions of AcNPV; C, cytoplasm; N, nucleus; i, inclusion. Bars represent 100 nm (a), 200 nm (c, d, e, g, and h), and 500 nm (b and f).

grams, no radioactivity was detected at the position of Pr55^{Gag} for *in40myr*⁺ and *in40myr*⁻, but a major signal was visible at 39 kDa (Fig. 4b and d). The same 39-kDa band was found in the WT and most of the mutant Gag patterns, and this was likely to represent a spontaneous cleavage product of the Gag precursor, consisting of the p24 capsid and p15 nucleocapsid domains. In their non-N-myristylated configuration, all mutant

Pr55^{Gag} other than *in40myr*⁻ bound to the viral RNA probe with similar efficiency (Fig. 4d). In their N-myristylated configuration, however, four mutants, besides *in40myr*⁺, showed no detectable RNA binding activity of Pr55^{Gag} (Fig. 4b). Among the five mutations analyzed, three (*in40myr*⁺, *in100myr*⁺, and *in120myr*⁺) were located in the p17 domain, one (*in143myr*⁺) was near the p17-p24 junction, and one (*in209myr*⁺) was in the N-terminal third of the CA domain. This suggested that domains other than the p15 nucleocapsid contributed significantly to the RNA binding of the full-length Gag precursor in the Northwestern blotting assay. To further analyze the role of C-terminal regions of the Gag precursor in RNA binding, C-truncated forms of Gag in both their N-myristylated and non-N-myristylated versions were also assayed by Northwestern blotting. The deletion mutants used were Pr47^{Gag} (p6 deleted), Pr41^{Gag} (p15 deleted), and Och184, a 22-kDa N-terminal Gag product consisting of the entire matrix domain and the first 50 amino acids from the capsid. As shown in Fig. 4, Och184 and Pr41^{Gag} did not bind to the genomic probe in either their N-myristylated or non-N-myristylated configuration, whereas p6-deleted Pr47^{Gag} bound to RNA with the same efficiency regardless of whether the myristyl group was present at its N terminus. This finding confirmed previous observations on the RNA binding function of the p7 nucleocapsid domain (9, 21).

However, the binding patterns shown by some of our insertion mutants implied a key role of the N-terminal moiety of Gag precursor in this function. This role would function only in the presence of the p6 domain, as inferred from the results obtained with N-myristylated and non-N-myristylated Pr47^{Gag} (Fig. 4). The difference observed in the Northwestern blotting assay between the N-myristylated mutants *in100myr*⁺, *in120myr*⁺, *in143myr*⁺, and *in209myr*⁺ on one hand and their non-N-myristylated counterparts *in100myr*⁻, *in120myr*⁻, *in143myr*⁻, and *in209myr*⁻ on the other hand suggested that the RNA binding capacity of Pr55^{Gag} could be both sequence and conformation dependent. In this hypothesis, the accessibility and binding efficiency of the RNA recognition sequence(s) in the p7 nucleocapsid domain would be significantly influenced by other upstream domains, located between residues 100 and 120 in the matrix and between positions 143 and 209 in the capsid.

Complementation and transdominance. Sf9 cells were coinfectd pairwise with the N-myristylated mutants, and extracellular Pr55^{Gag} was assayed in the cell culture supernatants for eventual complementation and rescue or, alternatively, negative transdominant effect on Gag budding and secretion. Table 2 shows the results of the complementation assays, performed on supernatant of cell cultures simultaneously infected by two recombinants. There was some complementation effect for Gag secretion in coinfections involving *in374myr*⁺ and *in426myr*⁺, but most of the coinfectd cells yielded less extracellular Gag than single WT recombinant-infected cells did. In most of the coinfections involving *in40myr*⁺ or *in120myr*⁺ as one of the pairs, a significantly lower Gag secretion was observed. A similar effect was observed with many pairs involving *in209myr*⁺.

FIG. 3. Extracellular release of WT and insertion mutants of Pr55^{Gag} by Sf9 cells. Aliquots of medium from cell cultures expressing N-myristylated (a) or non-N-myristylated (b) Pr55^{Gag} were withdrawn at 48 h p.i. and assayed for secretion of total (particulate plus soluble) Pr55^{Gag} (hatched columns) and for budding and release of membrane-enveloped Gag particles (filled columns). Gag secretion was expressed as the concentration of Pr55^{Gag} in the culture medium. Standard deviation was 10 to 15% of the reported value ($n = 3$). The insertions are positioned on the linear sequence of the Gag precursor (amino acids 1 to 500), represented by the x axis.

TABLE 2. Complementation for Gag secretion between N-myristylated insertion mutants^a

	WT	<i>in40</i>	<i>in100</i>	<i>in120</i>	<i>in143</i>	<i>in184</i>	<i>in209</i>	<i>in241</i>	<i>in334</i>	<i>in341</i>	<i>in357</i>	<i>in374</i>	<i>in426</i>	<i>in438</i>	<i>in462</i>
WT	100	79	59	67	71	52	53	72	67	72	83	66	96	73	81
<i>in40</i>		≤1	4	13	16	16	22	30	≤1	26	25	16	46	22	46
<i>in100</i>			31	43	42	29	62	77	39	33	28	110	91	70	43
<i>in120</i>				31	20	17	10	17	12	26	12	45	33	24	25
<i>in143</i>					42	25	44	36	19	16	14	27	37	41	36
<i>in184</i>						65	46	56	27	53	40	69	82	68	49
<i>in209</i>							23	18	13	13	3	6	21	32	22
<i>in241</i>								19	20	44	65	97	91	68	74
<i>in334</i>									23	19	21	53	24	13	75
<i>in341</i>										69	56	92	75	66	59
<i>in357</i>											49	53	75	61	40
<i>in374</i>												92	77	50	60
<i>in426</i>													78	68	59
<i>in438</i>														77	66
<i>in462</i>															45

^a Sf9 cells were coinfecting with the same MOI of each Gag recombinant (15 PFU per cell), and culture supernatants were assayed for Pr55^{Gag} secretion at 48 h p.i. Results are expressed as a percentage of WT Pr55^{Gag} secreted by Gag12myr⁺. Standard deviation represented 10 to 15% of the reported values (average of three determinations).

The transdominant negative effect on Gag secretion exerted by Gag mutants over the WT Gag was further analyzed by preinfecting the cells with each of the mutants 1 h before adding the WT Gag12myr⁺ inoculum. No drastic effect was observed when preinfections were performed with recombinants expressing the N-myristylated version of the GagPr55 mutants, and only a slight decrease in Gag secretion was observed for insertions located in a large region of Pr55^{Gag} overlapping the matrix and most of the capsid domain, until position 334 (Fig. 5, hatched columns). However, preexpression of non-N-myristylated, budding-defective insertion mutants resulted in a significant reduction of Gag secretion for some mutations located in two domains, the matrix and the nucleocapsid (Fig. 5, filled columns). The magnitude of the effect ranged from 5-fold with *in100myr*⁻, *in120myr*⁻, *in426myr*⁻, and *in438myr*⁻ to 10-fold with *in40myr*⁻.

DISCUSSION

A panel of two sets of 14 insertion mutants of HIV-1 Gag, one set carrying the normal N-myristylation signal and the other set constituting their non-N-myristylated versions, was isolated and expressed in baculovirus-infected cells. The phenotypes of these mutants are summarized in Table 3. The first picture which emerged from this phenotypic characterization was the essential role of the myristylated N terminus of Pr55^{Gag} in multiple biological functions, besides its role in membrane targeting and budding of Gag virus-like particles (8, 13, 15, 31, 34, 35). Three lines of experimental arguments suggested that this pleiotropic effect was mediated by an N-myristyl-dependent conformation of GagPr55.

(i) Comparison studies of our two sets of insertion mutants

suggested that the myristylated N terminus acted as a *cis*-dominant signal overriding various assembly mutations in the Pr55^{Gag} sequence. Mutations in non-N-myristylated GagPr55 which were detrimental to self-assembly of Gag cores within the cell were rescued to almost WT budding levels in the N-myristylated context. This was observed for insertions at positions 100, 120, 143, 184, 341, 374, 426, 438, and 462. A similar effect has been observed with p15-deleted, assembly-defective mutants of non-N-myristylated Pr41^{Gag}, in which the function of self-assembly and budding from the plasma membrane was restored upon myristylation of the N-terminal residue (21, 34, 35), and with an assembly-defective substitution mutant of Pr55^{Gag}, L322S, the assembly-defective phenotype of which was expressed only in the non-N-myristylated context (18). (ii) The processing of Pr55^{Gag} in vivo by HIV-1 protease coexpressed in *trans* was found to occur with different efficiency for N-myristylated and non-N-myristylated configurations of the same Pr55^{Gag} mutants, e.g., *in241myr*⁺ and *in241myr*⁻, and *in209myr*⁺ and *in209myr*⁻, respectively (Fig. 1). (iii) The binding of Pr55^{Gag} to a genomic RNA probe carrying the encapsidation signal (7, 17, 24) was abolished for four N-myristylated mutants (*in100myr*⁺, *in120myr*⁺, *in143myr*⁺, and *in209myr*⁺), whereas their non-N-myristylated counterparts showed a WT binding activity (Fig. 4 and Table 3). The deletion of the N-myristylation signal at the N terminus of Pr55^{Gag} mutants restored their normal RNA binding activity and thus acted as a common second-site suppressor mutation (11) for insertions localized in two separate domains. Such a global suppressor effect of mutations localized in both the matrix (*in100myr*⁻ and *in120myr*⁻) and the capsid (*in143myr*⁻ and *in209myr*⁻) was likely to result from a conformational change of the N-myristyl-deleted Pr55^{Gag}.

FIG. 4. Viral RNA binding analysis of parental and insertion mutants of Pr55^{Gag}, expressed in their N-myristylated (a and b) and non-N-myristylated (c and d) configurations. Mock-infected cells and cells infected with WT baculovirus (AcNPV) or AcNPV-Gag recombinants were harvested at 36 h p.i. After SDS-PAGE and electrical transfer to ECL membranes, proteins of cell lysates were analyzed by Western (a and c) and Northwestern (b and d) blotting, performed on the same membranes. In panels a and b are shown the blots after immunoreaction with anti-p17 MAb Epiclone 5003. The positions of the gag gene products Pr55, Pr47, Pr41, and Och184 are indicated by filled dots in Western blots and open dots in Northwestern blots. The position of the *in40* 39-kDa primary gag gene product is indicated by an open arrowhead. The 39-kDa band is not shown by the anti-p17 MAb, only by polyclonal anti-Gag (see Fig. 1) or by anti-p24 and anti-p6 MABs. Note the absence of specific radioactive Gag precursor signal in lanes corresponding to *in40myr*⁺, *in100myr*⁺, *in120myr*⁺, *in143myr*⁺, *in209myr*⁺, Och184myr⁺, and Pr41myr⁺ (b) as well as in some of their non-N-myristylated counterparts, *in40myr*⁻, Och184myr⁻, and Pr41myr⁻ (d). Controls for nonspecific binding of RNA are shown in lanes of mock-infected and WT baculovirus (AcNPV)-infected cell extracts. (m), size markers. Sizes are indicated in kilodaltons.

TABLE 3. Phenotype of GagPr55 insertion mutants^a

Recombinant Gag	Extracellular budding ^b	Cytoplasmic assembly ^c	Nuclear assembly ^c	Proteolytic processing ^d	Binding to vRNA probe ^e
Gag12myr ⁺ (WT)	+	0	0	+	+
<i>in40myr</i> ⁺ (II, III)	0 ^f	+/- ^g	+/- ^h	A	0 ^f
<i>in100myr</i> ⁺ (I)	+	0	0	+	0
<i>in120myr</i> ⁺ (I)	+	0	0	+	0
<i>in143myr</i> ⁺ (I)	+	0	0	+	0
<i>in184myr</i> ⁺ (I)	+	0	0	+	+
<i>in209myr</i> ⁺ (II)	0 ^f	0	0	A	0
<i>in241myr</i> ⁺ (II)	0	0	0	+	+
<i>in334myr</i> ⁺ (II)	0	0	0	+	+
<i>in341myr</i> ⁺ (I)	+	0	0	+	+
<i>in357myr</i> ⁺ (III, IV)	^g	0	0	+	+
<i>in374myr</i> ⁺ (I)	+	0	0	+	+
<i>in426myr</i> ⁺ (I)	+	0	0	+	+
<i>in438myr</i> ⁺ (I, III)	+	+/- ^j	0	+	+
<i>in462myr</i> ⁺ (I)	+	0	0	+	+
GagG2A (parental)	0	+/-	+	+	+
<i>in40myr</i> ⁻ (IV)	0	0	+/- ^h	A	0 ^f
<i>in100myr</i> ⁻ (II)	0	0	0	+	+
<i>in120myr</i> ⁻ (IV)	0	+/-	+/- ^h	+	+
<i>in143myr</i> ⁻ (IV)	0	+/-	+/- ^h	+	+
<i>in184myr</i> ⁻ (IV)	0	+/- ^k	+/- ^h	+	+
<i>in209myr</i> ⁻ (III)	0	+/- ^l	0	+	+
<i>in241myr</i> ⁻ (IV)	0	0	+/- ^h	A	+
<i>in334myr</i> ⁻ (II)	0	0	0	+	+
<i>in341myr</i> ⁻ (IV)	0	0	+/- ^h	+	+
<i>in357myr</i> ⁻ (IV)	0	0	+/- ^h	+	+
<i>in374myr</i> ⁻ (III)	0	+/-	0	+	+
<i>in426myr</i> ⁻ (III)	0	+/-	0	+	+
<i>in438myr</i> ⁻ (III)	0	+/-	0	+	+
<i>in462myr</i> ⁻ (III)	0	+/-	0	+	+

^a Each of the 14 insertion mutants was analyzed in both N-myristylated and non-N-myristylated configurations. The roman numerals in parentheses correspond to the phenotypic groups for Gag particle assembly. Mutant patterns differing from the WT are indicated in boldface.

^b +, occurrence of budding Gag particles; 0, absence of particles visible under the EM. Quantification of extracellular release of Gag is shown in Fig. 3.

^c Symbols refer to the density of Gag particles per unit surface area of cytoplasm or nucleus on EM sections: +, $\geq 3/\mu\text{m}^2$; +/-, 0.1 to $1.0/\mu\text{m}^2$; 0, not detected (34).

^d In vivo processing by HIV-1 protease was assayed by the occurrence of the specific p25-p24 doublet, indicated by +, resulting from the cleavage at the p17-p24, p24-p15, and p25-p15 junctions. Aberrant proteolytic pattern is indicated by A.

^e Northwestern blotting analysis. +, WT binding pattern; 0, drastic decrease in the RNA binding capacity of Pr55^{Gag}, as shown in Fig. 5.

^f Premature cleavage at the p17-p24 junction and absence of further cleavage of the intermediate 39-kDa Gag product.

^g Gag particles of reduced diameter compared with WT Gag12myr⁺ (65 to 70 nm versus 120 to 130 nm).

^h Rare individualized Gag particles within or around amorphous inclusions.

ⁱ Indentations and fractures in rigid plasma membrane.

^j Occurrence of numerous Gag particles within cytoplasmic vesicles.

^k Clusters of Gag particles within cytoplasmic vesicles.

^l Gag particles budding into the perinuclear cisterna.

Our finding that mutations in the matrix domain could interfere with viral RNA binding was reminiscent of recent data showing an association between matrix protein and the HIV-1 RNA genome in vivo (5). The intracellular p17-RNA interactions, and the critical role of the N-myristylated p17 domain in the conformational structure and physiology of full-length Gag precursor, could explain in part the requirement for a functionally intact matrix protein in the retrovirus capsid morphogenetic process (32, 33, 45, 46).

In the capsid domain of Pr55^{Gag}, one particular region was found to be critical for the assembly process of Gag particles. Mutations spanning residues 209 to 334 in N-myristylated Pr55^{Gag} were found to be detrimental to the assembly and budding of membrane-enveloped Gag particles, and insertions located at positions 209 and 241 in non-N-myristylated Pr55^{Gag} negatively affected soluble Gag secretion. The same area has recently been shown to be critical for recognition and cleavage of Gag precursor by HIV-1 protease coexpressed in bacterial cells, probably via mutation-induced conformational modifications (16, 27). In addition, an insertion mutant at position 241 (R1509 [27]) has been found to be incapable of incorporating

Gag-Pol polyprotein within particles (31a), and a double-serine insertion at the *SpeI* site (which overlaps the *RsaI* site used in our *in241* insertion) resulted in assembly of noninfectious Gag particles (43). Recent studies using substitution mutants in Pr55^{Gag} have also shown that positions 268 and 322 (18), 341 to 346, and 350 to 352 (41) are essential for Gag assembly and particle formation. On the other hand, our results with mutants *in426myr*⁺, *in438myr*⁺, and *in462myr*⁺ suggested that the nucleocapsid domain of Pr55^{Gag} was tolerant to a variety of mutations, confirming previous data (1, 43). Taken together, these data indicated that one crucial region for HIV-1 Gag precursor interactions and self-assembly consisted of the C-terminal moiety of the capsid domain (1, 10, 36, 43, 44).

In simultaneous pairwise coinfections, three budding-defective mutants, *in40myr*⁺, *in120myr*⁺, and *in209myr*⁺, appeared to exert some dominant negative effect on Gag secretion over most of the other insertion mutants (Table 2). In delayed coinfection experiments with WT and mutant Gag recombinants (Fig. 5), a negative effect in *trans* on the extracellular release of WT Pr55^{Gag} was found for insertions in two separate

FIG. 5. Analysis of *trans*-dominant effects of insertion mutants on the secretion of WT Pr55^{Gag} by Gag12myr⁺-infected cells. Sf9 cells were preinfected with each Gag mutant 1 h prior to the addition of Gag12myr⁺ inoculum at the same MOI (15 PFU per cell). Total Gag secretion (particulate plus soluble Pr55^{Gag}) was expressed as the concentration of Pr55^{Gag} in the culture media. Standard deviation was 10 to 15% of the reported value ($n = 3$). Hatched columns, coinfections with N-myristylated mutants; filled columns, coinfections with non-N-myristylated mutants. The insertions are positioned on the linear sequence of the Gag precursor (amino acids 1 to 500), represented by the x axis. The two leftmost columns correspond to control experiments using single Gag12myr⁺-infected cells (hatched column) and cells preinfected with parental G2A (myr⁻) before Gag12myr⁺ (filled column).

regions of the Pr55^{Gag} sequence, the p17 MA (*in40*, *in100*, and *in120*) and the p7 NC (*in426* and *in438*) domains. Insertions within the blunt restriction sites at positions 209 (A-209-IAM-E) and 241 (S-241-IDA-T) in the HIV-1 *gag* sequence have been characterized as being two dominant mutations which negatively affected the cellular capacity to support HIV replication and release of WT virus (38). The result of our study with baculovirus-expressed Gag insertion mutants implied that at least for the insertion at position 209, the overall dominant effect on virus replication and release could in part involve some step in the capsid morphogenesis and budding of Gag particles from the plasma membrane. As suggested by our coinfection experiments, other regions within the matrix and nucleocapsid domains should be investigated for possible transdominant mutations with negative effect on self-assembly and extracellular release of Gag particles.

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