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Single Amino Acid Changes in the Human Immunodeficiency Virus Type 1 Matrix Protein Block Virus Particle Production

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The matrix protein of human immunodeficiency virus type 1 is encoded by the amino-terminal portion of the Gag precursor and is postulated to be involved in a variety of functions in the virus life cycle. To define domains and specific amino acid residues of the matrix protein that are involved in virus particle assembly, we introduced 35 amino acid substitution mutations in the human immunodeficiency virus type 1 matrix protein. Using reverse transcriptase and radioimmunoprecipitation analyses and transmission electron microscopy, we assessed the mutants for their ability to form virus particles and to function in the infection process. This study has identified several domains of the matrix protein in which single amino acid substitutions dramatically reduce the efficiency of virus particle production. These domains include the six amino-terminal residues of matrix, the region of matrix between amino acids 55 and 59, and the region between amino acids 84 and 95. Single amino acid substitutions in one of these domains (between matrix amino acids 84 and 88) result in a redirection of the majority of virus particle formation to sites within cytoplasmic vacuoles.

The Gag proteins of human immunodeficiency virus type 1 (HIV-1) and other retroviruses are sufficient for the formation of virus particles. A number of studies have demonstrated that, in the absence of other virus-encoded proteins, the expression of Gag is capable of directing the assembly and release of virus particles (9, 22, 25, 27, 32, 34, 37, 38, 55, 57, 60, 61, 64, 73). Retrovirus particle formation typically occurs at the plasma membrane in the type C oncoretroviruses and the lentiviruses or within the cytoplasm in the type B and D retroviruses and the spumaviruses (for reviews, see references 20, 21, and 72). The HIV-1 Gag proteins are synthesized as a polyprotein precursor ($Pr55^{Gag}$) which is proteolytically processed by the viral protease into the mature Gag proteins: matrix (MA or p17), capsid (CA or p24), nucleocapsid (NC or p7), and p6 (36).

Several lines of evidence suggest that domains within retroviral MA proteins are involved in proper virus particle assembly. Most retroviral MA proteins are modified at their amino termini by the addition of a myristic acid moiety, which serves as a membrane attachment site for MA or the MA domain of the unprocessed Gag precursor protein (for a review, see reference 58). Modification by myristic acid occurs at the Gly residue which is the N-terminal residue of Gag after removal of the initiator Met. Mutation of the N-terminal Gly residue, which prevents myristylation, results in defects in particle assembly or transport for a number of retroviruses. In HIV-1, spleen necrosis virus, and murine leukemia virus expressed in mammalian cells, mutation of the N-terminal Gly blocks particle assembly (3, 7, 23, 30, 39, 59, 68). In the type D retrovirus Mason-Pfizer monkey virus, mutation of the N-terminal Gly blocks the intracellular transport of assembled particles to the plasma membrane (50).

It appears that determinants in MA other than the myristic acid moiety also participate in retrovirus particle production. The Gag proteins of some retroviruses, including avian leukosis virus, equine infectious anemia virus, and visna virus, are not modified by myristic acid but are efficiently targeted to the plasma membrane (63). Moreover, amino acid substitutions in the Mason-Pfizer monkey virus MA protein have been reported to affect virus particle assembly, transport, and budding (53), and a single amino acid change in the Mason-Pfizer monkey virus MA protein resulted in virus particle assembly at the plasma membrane rather than within the cytoplasm (51).

Deletion and insertion mutagenesis of retroviral MA genes has not generated consistent results with respect to virus assembly. Small deletions in the MA proteins of murine leukemia virus (31), Mason-Pfizer monkey virus (52), and Rous sarcoma virus (74) dramatically reduced virus particle production. Similarly, several small deletions and insertions in the central portion of the HIV-1 MA led to substantial reductions in the production of extracellular virus in mutant-transfected cells (6, 66, 76), as did the deletion of HIV-1 MA amino acids 22 to 32 (77). In contrast, deletion of HIV-1 MA amino acids 21 to 31 was reported to enhance particle production (76). A very large deletion, spanning amino acids 16 to 99 of the HIV-1 MA, was reported to cause a 10-fold decrease in particle release from transfected cells and in a redirection of assembly to the endoplasmic reticulum (13). A larger (100-amino-acid) deletion in HIV-1 MA, however, had only a slight effect on particle release in a COS7 cell overexpression system (67). In view of these discordant results, and because the vast majority of reported MA deletion mutations rendered HIV noninfectious (31, 52, 76), we chose to take the more subtle approach of using point mutagenesis to identify domains in the HIV-1 MA that are involved in virus particle assembly and release.

Several functions in addition to an involvement in virus particle production have been proposed for the HIV-1 MA protein. These include roles in the interaction with the viral envelope glycoprotein (11, 65, 76), early steps in virus entry (75), nuclear localization of the preintegration complex after virus entry (5), and RNA binding (4).

Despite the apparent importance of MA in the HIV-1 life

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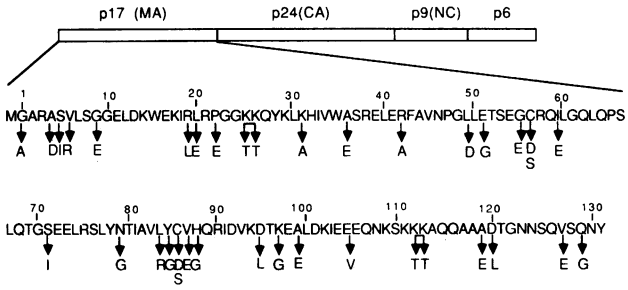


FIG. 1. Mutagenesis of the HIV-1 MA protein. The bars at the top represent the organization of the Gag precursor, $Pr55^{Gag}$. The predicted amino acid sequence of pNL4-3 MA is indicated, with amino acid positions shown above the sequence. Arrows indicate the changes made: single arrows represent single amino acid changes; double arrows (connected by bars) represent double amino acid changes.

cycle, little is known about the domains or specific amino acid residues involved in MA function. To identify and characterize domains of the HIV-1 MA that play a role in proper HIV-1 particle production, we introduced 35 amino acid substitutions throughout the protein. The mutants were analyzed for particle formation by reverse transcriptase (RT) and radioimmunoprecipitation analyses and by transmission electron microscopy. The mutants were also analyzed for their ability to establish a productive spreading infection in human T cells. The results of this study define specific amino acid residues of MA that are involved in the production of HIV-1 particles and identify a domain in which mutations redirect the majority of particle assembly to sites within the cytoplasm of virus-expressing cells.

Mutagenesis of the HIV-1 MA protein. To identify domains of the HIV-1 MA protein that are involved in proper MA function, we introduced 33 single amino acid substitutions and 2 double amino acid substitutions into the MA protein. The introduced changes are indicated in Fig. 1. A 1.6-kbp *Stu*I-*Sph*I fragment from the HIV-1 infectious molecular clone pNL4-3 (1), which includes the MA portion of the *gag* gene, was subcloned into M13mp19. Oligonucleotide-directed mutagenesis was performed as described previously (35). To prevent rapid reversion subsequent to transfection of T cells, we made all single amino acid substitutions by simultaneously changing two nucleotides. Following mutagenesis reactions, the mutagenized 0.7-kbp *Bss*HII-*Sph*I fragments were removed from the mutated M13 subclones and introduced back into pNL4-3. In each case, the entire *Bss*HII-*Sph*I fragment was sequenced to confirm the presence of the desired mutation and to determine that no undesired mutations had been introduced. Amino acid substitutions were introduced throughout the protein at highly conserved positions (41). Specific regions targeted for mutagenesis included the sequence at the N terminus of MA predicted to be required for myristylation (45, 58, 63), the highly basic regions between MA amino acids 17 and 32 and between residues 109 and 113, the residues surrounding and including the Cys at amino acids 56 and 86, and a sequence near the C terminus which bears homology to the picornavirus vp1 protein (2).

Effect of the MA mutations on virus replication in the CEM(12D-7) T-cell line. Following mutagenesis, we cloned the mutated MA genes into the full-length infectious HIV-1 molecular clone pNL4-3 (1). The mutant-containing proviral clones were then transfected, in parallel with wild-type pNL4-3, into the CEM(12D-7) T-cell line (54). CEM(12D-7) cells were transfected as follows by using the DEAE-dextran method (62). Five million cells were pelleted by centrifugation in a Sorvall RT6000B centrifuge for 5 min at 1,200 rpm. The cells were resuspended in 0.5 ml of DEAE-dextran (700 μg of DEAE-dextran per ml) in transfection buffer (25 mM Tris-HCl [pH 7.4], 0.6 mM Na_2HPO_4, 5 mM KCl, 140 mM NaCl, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$), and 5 μg of plasmid DNA was added per tube. DEAE-dextran was obtained from Pharmacia (molecular weight 500,000; catalog no. 17-0350-01). After a 3-h incubation at 37°C, the cells were washed once in 4 ml of warm transfection buffer and plated in 25-cm^2 flasks containing 6 ml of RPMI growth medium. Transfected CEM(12D-7) cells were split 1:3 every 2 days. Aliquots were reserved for RT assays prior to splitting. Production of RT activity in the supernatant of transfected cells was monitored over time to detect the presence of a spreading viral infection. RT assays were performed as described previously (71) with the addition of 1 mM EDTA to the RT cocktail.

The first set of mutations was introduced into the region of MA predicted to be involved in proper N-myristylation (8, 58, 63). As shown in Fig. 2A, mutation of the N-terminal Gly residue (1GA) blocked HIV-1 particle production in transfected cultures. Mutation of MA amino acid 5 (5SI) also rendered the virus unable to establish a productive infection (Fig. 2A). No virus replication was detected in 1GA- or 5SI-transfected cultures even after 3 months of continual passage (Fig. 2A; data not shown). Mutation at MA amino residues 4 (4AD) and 6 (6VR) resulted in a 10-day delay, relative to the wild type, in peak RT production (Fig. 2A). The 9GE mutant replicated with only a slight delay (2 days) relative to the wild type (Fig. 2A).

Several roles have been proposed for the highly basic stretch between HIV-1 MA amino acids 17 and 31 (Fig. 1). Wachinger et al. suggested that this region may be involved in the association between MA and the HIV-1 transmembrane envelope glycoprotein gp41 (65). Since deletions and mutations between amino acids 22 and 32 inhibited virus production and proper association of Gag with the plasma membrane, it was proposed that this domain plays a role in targeting HIV-1 Gag to the plasma membrane (77, 78). Gheysen et al. (22) and Delchambre et al. (9) proposed that the highly basic N-terminal sequence might function as a nuclear localization signal, and Bukrinsky et al. postulated that this putative karyophilic sequence serves as a nuclear localization signal for the HIV-1 preintegration complex and is thus essential for productive HIV-1 infection of nondividing cells (5). However, with the same mutation used by Bukrinsky et al. (which we term 25KT/26KT), we demonstrated that the two Lys residues at MA positions 25 and 26 are not required for growth of HIV-1 in primary human monocyte-derived macrophages (15). This result suggests that the pair of Lys residues at MA amino acids 25 and 26 are critical to nuclear localization only in certain cell or virus systems. To examine the effects of mutations in this domain on infection of T cells, we introduced a series of mutations in this highly basic region. Single amino acid substitutions at residues 19 (19RL) and 22 (22PE) had no effect on infectivity in CEM(12D-7) cells, whereas mutation at amino acid 20 (20LE) resulted in a modest and consistent reduction in peak virus production. The double amino acid substitution at residues 25 and 26 (25KT/26KT) and the single amino acid change at residue 31 (31KA) resulted in a reproducible 2-day delay in peak virus production relative to the wild type (data not shown). We are currently investigating further the potential role(s) of this highly basic stretch in HIV-1 MA function during virus particle production and infection of monocyte-derived macrophages.

Mutations between MA amino acids 30 and 50 had a range

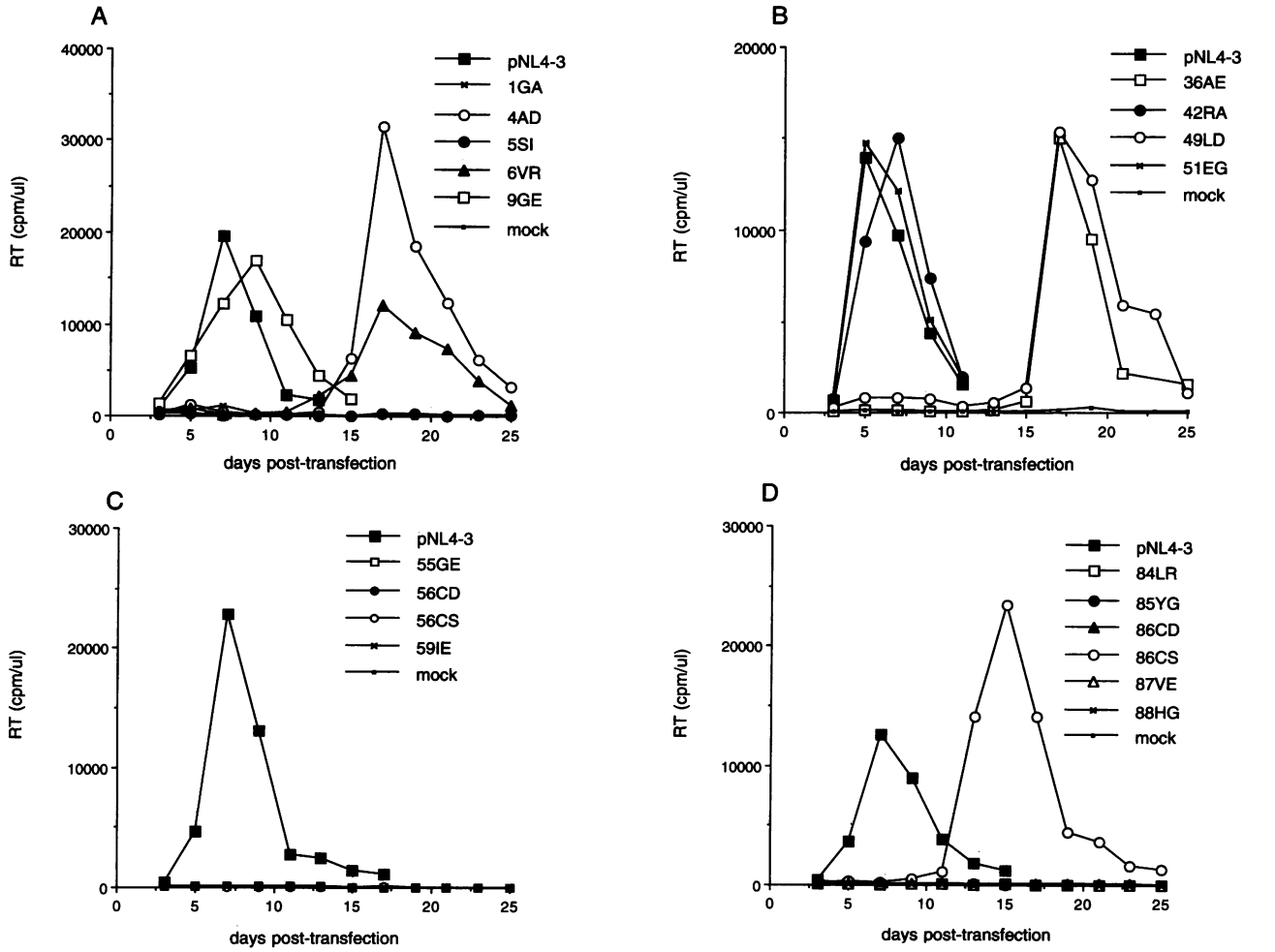


FIG. 2. Replication kinetics of MA mutants in the CEM(12D-7) T-cell line. CEM(12D-7) cells were transfected as described in the text. Cells were split 1:3 every 2 days, and supernatant RT activity was monitored at each time point.

of effects: the 36AE and 49LD mutations caused a 12-day lag (relative to pNL4-3) to peak RT production, whereas the 51EG mutation had no effect on virus replication kinetics in the CEM(12D-7) cell line (Fig. 2B). The 42RA mutation resulted in a 2-day lag in peak RT production. A series of mutations in the vicinity of the first Cys residue of MA (55GE, 56CD, 56CS, and 59IE) resulted in a complete absence of virus replication (Fig. 2C), even after 3 months of continual passage of the transfected cultures (data not shown). Substitutions at amino acids 71 (71SI) and 79 (79NG) had no effect on the establishment of a productive infection in CEM(12D-7) cells (data not shown).

The next group of mutations was introduced in the vicinity of the second Cys residue of MA. The 84LR, 85YG, 86CD, 87VE, and 88HG mutations completely blocked virus replication in the CEM(12D-7) cell line even after 3 months of continual passage of the transfected cultures (Fig. 2D; data not shown). A more conservative Cys→Ser mutation at residue 86 (86CS) resulted in a 1-week lag (relative to wild-type pNL4-3) to peak RT production (Fig. 2D). Mutations at amino acids 95 and 99 completely blocked virus replication, whereas single amino acid changes at residues 97 and 105 had no significant effect on the kinetics of virus replication (data not shown). A double amino acid substitution in the highly basic domain between MA amino acids 109 and 113 had no effect on the establishment of a productive infection (data not shown).

It has been previously reported that the region of HIV-1 MA between amino acids 118 and 127 bears homology to the picornavirus protein vp1 and the flavivirus E protein (2). This

TABLE 1. Infectivity of MA mutants in the CEM(12D-7) T-cell line

Mutant	Progeny virus production[a]	Mutant	Progeny virus production[a]
Wild type	+	59IE	−
1GA	−	71SI	+
4AD	D	79NG	+
5SI	−	84LR	−
6VR	D	85YG	−
9GE	+	86CD	−
19RL	+	86CS	D
20LE	+	87VE	−
22PE	+	88HG	−
25KT/26KT	+	95DL	−
31KA	+	97KG	+
36AE	D	99AE	−
42RA	+	105EV	+
49LD	D	112KT/113KT	+
51EG	+	119AE	+
55GE	−	120DL	+
56CD	−	127VE	+
56CS	−	129QG	+

[a] +, significant virus production at the peak RT activity of the wild type; D, delayed infection kinetics relative to the wild type; −, no detectable virus production by RT.

region of vp1 is thought to play a role in picornavirus entry (18), and deletion of this sequence from HIV-1 MA was reported to impair virus entry (75). To test whether single amino acid changes in the most highly conserved residues of this sequence (2) affected virus replication, we introduced nonconservative substitutions at positions 119 (119AE), 120 (120DL), and 127 (127VE). These mutations either had no effect on replication kinetics (127VE, 119AE) or modestly reduced peak RT production (120DL) (Fig. 2E; data not shown). We also introduced a mutation at residue 129; this mutation resulted in a slight reduction in peak virus production (Fig. 2E).

The cumulative results from the analysis of virus replication identify several domains in MA that are critical for the establishment of a productive infection in CEM(12D-7) cells. A summary of these data is presented in Table 1. It is possible that reductions in the levels of virion-associated gp120, which have been observed for other MA mutations (11, 76), affect the infectivity of some of our MA mutants. This issue is currently under examination.

Several mutants (4AD, 6VR, 36AE, 49LD, and 86CS) initially did not produce detectable virus in cultures of transfected CEM(12D-7) cells, but, after a lag of greater than 1 week relative to the wild type, significant levels of virus production were observed (Fig. 2). Our preliminary results suggest that the virus replication detected in these cultures is due to the emergence of revertant viruses (28).

Effect of the MA mutations on virus particle production. To examine whether the altered infectivity of a number of the MA mutants (Fig. 2) might be due to an effect on virus particle production, we transfected HeLa cells in parallel with wild-type or MA mutant-containing proviral clones and assayed for particle-associated RT activity in the transfected-cell medium. HeLa cells were transfected by the calcium phosphate precipitation method as described previously (17). To obtain virus particles for RT analysis, we pelleted 100 μl of transfected-cell medium for 1 h at 14,000 rpm in a Tomy high-speed microcentrifuge. The supernatant was removed and replaced with 100 μl of cold phosphate-buffered saline (PBS), and the tubes were centrifuged again for 1 h at 14,000 rpm. The supernatant from this step was removed, and the virus pellet was resuspended in 100 μl of PBS. The efficient pelleting of HIV-1 from small volumes (200 μl or less) of virus suspensions in a microcentrifuge has been described previously (16, 70). The results of this analysis are presented in Fig. 3. As expected from earlier work (3, 23, 43), mutation of the myristylated N-terminal Gly resulted in essentially a complete loss of virus particle production. Mutations in residues 4, 5, and 6, predicted to lie within the *N*-myristoyl transferase recognition sequence (45, 63), also substantially diminished the amount of particle-associated RT activity present in the medium of transfected cells. Mutation of the more distally located residue 9 had no effect on virus particle production.

Mutations in the first highly basic stretch of MA (residues 17 to 31) either had no significant effect on the release of particle-associated RT activity from transfected HeLa cells (19RL and 22PE) or displayed a 25% (20LE and 31KA) to 50% (25KT/26KT) reduction in particle production. The 36AE mutation led to a fivefold reduction in particle-associated RT activity, whereas mutations at residues 42, 49, and 51 each resulted in essentially wild-type activity for particle assembly and release.

The mutations at MA amino acids 55, 56, and 59, which blocked virus replication in the CEM(12D-7) cell line (Fig. 2), profoundly reduced the amount of particle-associated RT activity in the supernatant of transfected HeLa cells. The magnitude of this effect was greater than 10-fold. The 71SI and 79NG mutations, which did not affect infectivity, had no effect on virus particle assembly and release. Mutations at residues 84, 85, 86, 87, 88, 95, and 99 resulted in significant decreases in virus production. The 86CD mutation had a more profound effect on particle assembly and release than did the more subtle 86CS mutation. Mutations at residues 97, 105, 112/113, 119, 120, 127, and 129, which had no substantial effect on virus infectivity in the CEM(12D-7) cell line, did not significantly affect virus particle assembly and release in transfected HeLa cells.

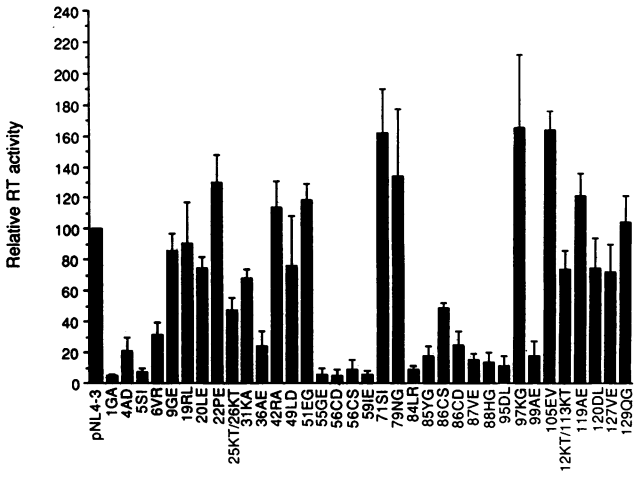


FIG. 3. Release of particle-associated RT activity from HeLa cells transfected with MA mutants. HeLa cells were transfected in parallel with wild-type or MA mutant-containing proviral clones. At 2 days posttransfection, the HIV-1 virions were pelleted from the transfected-cell medium and RT analysis was performed on the viral pellets. The data represent the means of at least three assays; the error bars represent the standard deviations.

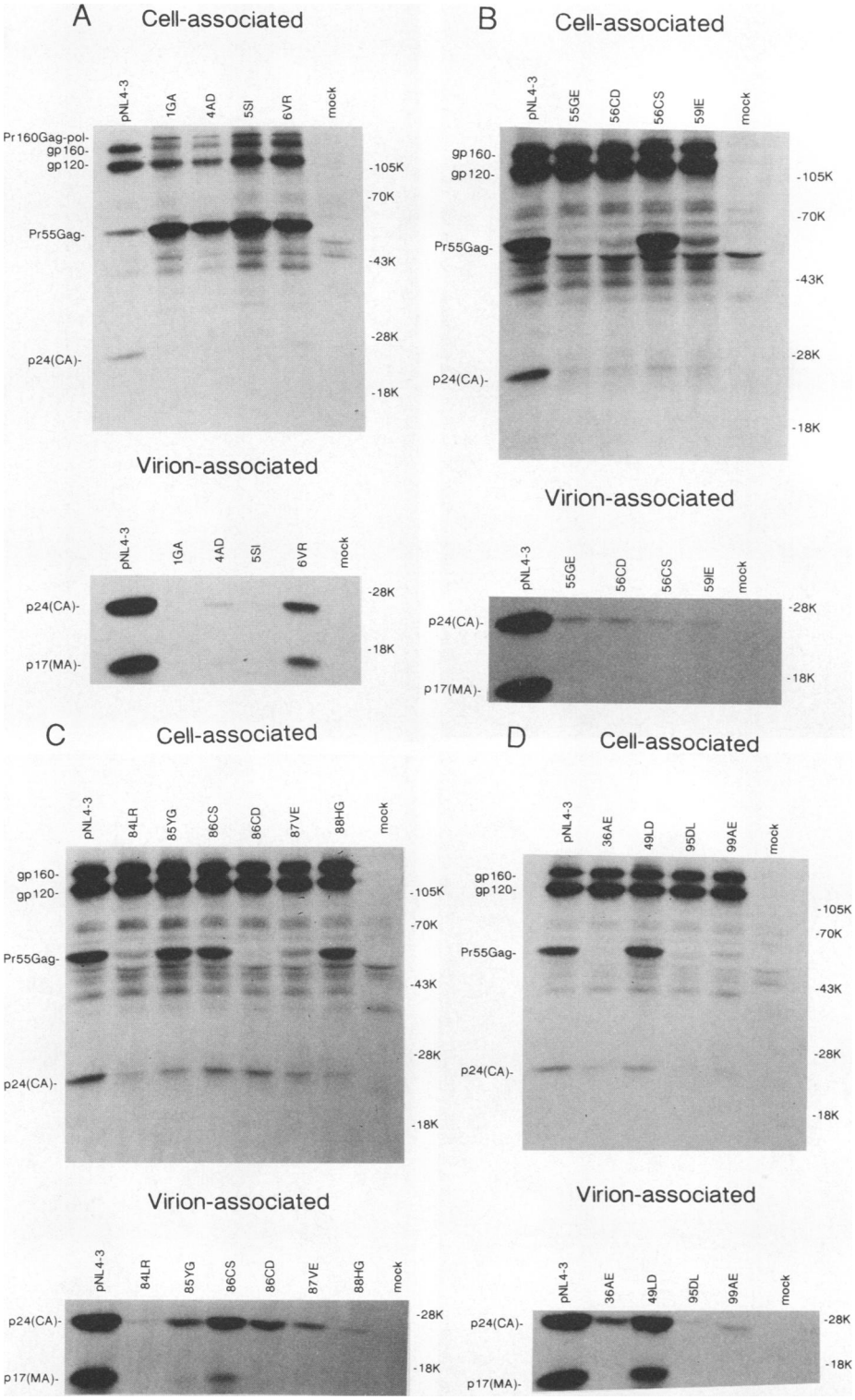
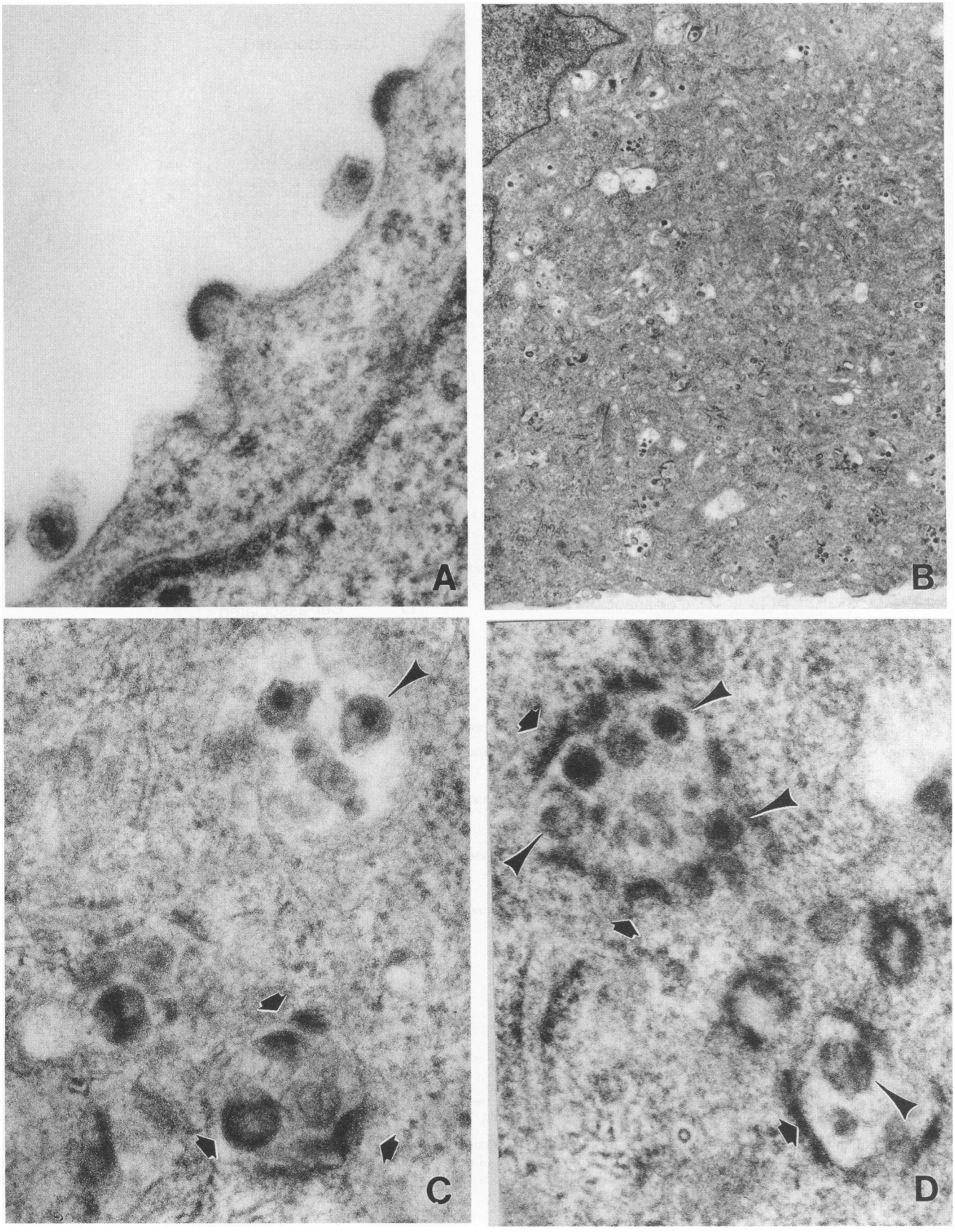


FIG. 4. Radioimmunoprecipitation analysis of MA mutant expression. HeLa cells were transfected in parallel with wild-type or MA mutant-containing proviral clones. Transfected-cell cultures were metabolically labeled with [^{35}S]Cys; cell-associated and virion-associated proteins were immunoprecipitated with sera from AIDS patients. The positions of virus-encoded proteins are shown on the left; the positions of the molecular weight standards (in thousands) are shown on the right.


A
B
C
D

Cell- and virion-associated expression of mutant Gag proteins. The possibility existed that the absence of particle-associated RT activity observed with some of the MA mutants (Fig. 3) was due not to a decrease in particle release but to a defect in the enzymatic activity of RT in mutant virus particles. We therefore decided that it was necessary to determine the effects of the MA mutations on Gag expression and processing within mutant-expressing cells, as well as the amount of particle-associated Gag proteins produced by the various MA mutants (Fig. 4). HeLa cells were transfected in parallel with wild-type pNL4-3 or mutant-containing derivatives and metabolically labeled with [^{35}S]Cys. Labeled virus was prepared by filtering supernatant through a 0.45-μm-pore-size filter to remove unattached cells and cell debris and pelleting the virus in a Tomy microcentrifuge as described above. Metabolic labeling with [^{35}S]Cys was performed as described previously (16). Virus pellets and metabolically labeled cells were lysed and immunoprecipitated with sera from AIDS patients as described previously (69). The sera were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (human HIV immune globulin; catalog no. 192).

Mutation of the N-terminal Gly, which blocks myristylation, impairs the processing of both Pr160$^{Gag\text{-}Pol}$ and Pr55^{Gag} (Fig. 4A). As a result, both species accumulate intracellularly and a reduction in the level of cell-associated p24 is observed. This increase in Gag precursor stability has also been reported by others for retroviral myristylation mutants (3, 23, 59, 68). The mutations at residues 4, 5, and 6, which impair virus particle production, also result in increased levels of Pr160$^{Gag\text{-}Pol}$ and Pr55^{Gag}. The similarity in the pattern of Gag processing observed with mutations in the six amino-terminal residues of MA suggests that these mutants are at least partially blocked for myristylation. This result is consistent with previous reports demonstrating that proper N-myristylation involves the recognition of residues at the N-terminal end of myristylated proteins by *N*-myristyl transferase (63). It is also worth noting that the 1GA mutant, which is presumably incapable of being modified by myristic acid, is not completely blocked for Gag precursor processing; some p24(CA) production is observed after a longer exposure of the gel in Fig. 4A (data not shown). This observation indicates that HIV-1 Gag precursor processing is not absolutely dependent on particle formation or membrane association, in agreement with results of previous studies (23, 33, 39, 44).

Mutations at MA residues 55, 56, and 59, which dramatically reduce virus particle production, also result in decreased synthesis of cell-associated p24 (Fig. 4B). Interestingly, some of these mutants (55GE, 56CD, and 59IE) also express less cell-associated Pr55^{Gag}, whereas the 56CS-transfected cells contain wild-type levels of Pr55^{Gag}. Pulse-chase analysis of the 55GE mutant, in parallel with the wild type, indicates that the half-life of 55GE Pr55^{Gag} is significantly reduced relative to that of the wild type and that the antiserum used is capable of efficiently recognizing the mutant Pr55^{Gag} (14). A similar reduction in Gag precursor half-life was observed previously for a number of retrovirus MA deletion and insertion mutants (6, 31, 76).

Mutations between MA amino acids 84 and 88 (Fig. 4C) and at residues 36, 49, 95, and 99 (Fig. 4D) also result in reduced amounts of cell-associated p24. In some cases (mutants 36AE, 84LR, 86CD, 87VE, 95DL, and 99AE) reductions in the level of Pr55^{Gag} are also observed, whereas in other cases (85YG, 86CS, and 88HG) the reduction in cell-associated p24 production is not accompanied by reduced levels of Pr55^{Gag}.

To examine virus particle release directly, we immunoprecipitated the pelleted, particle-associated material released into the medium of transfected, metabolically- labeled cells as described above. The results of this analysis are presented in the "virion-associated" panels in Fig. 4. Virion proteins were quantified by using a Fujix Bioimage phosphoimager. The amount of cell-associated gp120, which is not influenced by the MA mutations, provides an internal control for transfection efficiency. The results of this analysis (data not shown) agree closely with those obtained by analyzing particle-associated RT activity (Fig. 3); the majority of mutations that result in a dramatic impairment in the release of virion-associated proteins are located at the N terminus of MA and between amino acids 55 and 59 and between amino acids 84 and 95.

Ultrastructural analysis of mutant particle formation by electron microscopy. Several explanations could account for the dramatic reduction in the amount of virus particle-associated material released into the medium of cells transfected with MA mutants. The mutations could (i) prevent particle assembly, (ii) redirect particle assembly to sites within the cytoplasm, or (iii) block the release of virus particles from the plasma membrane. Each of these effects has been previously reported for mutations in retroviral Gag proteins (3, 23, 24, 43, 51, 53, 59, 68, 77). The experiments described above measure steady-state levels of Gag synthesis and particle release and, as a consequence, do not allow us to distinguish among these various alternatives. We therefore examined, by electron microscopy, cells expressing MA mutants which displayed significant reductions in virus particle production. HeLa cells were plated at 3.5×10^5 cells per well in six-well tissue culture dishes and transfected with 10 μg of DNA as described above. At 2 days posttransfection, the medium was removed, the cells were washed once with cold PBS, and 3 ml of a 2.5% solution of glutaraldehyde in PBS was added to each well. Glutaraldehyde was obtained as a 25% solution (no. G5882; Sigma). The cells were allowed to fix at 4°C at least overnight before being gently scraped from the dishes with the tip of a wide-mouth, soft-plastic, dropper-type pipette. The cell suspension was processed into Spurr's epoxy after being hardened into agar. During alcohol dehydration, the cells were block uranyl acetate stained. Sections (600 Å [60 nm]) were cut with a diamond knife, stained with uranyl acetate and lead citrate, and viewed in a Zeiss transmission electron microscope operating at 60 kV.

As expected, high levels of virus particles associated with and budding from the plasma membrane were visualized in cells transfected with wild-type pNL4-3 (Fig. 5A). Consistent

FIG. 5. Transmission electron microscopy of transfected cells. (A) A portion of the plasma membrane of a pNL4-3-transfected cell shows budding and mature HIV-1 virions. Magnification, ×110,000. (B) The perinuclear Golgi region of an 87VE-transfected cell is rich in vacuoles containing numerous darkly staining aberrant virus particles. The nucleus is in the upper left of the field. Magnification, ×10,000. (C) High magnification of a small portion of the Golgi region of the 87VE-transfected cell in panel B shows the presence of particles (long arrowheads). The electron-dense structures in the lower vacuole represent virions in various stages of budding (short arrows). Magnification, ×100,000. (D) High magnification of a small portion of the Golgi region of an 85YG-transfected cell shows that portions of the membranes of both vacuoles have electron-dense areas consistent with early-budding virions (short arrows). Immature and mature virus particles are evident (long arrowheads). Magnification, ×114,000.

with previous reports (3, 23, 43), mutation of the N-terminal Gly residue (the site of N-myristylation) resulted in completely abrogated particle assembly as monitored by electron microscopy (data not shown). Mutations in the vicinity of the N-terminal Gly, which would also be predicted to adversely affect myristylation (45, 46), also severely impaired particle assembly (data not shown). Mutations at MA amino acids 55, 56, and 59 also largely blocked particle assembly (data not shown).

Interestingly, and in contrast to mutations at the N- terminus or between residues 55 and 59, mutations between MA amino acids 84 and 88 resulted in disproportionately high levels of particle assembly within cytoplasmic vacuoles of transfected cells (Fig. 5B to D). These vacuoles appear to be Golgi elements (42). Many of these particles were in the process of budding, and both budding and mature particles exhibited a highly aberrant morphology. Relatively little assembly was evident at the plasma membrane with these mutants. These results suggest that the region of HIV-1 MA which includes amino acid residues 84 to 88 may play a role in determining the site of particle assembly within virus-expressing cells.

Conclusions. This study identifies domains and specific amino acid residues of HIV-1 MA that are involved in virus particle production. Several explanations could account for the failure of these MA mutants to properly produce virus particles. (i) The mutations could result in a retargeting of $Pr55^{Gag}$. This could occur either directly as a result of the removal or alteration of MA-encoded targeting signals or indirectly from the failure of MA mutants to associate with cellular carrier or chaperone proteins involved in proper Gag transport and targeting (12, 26, 29, 56, 72). (ii) The MA mutations could fail to associate with an as yet unidentified membrane receptor for Gag. The identification of such a receptor has been reported for another myristylated protein, $p60^{src}$ (49). (iii) The mutations could affect interactions between MA proteins or between MA and other Gag proteins. The results of studies involving chimeras between HIV-1 and murine leukemia virus Gag proteins and of cross-linking analyses performed with avian and murine retroviruses suggest that MA may mediate Gag protein interactions during the assembly of virus particles (10, 19, 40, 47, 48). Future studies will further elucidate the role of MA in HIV-1 Gag trafficking and virus particle formation.

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REFERENCES

1. **Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin.** 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. **59:**284–291.
2. **Blomberg, J., and P. Medstrand.** 1990. A sequence in the carboxyl terminus of the HIV-1 matrix protein is highly similar to sequences in membrane-associated proteins of other RNA viruses: possible functional implications. New Biol. **2:**1044–1046.
3. **Bryant, M., and L. Ratner.** 1990. Myristoylation-dependent replication and assembly of human immunodeficiency virus 1. Proc. Natl. Acad. Sci. USA **87:**523–527.
4. **Bukrinskaya, A. G., G. K. Vorkunova, and Y. Y. Tentsov.** 1992. HIV-1 matrix protein p17 resides in cell nuclei in association with genomic RNA. AIDS Res. Hum. Retroviruses **8:**1795–1801.
5. **Bukrinsky, M. I., S. Haggerty, M. P. Dempsey, N. Sharova, A. Adzhubei, L. Spitz, P. Lewis, D. Goldfarb, M. Emerman, and M. Stevenson.** 1993. A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. Nature (London) **365:**666–669.
6. **Chazal, N., C. Carriere, B. Gay, and P. Boulanger.** 1994. Phenotypic characterization of insertion mutants of the human immunodeficiency virus type 1 Gag precursor expressed in recombinant baculovirus-infected cells. J. Virol. **68:**111–122.
7. **Copeland, N. G., N. A. Jenkins, B. Nexo, A. M. Schultz, A. Rein, T. Mikkelsen, and P. Jorgensen.** 1988. Poorly expressed endogenous ecotropic provirus of DBA/2 mice encodes a mutant $Pr65^{gag}$ protein that is not myristylated. J. Virol. **62:**479–487.
8. **Cross, F. R., E. A. Garber, D. Pellman, and H. Hanafusa.** 1984. A short sequence in the $p60^{src}$ N terminus is required for $p60^{src}$ myristylation and membrane association and for cell transformation. Mol. Cell. Biol. **4:**1834–1842.
9. **Delchambre, M., D. Gheysen, D. Thines, C. Thiriart, E. Jacobs, E. Verdin, M. Horth, A. Burny, and F. Bex.** 1989. The Gag precursor of simian immunodeficiency virus assembles into virus-like particles. EMBO J. **8:**2653–2660.
10. **Deminie, C. A., and M. Emerman.** 1993. Incorporation of human immunodeficiency virus type 1 Gag proteins into murine leukemia virus virions. J. Virol. **67:**6499–6506.
11. **Dorfman, T., F. Mammano, W. A. Haseltine, and H. G. Gottlinger.** 1994. Role of the matrix protein in the virion association of the human immunodeficiency virus type 1 envelope glycoprotein. J. Virol. **68:**1689–1696.
12. **Edbauer, C. A., and R. B. Naso.** 1984. Cytoskeleton-associated Pr65gag and assembly of retrovirus temperature-sensitive mutants in chronically infected cells. Virology **134:**389–397.
13. **Facke, M., A. Janetzko, R. L. Shoeman, and H.-G. Krausslich.** 1993. A large deletion in the matrix domain of the human immunodeficiency virus *gag* gene redirects virus particle assembly from the plasma membrane to the endoplasmic reticulum. J. Virol. **67:**4972–4980.
14. **Freed, E. O., and M. A. Martin.** Unpublished results.
15. **Freed, E. O., and M. A. Martin.** 1994. HIV-1 infection of non-dividing cells. Nature (London) **369:**107–108.
16. **Freed, E. O., and M. A. Martin.** 1994. Evidence for a functional interaction between the V1/V2 and C4 domains of human immunodeficiency virus type 1 envelope glycoprotein gp120. J. Virol. **68:**2503–2512.
17. **Freed, E. O., and R. Risser.** 1987. The role of envelope glycoprotein processing in murine leukemia virus infection. J. Virol. **61:**2852–2856.
18. **Fricks, C. E., and J. M. Hogle.** 1990. Cell-induced conformational change in poliovirus: externalization of the amino terminus of VP1 is responsible for liposome binding. J. Virol. **64:**1934–1945.
19. **Gebhardt, A., J. V. Bosch, A. Ziemiecki, and R. R. Friis.** 1984. Rous sarcoma virus p19 and gp35 can be chemically crosslinked to high molecular weight complexes. An insight into virus assembly. J. Mol. Biol. **174:**297–317.
20. **Gelderblom, H. R.** 1991. Assembly and morphology of HIV: potential effect of structure on viral function. AIDS **5:**617–638.
21. **Gelderblom, H. R., M. Ozel, and G. Pauli.** 1989. Morphogenesis and morphology of HIV. Structure-function relations. Arch. Virol. **106:**1–13.
22. **Gheysen, D., E. Jacobs, F. de Foresta, C. Thiriart, M. Francotte, D. Thines, and M. De Wilde.** 1989. Assembly and release of HIV-1 precursor Pr55gag virus-like particles from recombinant baculovirus-infected insect cells. Cell **59:**103–112.
23. **Gottlinger, H. G., T. Dorfman, J. G. Sodroski, and W. A. Haseltine.** 1989. Role of capsid precursor processing and myristylation in morphogenesis and infectivity of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **86:**5781–5785.
24. **Gottlinger, H. G., T. Dorfman, J. G. Sodroski, and W. A. Haseltine.** 1991. Effect of mutations affecting the p6 gag protein on human immunodeficiency virus particle release. Proc. Natl. Acad. Sci. USA **88:**3195–3199.
25. **Haffar, O., J. Garrigues, B. Travis, P. Moran, J. Zarling, and S.-L. Hu.** 1990. Human immunodeficiency virus-like, nonreplicating, *gag-env* particles assemble in a recombinant vaccinia virus expression system. J. Virol. **64:**2653–2659.
26. **Hansen, M., L. Jelinek, S. Whiting, and E. Barklis.** 1990. Transport and assembly of *gag* proteins into Moloney murine leukemia virus. J. Virol. **64:**5306–5316.
27. **Hu, S.-L., B. M. Travis, J. Garrigues, J. M. Zarling, P. Sridhar, T.**

Dykers, J. W. Eichberg, and C. Alpers. 1990. Processing, assembly, and immunogenicity of human immunodeficiency virus core antigens expressed by recombinant vaccinia virus. Virology **179:**321–329.

28. **Huang, M., M. A. Martin, and E. O. Freed.** Unpublished results.
29. **Jones, T. A., G. Blaug, M. Hansen, and E. Barklis.** 1990. Assembly of *gag*-β-galactosidase proteins into retrovirus particles. J. Virol. **64:**2265–2279.
30. **Jorgensen, E. C., N. O. Kjeldgaard, F. S. Pedersen, and P. Jorgensen.** 1988. A nucleotide substitution in the *gag* N terminus of the endogenous ecotropic DBA/2 virus prevents $Pr65^{gag}$ myristylation and virus replication. J. Virol. **62:**3217–3223.
31. **Jorgensen, E. C. B., F. S. Pedersen, and P. Jorgensen.** 1992. Matrix protein of Akv murine leukemia virus: genetic mapping of regions essential for particle formation. J. Virol. **66:**4479–4487.
32. **Jowett, J. B. M., D. J. Hockley, M. V. Nermut, and I. M. Jones.** 1992. Distinct signals in human immunodeficiency virus type 1 Pr55 necessary for RNA binding and particle formation. J. Gen. Virol. **73:**3079–3086.
33. **Kaplan, A. H., and R. Swanstrom.** 1991. Human immunodeficiency virus type 1 Gag proteins are processed in two cellular compartments. Proc. Natl. Acad. Sci. USA **88:**4528–4532.
34. **Karacostas, V., K. Nagashima, M. A. Gonda, and B. Moss.** 1989. Human immunodeficiency virus-like particles produced by a vaccinia virus expression vector. Proc. Natl. Acad. Sci. USA **86:**8964–8967.
35. **Kunkle, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154:**367–382.
36. **Leis, J., D. Baltimore, J. M. Bishop, J. Coffin, E. Fleissner, S. P. Goff, S. Oroszlan, H. Robinson, A. M. Skalka, H. M. Temin, and V. Vogt.** 1988. Standardized and simplified nomenclature for proteins common to all retroviruses. J. Virol. **62:**1808–1809.
37. **Luo, L., Y. Li, and C. Y. Kang.** 1990. Expression of gag precursor proteins and secretion of virus-like gag particles of HIV-2 from recombinant baculovirus-infected insect cells. Virology **179:**874–880.
38. **Madisen, L., B. Travis, S.-L. Hu, and A. F. Purchio.** 1987. Expression of the human immunodeficiency virus gag gene in insect cells. Virology **158:**248–250.
39. **Mergener, K., M. Facke, R. Welker, V. Brinkmann, H. R. Gelderblom, and H.-G. Krausslich.** 1992. Analysis of HIV particle formation using transient expression of subviral constructs in mammalian cells. Virology **186:**25–39.
40. **Montelaro, R. C., S. J. Sullivan, and D. P. Bolognesi.** 1978. An analysis of type-C retrovirus polypeptides and their associations in the virion. Virology **84:**19–31.
41. **Myers, G., S. Wain-Hobson, B. Korber, R. F. Smith, and G. N. Pavlakis (ed.).** 1993. Human retroviruses and AIDS. A compilation and analysis of nucleic acid and amino acid sequences. Los Alamos National Laboratory, Los Alamos, N.Mex.
42. **Orenstein, J. M., M. S. Meltzer, T. Phipps, and H. E. Gendelman.** 1988. Cytoplasmic assembly and accumulation of human immunodeficiency virus types 1 and 2 in recombinant human colony-stimulating factor 1-treated human monocytes: an ultrastructural study. J. Virol. **62:**2578–2586.
43. **Pal, R., M. S. Reitz, Jr., E. Tschachler, R. C. Gallo, M. G. Sarngadharan, and F. D. M. Veronese.** 1990. Myristylation of gag proteins of HIV-1 plays an important role in virus assembly. AIDS Res. Hum. Retroviruses **6:**721–730.
44. **Park, J., and C. D. Morrow.** 1991. Overexpression of the *gag-pol* precursor from human immunodeficiency virus type 1 proviral genomes results in efficient proteolytic processing in the absence of virion production. J. Virol. **65:**5111–5117.
45. **Pellman, D., E. A. Garber, F. R. Cross, and H. Hanafusa.** 1985. Fine structure mapping of a critical NH_2-terminal region of p60src. Proc. Natl. Acad. Sci. USA **82:**1623–1627.
46. **Pellman, D., E. A. Garber, F. R. Cross, and H. Hanafusa.** 1985. An N-terminal peptide from p60src can direct myristylation and plasma membrane localization when fused to heterologous proteins. Nature (London) **314:**374–377.
47. **Pepinsky, R. B., D. Cappiello, C. Wilcowski, and V. M. Vogt.** 1980. Chemical crosslinking of proteins in avian sarcoma and leukemia viruses. Virology **102:**205–210.
48. **Pepinsky, R. B., and V. M. Vogt.** 1984. Fine-structure analyses of lipid-protein and protein-protein interactions of *gag* protein by p19 of the avian sarcoma and leukemia viruses by cyanogen bromide mapping. J. Virol. **52:**145–153.
49. **Resh, M. D., and H.-P. Ling.** 1990. Identification of a 32K plasma membrane protein that binds to the myristylated amino-terminal sequence of $p60^{v\text{-}src}$. Nature (London) **346:**84–86.
50. **Rhee, S. R., and E. Hunter.** 1987. Myristylation is required for intracellular transport but not for assembly of D-type retrovirus capsids. J. Virol. **61:**1045–1053.
51. **Rhee, S. R., and E. Hunter.** 1990. A single amino acid substitution within the matrix protein of a type D retrovirus converts its morphogenesis to that of a type C retrovirus. Cell **63:**77–86.
52. **Rhee, S. R., and E. Hunter.** 1990. Structural role of the matrix protein of type D retroviruses in Gag polyprotein stability and capsid assembly. J. Virol. **64:**909–917.
53. **Rhee, S. R., and E. Hunter.** 1991. Amino acid substitutions within the matrix protein of type D retroviruses affect assembly, transport, and membrane association of a capsid. EMBO J. **10:**535–546.
54. **Ross, E. K., A. J. Buckler-White, A. B. Rabson, G. Englund, and M. A. Martin.** 1991. Contribution of NF-κB and Sp1 binding motifs to the replicative capacity of human immunodeficiency virus type 1: distinct patterns of viral growth are determined by T-cell types. J. Virol. **65:**4350–4358.
55. **Ross, E. K., T. R. Fuerst, J. M. Orenstein, T. O'Neill, M. A. Martin, and S. Venkatesan.** 1991. Maturation of human immunodeficiency virus particles assembled from the gag precursor protein requires in situ precessing by gag-pol protease. AIDS Res. Hum. Retroviruses **7:**475–483.
56. **Rothman, J. E.** 1989. Polypeptide chain binding proteins: catalysts of protein folding and related processes in cells. Cell **59:**591–601.
57. **Royer, M., S.-S. Hong, B. Gay, M. Cerutti, and P. Boulanger.** 1992. Expression and extracellular release of human immunodeficiency virus type 1 Gag precursor proteins by recombinant baculovirus-infected cells. J. Virol. **66:**3230–3235.
58. **Schultz, A. M., L. E. Henderson, and S. Oroszlan.** 1988. Fatty acylation of proteins. Annu. Rev. Cell Biol. **4:**611–647.
59. **Schultz, A. M., and A. Rein.** 1989. Unmyristylated Moloney murine leukemia virus $Pr65^{gag}$ is excluded from virus assembly and maturation events. J. Virol. **63:**2370–2373.
60. **Shioda, T., and H. Shibuta.** 1992. Production of human immunodeficiency virus (HIV)-like particles from cells infected with recombinant vaccinia viruses carrying the gag gene of HIV. Virology **175:**139–148.
61. **Smith, A. J., M.-I. Cho, M.-L. Hammarskjold, and D. Rekosh.** 1990. Human immunodeficiency virus type 1 $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ expressed from a simian virus 40 late replacement vector are efficiently processed and assembled into viruslike particles. J. Virol. **64:**2743–2750.
62. **Sompayrac, L. M., and K. J. Danna.** 1981. Efficient infection of monkey cells with DNA of simian virus 40. Proc. Natl. Acad. Sci. USA **78:**7575–7578.
63. **Towler, D. A., J. I. Gordon, S. P. Adams, and L. Glaser.** 1988. The biology and enzymology of eukaryotic protein acylation. Annu. Rev. Biochem. **57:**69–99.
64. **Vernon, S. K., S. Murthy, J. Wilhelm, P. K. Chanda, N. Kalyan, S.-G. Lee, and P. P. Hung.** 1991. Ultrastructural characterization of human immunodeficiency virus type 1 Gag-containing particles assembled in a recombinant adenovirus vector system. J. Gen. Virol. **72:**1243–1251.
65. **Wachinger, G., H. Nitschko, M. Wachinger, and K. von der Helm.** 1991. The p17 stretch at the N terminus of the HIV-1-gag precursor and the intracellular part of HIV-1-env gp41 have heptad repeat sequences that could bind to each other in a coiled coil. Protein Sequences Data Anal. **4:**375–376.
66. **Wang, C.-T., and E. Barklis.** 1993. Assembly, processing, and infectivity of human immunodeficiency virus type 1 Gag mutants. J. Virol. **67:**4264–4273.
67. **Wang, C.-T., Y. Zhang, J. McDermott, and E. Barklis.** 1993. Conditional infectivity of a human immunodeficiency virus matrix domain deletion mutant. J. Virol. **67:**7067–7076.
68. **Weaver, T. A., and A. T. Panganiban.** 1990. N myristoylation of the

spleen necrosis virus matrix protein is required for correct association of the Gag polyprotein with intracellular membranes and for particle formation. J. Virol. **64:**3995–4001.

69. **Willey, R. L., J. S. Bonifacino, B. J. Potts, M. A. Martin, and R. D. Klausner.** 1988. Biosynthesis, cleavage and degradation of the human immunodeficiency virus type 1 envelope glycoprotein gp160. Proc. Natl. Acad. Sci. USA **85:**9580–9584.
70. **Willey, R. L., M. A. Martin, and K. W. C. Peden.** 1994. Increase in soluble CD4 binding to and CD4-induced dissociation of gp120 from virions correlates with infectivity of human immunodeficiency virus type 1. J. Virol. **68:**1029–1039.
71. **Willey, R. L., D. H. Smith, L. A. Lasky, T. S. Theodore, P. L. Earl, B. Moss, D. J. Capon, and M. A. Martin.** 1988. In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity. J. Virol. **62:**139–147.
72. **Wills, J. W., and R. C. Craven.** 1991. Form, function, and use of retroviral Gag proteins. AIDS **5:**639–654.
73. **Wills, J. W., R. C. Craven, and J. A. Achacoso.** 1989. Creation and expression of myristylated forms of Rous sarcoma virus Gag protein in mammalian cells. J. Virol. **63:**4331–4343.
74. **Wills, J. W., R. C. Craven, R. A. Weldon, Jr., T. D. Nelle, and C. R. Erdie.** 1991. Suppression of retroviral MA deletions by the amino-terminal membrane-binding domain of $p60^{src}$. J. Virol. **65:**3804–3812.
75. **Yu, X., Q.-C. Yu, T.-H. Lee, and M. Essex.** 1992. The C terminus of human immunodeficiency virus type 1 matrix protein is involved in early steps of the virus life cycle. J. Virol. **66:**5667–5670.
76. **Yu, X., X. Yuan, Z. Matsuda, T.-H. Lee, and M. Essex.** 1992. The matrix protein of human immunodeficiency virus type 1 is required for incorporation of viral envelope protein into mature virions. J. Virol. **66:**4966–4971.
77. **Yuan, Y., X. Yu, T.-H. Lee, and M. Essex.** 1993. Mutations in the N-terminal region of human immunodeficiency virus type 1 matrix protein block intracellular transport of the Gag precursor. J. Virol. **67:**6387–6394.
78. **Zhou, W., L. J. Parent, J. W. Wills, and M. D. Resh.** 1994. Identification of a membrane-binding domain within the amino-terminal region of human immunodeficiency virus type 1 Gag protein which interacts with acidic phospholipids. J. Virol. **68:** 2556–2569.