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Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus type 1

(virion formation/maturation/protease/gag cleavage sites)

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ABSTRACT **The effects on human immunodeficiency virus type 1 virion morphogenesis and on virus replication of mutations that affect posttranslational processing of the capsid precursor protein are described. A change in the glycine residue at position two from the N terminus abolishes the myristoylation of the precursor proteins and also prevents virus particle release. Mutations in the viral protease gene abolish proteolytic cleavage of the capsid precursor but do not prevent the formation and budding of virion particles of immature appearance. Mutations that alter the sequence of the sites normally used for cleavage of the major capsid protein p24 from the capsid precursor alter virion morphogenesis and prevent virus replication.**

Electron microscopy of infected cells reveals that particle formation of the human immunodeficiency virus type 1 (HIV-1) begins when an electron-dense structure assembles in patches under the cell membrane (1–3). As the structure assumes a more spherical shape, a portion of the cell membrane pinches off to surround the budding structure. During the course of budding, no clear central core of the virus particle is apparent. After release from the cell, a cone-shaped inner core particle is formed (2).

What features of the capsid proteins and their precursor polypeptides determine this maturation process? The precursor of the virion capsid proteins is a 55-kDa polyprotein (p55) encoded by the *gag* region of the viral genome (4). A 160-kDa polyprotein, which is made as a consequence of a −1 frameshift between the *gag* and *pol* frames (5), contains precursor forms of the enzymes necessary for virus replication in addition to the capsid proteins. The N terminus of both the 55-kDa and 160-kDa proteins is myristoylated (6). Addition of myristic acid to proteins typically occurs cotranslationally (7) and is specified by the sequence of the N terminus of the protein (8).

Cleavage of both the capsid precursor to yield the mature p17, p24, p9, and p6 capsid proteins as well as the precursor of the replication activities is at least in part specified by the viral protease (9, 10). The viral protease is encoded by the N terminus of the *pol* reading frame. The protease is reported to be active only as a dimer (11) and has been demonstrated to exhibit a preference for certain natural cleavage sites over others (12).

The effects of mutations that prevent the capsid protein myristoylation and the protease activity of HIV-1 on virus assembly and virion morphology have not been reported. The experiments reported here reveal the effects on capsid protein processing, virion morphogenesis, and virus replication of a series of mutations that prevent myristoylation, inactivate the viral protease, or alter the sequence of the cleavage sites.

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MATERIALS AND METHODS

Construction of Mutants. A 1.3-kilobase (kb) *Sac* I–*Apa* I *gag* fragment from pHXB-SV (13) was subcloned into pBluescriptSK(+) (Stratagene) to generate pSK$^+$gag as a target plasmid for the mutagenesis of the myristoylation site and the N-terminal p24 cleavage site. A 4.3-kb *Sph* I–*Sal* I *gag–pol* fragment from pHXB-SV was inserted into pGEM-5Zf(+) (Promega) to create pGEMgag-pol for the mutagenesis of the C-terminal p24 cleavage site and the protease region. Site-directed mutagenesis was performed as described (14) by using the mutagenic oligonucleotides shown in Fig. 1. The presence of the mutation was confirmed by DNA sequence analysis. To regenerate complete proviral clones, fragments carrying the desired mutation were reinserted into the parental vector pHXB-SV. A 0.7-kb *Bss*HII–*Sph* I fragment was transferred from the mutagenesis vector to create the mutant proviral plasmid pSVC-H2; a 1.3-kb *Bss*HII–*Apa* I fragment was used to create pSVC-C1, and 4.3-kb *Sph* I–*Sal* I fragments were used to create pSVC-C2, pSVC-P1, and pSVC-P2 (Fig. 1).

Cell Culture, Transfection, and Infections. COS-7 and Jurkat cells were propagated and transfected as described (15). To test for the infectivity of virions released from COS-7 cells, 0.3×10^7 Sup-T1 cells (16) were pelleted and resuspended in 3 ml of clarified supernatant derived from COS-7 cells transfected 60 hr earlier. After 24 hr, 12 ml of fresh medium was added and the cells were then split at 48-hr intervals.

Labeling of Cells and Immunoprecipitation. Metabolic labeling of cells with [^{35}S]cysteine and [^{35}S]methionine and immunoprecipitation were performed as described (17). For myristic acid labeling, [9,10-^{3}H]myristic acid was dissolved in dimethyl sulfoxide and added to the culture medium (final concentration = 250 μCi/ml; 1 Ci = 37 GBq).

Reverse Transcriptase Assay. Particle-associated reverse transcriptase activity in culture supernatants was determined as described (17).

Electron Microscopy. Starting 60 hr posttransfection, COS-7 cells were fixed for 12 hr in 4% (vol/vol) glutaraldehyde and 3% (wt/vol) paraformaldehyde in phosphate-buffered saline. Further processing for thin-section electron microscopy was done as described (17).

RESULTS

A Myristoylation-Defective Virus. A two-nucleotide mutation that was predicted to substitute an alanine for a glycine at residue two of the capsid protein precursor of HIV-1 was introduced into the HXBc2 provirus (Fig. 1, position 1). A full complement of viral proteins labeled with [^{35}S]cysteine and [^{35}S]methionine was produced by both wild-type and mutant

Abbreviations: HIV-1, human immunodeficiency virus type 1; Mo-MuLV, Moloney murine leukemia virus; MPMV, Mason–Pfizer monkey virus.

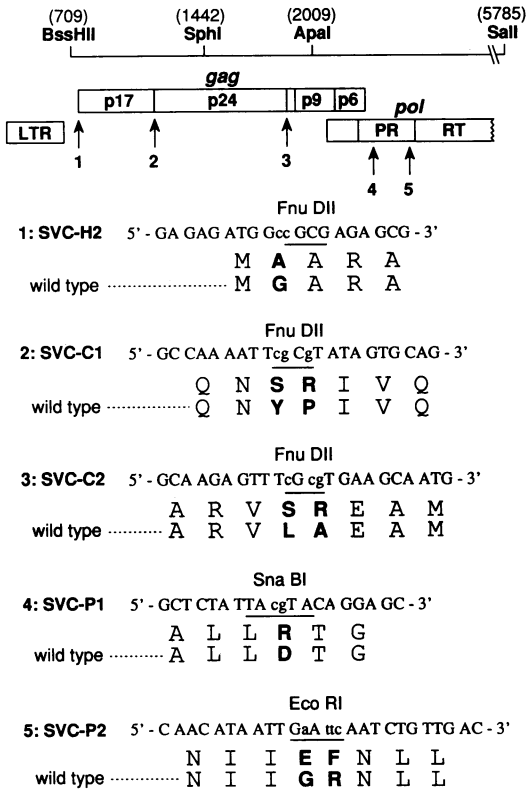

FIG. 1. Schematic representation of the *gag* and part of the *pol* region of HIV-1. The restriction sites used for reconstruction of the mutant proviral plasmids are indicated (numbers represent positions in the proviral DNA). The locations of the mutations are marked by arrows. The mutating oligonucleotides are shown below; the changed nucleotides are written in lowercase letters and the sequences of the newly generated restriction sites are underlined. The mutated amino acid sequence is compared to the wild-type sequence, with sequence changes indicated in boldface letters. The one-letter amino acid code is used. LTR, long terminal repeat; RT, reverse transcriptase; PR, protease.

provirus in transfected COS-7 cells (Fig. 2*A*, lanes 3 and 5). Evidently, proteolytic processing of the capsid precursor was not blocked by the mutation. Both wild-type and mutant DNA yielded about the same amount of the processed capsid proteins p24 and p17. In repeated experiments, the capsid precursor was observed to be more abundant in cells transfected with the mutant provirus.

After metabolic labeling with [^{3}H]myristic acid, the matrix protein p17 was precipitated as a prominent band from cells transfected with the wild-type provirus (Fig. 2*B*, lane 1). Weak bands corresponding to the p55 capsid precursor and p41, a processing intermediate composed of p17 and p24, were also visible. In contrast, no labeled myristic acid was incorporated into the p55, p41, or p17 of the mutant virus (Fig. 2*B*, lane 2).

The supernatant fractions were examined to determine if viral proteins were released from the transfected cells. Both the envelope glycoprotein gp120 and the processed capsid proteins p24 and p17 were precipitated from the supernatants after transfection with the wild-type provirus (Fig. 2*A*, lane 4). In contrast, only gp120 was obtained from the supernatant of COS-7 cells transfected with the myristoylation-site mutant (Fig. 2*A*, lane 6). No reverse transcriptase activity was detected in the supernatant of COS-7 cells transfected with the mutant provirus plasmid (Fig. 3*A*, bar 7), in contrast to supernatant of cells transfected with the wild-type provirus (Fig. 3*A*, bar 2).

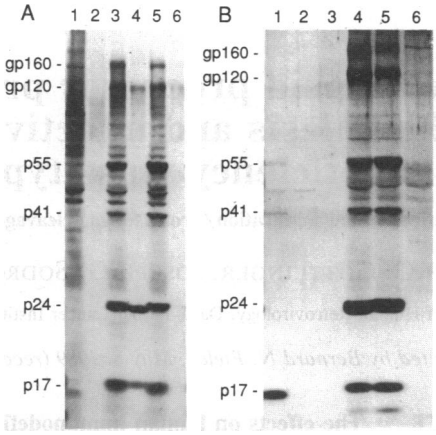

FIG. 2. Absence of capsid protein release (*A*) and capsid protein myristoylation (*B*) in cells transfected with the SVC-H2 provirus. (*A*) COS-7 cells were mock-transfected (lanes 1 and 2) or transfected with the wild-type provirus HXB-SV (lanes 3 and 4) or the myristoylation-site mutant SVC-H2 (lanes 5 and 6). The cells were labeled with [^{35}S]cysteine and [^{35}S]methionine from 48 to 60 hr after transfection. Viral proteins were immunoprecipitated from the cell lysates (lanes 1, 3, and 5) or the culture medium (lanes 2, 4, and 6) by serum from an HIV-1-infected patient and resolved by SDS/polyacrylamide gel electrophoresis. (*B*) COS-7 cells were transfected with HXB-SV (lanes 1 and 4) or SVC-H2 (lanes 2 and 5) or were mock-transfected (lanes 3 and 6). The cultures were split and labeled with [^{3}H]myristic acid (lanes 1–3) or [^{35}S]cysteine and [^{35}S]methionine (lanes 4–6) from 48 to 60 hr after transfection.

Budding and release of virus particles were readily detected by thin-section electron microscopy in COS-7 monolayer preparations transfected with the wild-type provirus (see Fig. 5*A*). In several independent experiments, no virus particle formation was observed in cells transfected with the mutant provirus, and no cell-free virus particles were detected.

To test for the release of infectious virus, Sup-T1 cells were incubated with supernatant taken from COS-7 cells 48 hr after transfection. Syncytia were observed within 2 days by using supernatant from cells transfected with the wild-type virus.

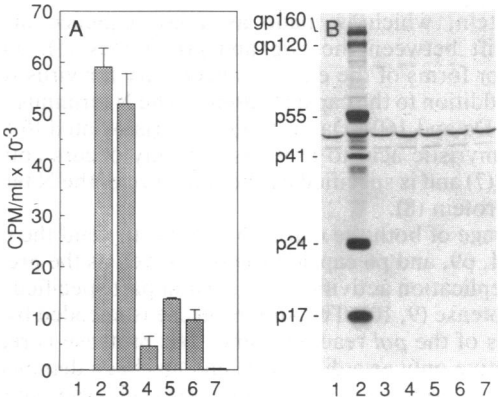

FIG. 3. Reverse transcriptase activity (*A*) and viral infectivity (*B*) of culture supernatants from COS-7 cells transfected with proviral DNAs. (*A*) Particle-associated reverse transcriptase activity released into the culture medium was measured 60 hr posttransfection. Values represent the means of three independent experiments, and error bars indicate the standard deviations obtained. (*B*) Viral replication in permissive cells was determined by cultivating Sup-T1 cells in culture supernatant derived from COS-7 cells that had been transfected with proviral DNAs. Two weeks after infection, the cells were assayed for the expression of viral proteins by immunoprecipitation and SDS/polyacrylamide gel electrophoresis. The transfected DNAs were 1, salmon sperm; 2, HXB-SV; 3, SVC-C1; 4, SVC-C2; 5, SVC-P1; 6, SVC-P2; 7, SVC-H2.

No cytopathic effect was observed during 2 weeks of incubation with similar supernatants from cells transfected with the mutant provirus. Metabolic labeling showed abundant viral protein expression in the Sup-T1 cells infected with the wild-type virus (Fig. 3*B*, lane 2). No viral protein was detectable in the Sup-T1 cells exposed to supernatant from COS-7 cells transfected with the myristoylation-site mutant (Fig. 3*B*, lane 7). Also, no evidence for virus replication was observed after transfection of the myristoylation-site mutant directly into Jurkat cells (data not shown).

Protease-Defective Mutants. To create a provirus incapable of making active protease, the codon that specifies the aspartic residue at position 25 of the HIV protease, which is believed to form the catalytic residue (18), was replaced by a codon that specifies arginine (Fig. 1, position 4). A mutation was also introduced into a second highly conserved stretch of amino acids. The sequence of the mutated protein specifies Glu-Phe instead of Gly-Arg (HIV-1 protease amino acids 86 and 87) (Fig. 1, position 5).

The p55 capsid precursor was abundant in COS-7 cells transfected with both mutant proviral DNAs (Fig. 4*A*, lanes 5 and 6). Processed capsid protein products were absent. Also missing were the 31-kDa and 66-kDa products of the *pol* gene. A 100-kDa protein that is evident in lysates of cells transfected with the wild-type provirus and is likely to be an intermediate in the processing of the *pol* precursor is also absent from cells transfected with these mutants. Processing of the *env* precursor was not affected. Immunoprecipitation from the cell-free supernatant of cells transfected with the mutants showed that the unprocessed capsid precursor was transported into the growth medium (Fig. 4*B*, lanes 5 and 6).

The appearance of the capsid precursor and of particle-associated reverse transcriptase activity in the supernatant (Fig. 3*A*, bars 5 and 6) indicated that virus particles are made by the protease-defective mutants. By electron microscopy, the virions produced by both of the protease-defective mutants have the appearance of wild-type virions newly released from infected cells (Fig. 5 *B* and *C*). The particles are, on the average, larger (121 ± 14 nm) as compared to the wild-type virions (102 ± 11 nm, $n = 20$). The center of the virus particles is electron-lucent. At the inner circumference of the moderately electron-dense, 17- to 19-nm-thick envelope shell, there is a thin band of higher electron density. The mutant virions are more uniformly decorated with envelope spikes than are the wild-type mature virions. Aberrant particles formed by multiple budding were frequently seen (Fig. 5*D*).

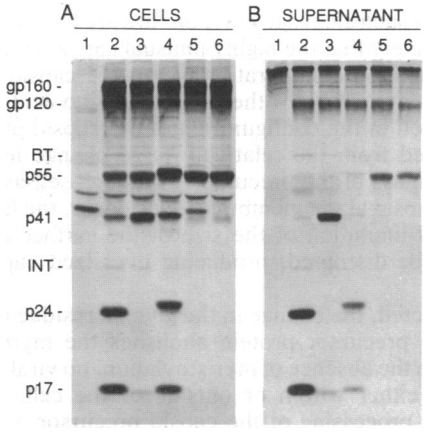

FIG. 4. Protein profile expressed by proviruses with a defect in capsid precursor processing. COS-7 cells were labeled with [^{35}S]-cysteine and [^{35}S]methionine 48–60 hr posttransfection, and the viral proteins were immunoprecipitated from the cell lysates (*A*) or the culture supernatant (*B*). The transfected DNAs were salmon sperm (lane 1), HXB-SV (lane 2), SVC-C1 (lane 3), SVC-C2 (lane 4), SVC-P1 (lane 5), and SVC-P2 (lane 6). RT, reverse transcriptase; INT, integrase.

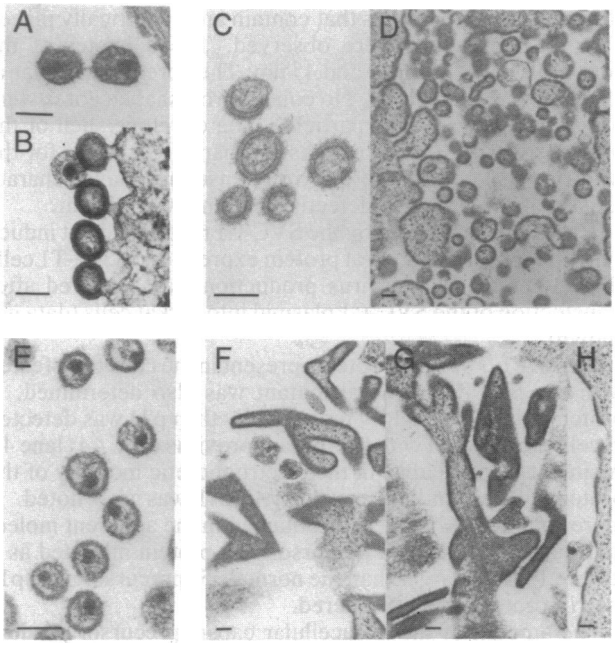

FIG. 5. Virion and capsid structures produced by processing-defective proviruses. (*A* and *C–H*) COS-7 cell monolayers were fixed 60 hr posttransfection and prepared for thin-section electron microscopy. The transfected proviruses were HXB-SV (*A*), SVC-P1 (*C* and *D*), SVC-C1 (*E*), and SVC-C2 (*F–H*). (*B*) HXBH10, a vpu-positive wild-type HIV-1 virus (24) (kindly provided by Ernest Terwilliger, Dana–Farber Cancer Institute). (Bar = 100 nm.)

When Sup-T1 cells were exposed to the supernatants containing the protease-defective mutant virions, no syncytia were observed 2 weeks after exposure. No viral protein was present in [^{35}S]cysteine and [^{35}S]methionine-labeled cell lysates (Fig. 3*B*, lanes 5 and 6). No evidence of virus replication was observed after transfection of Jurkat cells with the mutant proviral DNAs (data not shown).

Alteration of Protease Recognition Sites. To alter selectively the processing sites of the capsid protein p24, the codons that specify the two residues flanking the N- or C-terminal scissile bond (N terminus: Tyr-Pro; C terminus: Leu-Ala) were changed to codons that specify Ser-Arg to yield the mutant proviruses designated SVC-C1 and SVC-C2, respectively (Fig. 1, positions 2 and 3). The changes are predicted to alter the cleavage sites between p17 and p24 and between p24 and the C-terminal capsid proteins.

Immunoprecipitation of viral proteins from cells transfected with the p17–p24 cleavage mutant SVC-C1 produced a prominent 41-kDa band, the size expected for a p17–p24 fusion protein (Fig. 4*A*, lane 3). A weak band of slightly lower molecular mass than wild-type p17 (p16.8) was also obtained in some experiments. However, the total absence of p24 in the cell lysates demonstrated that further processing of p41 into the mature capsid proteins p17 and p24 did not occur.

The p41 fusion protein was the major capsid protein released into the supernatant, together with a trace amount of the 16.8-kDa protein (Fig. 4*B*, lane 3). The mature capsid protein p24 was not detected in cell supernatants. The total amount of the p66 and p31 proteins was similar to that produced by wild-type virus. The amount of reverse transcriptase activity was also similar (Fig. 3*A*, bar 3). Apparently, the mutation does not decrease the efficiency of virus budding.

The virions released from cells transfected with the p17–p24 cleavage mutant had an average diameter of 101 ± 9 nm ($n = 20$), which is similar to the size of the wild-type particles

(Fig. 5*E*). Virus particles that contain an eccentrically placed electron-dense core were observed. The diameter of the cores varied between 35 and 45 nm. The core was circular in all cross-sectional views. No cone- or rod-shaped cores were observed in these virus particles. The envelope shell of the particles was 9–10 nm thick, as compared to 5–6 nm for the wild-type envelope. The electron-dense inner layer characteristic of the protease-defective mutants was absent.

The virions produced by the SVC-C1 mutant did not induce syncytia formation or viral protein expression in Sup-T1 cells (Fig. 3*B*, lane 3). No virus production was observed after transfection of the SVC-C1 plasmid into Jurkat cells (data not shown).

The size of the viral proteins present in the cells transfected with the SVC-C2 proviral mutant was also determined. A protein migrating slightly more slowly than p24 was detected in cells transfected with the mutant provirus (Fig. 4*A*, lane 4). A similar small change in the electrophoretic mobility of the protein corresponding to wild-type p41 was also noted. A more significant change was obtained in the apparent molecular mass of the capsid precursor: the protein migrated as if it were 1–2 kDa larger than the normal p55 precursor. The p17 matrix protein was not altered.

The amount of the intracellular capsid precursor product was substantially larger in cells transfected with the SVC-C2 mutant provirus than in cells transfected with the wild-type virus. In the same cells, the amount of the viral envelope proteins was comparable. The p17 and slightly altered p24 (Fig. 4*B*, lane 4) as well as reverse transcriptase activity (Fig. 3*A*, bar 4) were present in the cell supernatant albeit at greatly reduced amounts.

The morphology of the viral structures synthesized by cells transfected with this mutant is remarkably different from that obtained with wild-type provirus or the other mutants (Fig. 5 *F–H*). Large linear electron-dense structures were observed at the inner layer of the cell membrane. Slices parallel to the cell membrane revealed broad sheets of electron-dense areas. These structures did not assume the uniform curvature found with normal budding virus particles. Long tube-like structures with spherical tips were observed, apparently budding from the cell membrane, as well as triangular and other geometric forms. A fringe of envelope spikes was observed over the surface of the electron-dense patches. The appearance of the wall of the particles resembled that of normal budding viruses. Spherical budding particles were occasionally seen, especially at the tips of cellular protuberances.

The supernatant of COS-7 cells transfected with the SVC-C2 provirus did not induce a productive infection in Sup-T1 cells (Fig. 3*B*, lane 4). No evidence of virus replication was observed in Jurkat cells transfected with the SVC-C2 provirus.

DISCUSSION

The experiments reported here demonstrate that proteolytic cleavage and myristoylation of the HIV-1 capsid precursor are required for the production of infectious virus particles. As expected, mutations that alter conserved sequences in the protease prevent proteolytic processing of the capsid precursor. However, the mutations do not prevent assembly of the capsid structure or virus particle budding. Viral DNA polymerase activity is detected in preparations of protease-defective virus particles. A DNA polymerase activity was also present in virus particles produced by a protease-defective mutant of the Moloney murine leukemia virus (Mo-MuLV) (19).

The failure of a provirus with a mutation in the protease to replicate and process the p55 precursor was reported previously (20). However, the studies reported here demonstrate that virions are formed by protease-defective mutants and describe the morphology and the DNA polymerase activity present in such virions.

The change in the sequence of the cleavage site between the p17 and p24 capsid proteins eliminates processing at this site. There is no residual susceptibility of the altered site to cleavage by the viral protease, as p24 is clearly absent. The trace amount of the 16.8-kDa protein observed in the virions may correspond to a C-terminal fragment of the capsid precursor generated by cleavage of the Leu-Ala sequence at the C terminus of p24. Such a product has been found to exhibit an electrophoretic mobility similar to that of p17 (11).

The virions produced by the mutant contain a homogeneously electron-dense, circular core. The morphology of the core as well as the increased thickness of the virus shell as compared to wild-type virions implies that the p24 protein remains attached to the inner surface of the viral membrane and that the condensation of the C-terminal capsid proteins and the genomic RNA to form a circular nucleoid occurs independently of the formation of a tubular core shell. The eccentric location of the core, which is a characteristic of the lentivirus family, is preserved despite the absence of the core shell.

The virions produced by the p17–p24 cleavage mutant are not infectious, despite the presence of wild-type levels of reverse transcriptase activity and a condensed core. It was recently observed that the major capsid protein of Mo-MuLV is found associated with proviral DNA both in the cytoplasm and the nucleus prior to integration (P. Brown, personal communication). Absence of p24 from the intracellular HIV-1 nucleic acid complex may prevent the synthesis and/or integration of a nascent provirus.

Highly unusual viral structures were produced by a mutant with a defect in the C-terminal cleavage site of p24. The normal cleavage site for the generation of p24 (21) is apparently not used in the mutant precursor as both the p24 and the p17–p24 cleavage intermediate migrate with slightly reduced electrophoretic mobility as compared to the wild-type proteins. Cleavage has been reported to occur also at a Met-Ser as well as a Met-Met sequence near the C terminus of p24 (21). Cleavage at the first of these sites would result in an increase in the length of the p24 protein by four amino acids, a change consistent with the apparent change in molecular mass.

The dramatic alteration of the capsid morphogenesis is probably not attributable to the addition of residues at the N terminus of p24, as similar aberrant capsid forms are not found when such cleavage reactions are abolished altogether. It is more likely that the highly unusual capsid structures are due to an altered configuration of the p55 capsid precursor that occurs as a result of the changed amino acid sequence. An alteration in the configuration of the capsid precursor is also inferred from the relatively large change in apparent molecular mass of the precursor. Although self-assembly of the protocapsid at the membrane does occur, the factors that lead to self-limitation of the size of the surface aggregates appear to be disrupted, producing oversized capsid structures.

As predicted, the change in the glycine residue at position two of the precursor protein abolishes the myristoylation reaction. In the absence of myristoylation, no viral structures are found either within or outside of the cell. However, proteolytic processing of the capsid precursor occurs. The effects of the myristoylation-defective phenotype on HIV-1 morphogenesis differ from the effects of similar mutations on the morphogenesis of the type C Mo-MuLV and the type D Mason–Pfizer monkey virus (MPMV) (22, 23). Assembly of the capsid protein into discrete particles occurs at the cell membrane for wild-type HIV-1 and Mo-MuLV. Assembly of such structures is not observed in both viruses if mutations are introduced that prevent myristoylation of the capsid

precursor. However, very little processing of the capsid precursor is observed in the myristoylation-defective Mo-MuLV in contrast to the results reported here (22). In the case of MPMV, assembly of the capsid occurs within the cytoplasm both in the presence and absence of myristoylation of the capsid precursor (23). However, in the absence of myristoylation, the capsid does not migrate to the cell surface nor is it budded from the cell. No proteolytic processing of the capsid precursors is observed for myristoylation-defective MPMV.

These differences imply that the requirements for the activation of proteolytic processing differ among these viruses. For both Mo-MuLV and MPMV, it is proposed that proteolytic cleavage of the virus capsid occurs only when the virus particle is released from the cell. Evidently, the HIV-1 protease is not dependent on virus budding for activity.

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