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Myristoylation of *gag* Proteins of HIV-1 Plays an Important Role in Virus Assembly

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ABSTRACT

The *gag* proteins of HIV-1 are modified by the addition of myristic acid to the amino terminal glycine residue. Site-directed mutagenesis was used to construct a mutant of HIV-1 in which this glycine residue was changed to an alanine. Upon transfection into *cos*-1 cells, the mutant genome directed the synthesis of the full complement of HIV-1 proteins, but p17 and p17-containing polyproteins were not myristoylated. The cells transfected with the mutant DNA did not release any virus particles and no viral cores were visible by electron microscopy. Furthermore, supernatant from these transfected cells failed to infect CEM cells. The expression and function of gp120 on the surface of cells transfected with the mutant DNA was unaffected as these cells formed syncytia comparable in both size and number to the ones obtained with wild-type DNA.

INTRODUCTION

THE PRIMARY *gag* GENE PRODUCT of human immunodeficiency virus type 1 (HIV-1) is synthesized using an initiation near the 5′ end of the viral genomic RNA. This precursor polyprotein $Pr53^{gag}$ is proteolytically processed into proteins of molecular weight 17 (p17), 24 (p24), 7 (p7), and 6 (p6) kD.[1,2] The *gag* protein p17 is modified by the addition of a myristic acid residue through an acyl linkage to the amino terminal glycine residue.[1] Similar modification of the *gag* gene product has been described for other type C and type D retroviruses.[3–5] It has been shown that in Moloney murine leukemia virus, myristoylation of $Pr65^{gag}$ is essential for virus particle formation.[3,5] In Mason-Pfizer monkey virus, myristate has been shown to be critical for intracytoplasmic transport of completed viral capsids to their normal site of budding on the plasma membrane.[4]

Cerulenin, an inhibitor of de novo sterol and fatty acid biogenesis, has been shown to inhibit the myristoylation and proteolytic cleavage of $Pr53^{gag}$ in HIV-1-infected cells.[6] However, the toxicity of this drug prohibits its use to study the effect of myristoylation of *gag* proteins on HIV-1 assembly. In this communication we show that the substitution of the N-terminal glycine of $Pr53^{gag}$ with alanine by

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site-directed oligonucleotide mutagenesis resulted in the synthesis of unmyristoylated *gag* proteins. This mutation allowed us to study the role of myristoylation in the morphogenesis of HIV-1.

MATERIALS AND METHODS

Cells

Cos-1 cells, CEM cells, and CD4-expressing HeLa cells[7] were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum, 1 mM glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin.

Construction of myr(−) *HIV-1 mutant*

The *myr*(−) mutant was constructed by oligonucleotide-directed mutagenesis as described by Zoller and Smith.[8] The insert of the HIV-1 clone lambda HXB2[9] was excised by digestion with XbaI and cloned into an sp65*gpt* vector,[10] designated sp65*gpt*X into which an XbaI site was substituted for the EcoRI site in the polylinker. The sp65*gpt* vector contains an SV40 replication origin to ensure a high transient copy number after transfection into *cos*-1 cells. The proviral construct, designated pHXB2-X, was transfected into a *dam*- strain of *Escherichia coli,* GM161, to demethylate the ClaI site within the *gag* gene, and then digested with XbaI plus ClaI. The 1.7-Kb fragment containing the 5′ flanking region and long terminal repeat (LTR) and the 5′ end of the *gag* gene was cloned into the M13 vector Phagescript SK (Stratagene, Inc., La Jolla, CA) at the XbaI–ClaI sites. The resultant single-stranded DNA was mutagenized with an antisense primer 5′-CGCTCTCGCA*G*CCATCTCTC-3′, where the italicized base change converted the *Gly* residue of the *gag* polyprotein to an *Ala*. Mutants were selected by differential screening using ^{32}P-labeled mutagenic oligomer and verified by DNA sequence analysis using the method of Sanger et al.[11] The RF form of the mutant plasmid was grown, the insert purified by digestion with XbaI and XhoI, and cloned into pBluescript SK- (Stratagene) at the XbaI and XhoI sites. This construct was transfected into GM161 to again demethylate the ClaI site and the mutagenized 1.7-Kb ClaI-XbaI fragment was excised and purified. The 10-Kb ClaI–XbaI fragment, which contained the carboxy portion of the *gag* gene, all downstream proviral sequences, and the 3′ flanking region, was purified from pHXB2-X grown in GM161 and coligated together with the 1.7-Kb mutagenized ClaI–XbaI fragment into the XbaI site of sp65*gpt*X to give the *myr*(−) biologically active mutant clone pHXB2-*myr*(−). Mutants were again selected by differential screening using the mutagenic oligomer as a probe. The identity of this mutant was also verified by directly sequencing the final plasmid construct by the method of Sanger et al.[11]

DNA transfection

The biologically active molecular clone pHXB2-X or the pHXB2-*myr*(−) mutant were transfected into *cos*-1 or HeLa CD4 cells by electroporation.[12] Briefly, 30 μg of plasmid DNA was used to transfect 10^7 cells resuspended in 0.8 ml of sucrose electroporation buffer (272 mM sucrose, 7 mM sodium phosphate buffer, pH 7.4, 1 mM $MgCl_2$). For electroporation, the Gene Pulser™ apparatus (BioRad) was used at a voltage setting of 0.3 kV for *cos*-1 cells and 0.25 kV for HeLa CD4 cells. Capacitance setting for both cell lines was 25 μF. After transfection, cells were resuspended in 20 ml of complete medium and plated either in T75 tissue culture flasks (Costar) or in Lab-Tek® chamber slides.

Virus infectivity assay

Infectivity assays were performed either with cell-free supernatant harvested from transfected *cos*-1 cells, or with pelleted virus from the same supernatant using CEM as the target cell. Culture medium obtained from the transfected *cos*-1 cells was filtered through a 0.45-μm Millipore filter and a portion of it was centrifuged at 150,000 × g for 90 min. The virus pellet was resuspended in RPMI-1640 medium. The cell-free

supernatant or the resuspended pellet virus was added at several dilutions to 1×10^5 CEM cells in 0.1 ml RPMI-1640 medium. After 1 h of incubation at 37°C, the medium was removed and the cells were cultured with fresh medium. The reverse transcriptase activity in the supernatant was determined after 7 days as described elsewhere.[13]

Syncytia assay

The syncytia assay was performed in 96-well plates using CD4 expressing HeLa cells. Wild-type and mutant DNA were transfected into HeLa cells expressing CD4 by electroporation and the cells were cultured for 16 h at 37°C in a CO_2 incubator. The multinucleated giant cells were detected by microscopic examination.

Radiolabeling of cells and immunoprecipitation of HIV-1 proteins

Transfected *cos*-1 cells were labeled with [^{35}S]cysteine or [^{3}H]myristic acid and radioimmunoprecipitation assays were performed as described before.[1] Briefly, [^{35}S]cysteine was added to a final concentration of 200 μCi/ml in cysteine-free medium and the cells were incubated for either 7 h or overnight at 37°C. [^{3}H]Myristic acid was added to a final concentration of 1 mCi/ml in RPMI-1640 medium and the cells were incubated for 5 h. After labeling, the cells were washed in phosphate-buffered saline (PBS) and disrupted in PBS containing 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, and 0.5% sodium deoxycholate (PBS-TDS). The lysates were then absorbed for 3 h at room temperature with protein A-Sepharose (PAS) and an aliquot of normal human sera. Portions of the lysates to be immunoprecipitated with monoclonal antibody were instead preabsorbed with PAS bound to rabbit antiserum to mouse κ light chain (κ-PAS). The lysates were then clarified by centrifugation. Radioimmunoprecipitation analysis was performed by the addition of 10 μl of serum or 2 μl of ascites fluid and 0.2 ml of a 10% suspension of protein A-Sepharose to 1 ml of labeled extract. The samples were incubated at 4°C for 18 h. The immunoprecipitates were collected by centrifugation, washed repeatedly in PBS-TDS, resuspended in Laemmli sample buffer, heated for 3 min at 90°C, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

Subcellular fractionation

Transfected *cos*-1 cells were labeled with [^{35}S]cysteine (200 μCi/ml) for 7 h at 37°C as described above. The monolayers were then rinsed with PBS and the cells were scraped into swelling buffer (10 mM phosphate buffer, pH 7.2, 0.1 mM phenylmethylsulfonyl fluoride). After 20 min of incubation in ice, the cells were homogenized with a tight-fitting glass Dounce homogenizer and the nuclei were pelleted by centrifuging at $1{,}000 \times g$ for 5 min. Four volumes of PBS were added to the supernatant and the suspension was centrifuged for 60 min at $100{,}000 \times g$ to separate membrane and cytoplasmic fractions. The membrane pellet and the cytosol were then solubilized with PBS-TDS and the viral proteins were immunoprecipitated and analyzed by SDS-PAGE using a mixture of monoclonal antibodies to gp120, p24, and p17 as described above.

RESULTS

Mutant gag *proteins are not myristoylated*

We introduced a mutation in the HIV-1 clone lambda HXB2 that resulted in substitution of a glycine residue for an alanine at position 2 of the *gag* precursor polyprotein p53 ($Pr53^{gag}$) (Fig. 1). The mutant clone was prepared as described under Methods. The *cos*-1 cells were transfected with pHXB2-*myr*(−) and pHXB2-X (wild type), and 48 h after transfection, the cells were labeled with either [^{35}S]cysteine (200 μCi/ml) for 18 h or with [^{3}H]myristic acid (1 mCi/ml) for 5 h. The proteins in the cell lysate were precipitated with either HIV-1 antibody-positive human serum or with monoclonal antibodies to HIV-1 p17 and p24. The human serum efficiently immunoprecipitated similar amounts of [^{35}S]cysteine-labeled proteins and the precipitates contained $Pr53^{gag}$ and $Pr39^{gag}$ as well as the processed products p17 and p24 from both wild-type and *myr*(−)

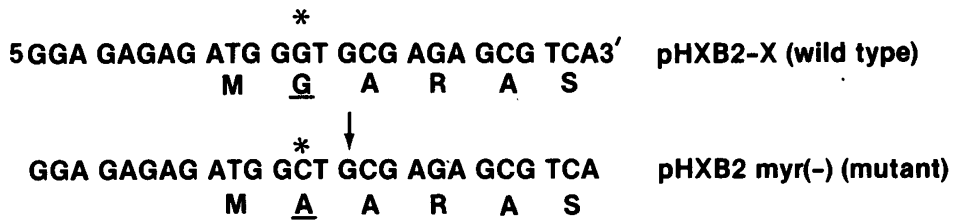


FIG. 1. Schematic representation of the nucleotide sequence in the HIV-1 *gag* gene subjected to site-directed mutagenesis. Amino acids corresponding to the codons are shown below. The mutated nucleotide is indicated by an asterisk and the corresponding amino acid is underlined.

mutant-transfected cells (Fig. 2, panel A). Similar results were obtained with the monoclonal antibodies to p17 and p24.[6] These findings suggested that the absence of this post translational modification does not interfere with proteolytic processing of the *gag* precursors. After labeling with [^{3}H]myristic acid, the human serum and the monoclonal antibodies immunoprecipitated Pr53^{gag}, Pr39^{gag}, and p17 from lysates of *cos*-1 cells transfected with the wild-type DNA (Fig. 2, panel B). In contrast, but as expected, protein immunoprecipitated from *cos*-1 cells transfected with the mutant virus contained no myristate.

Localization of gag *proteins in the subcellular fractions*

The hydrophobic myristate residue on the HIV-1 *gag* proteins may determine their association with the intracellular membrane in infected cells. To determine the distribution of *gag* proteins in the subcellular

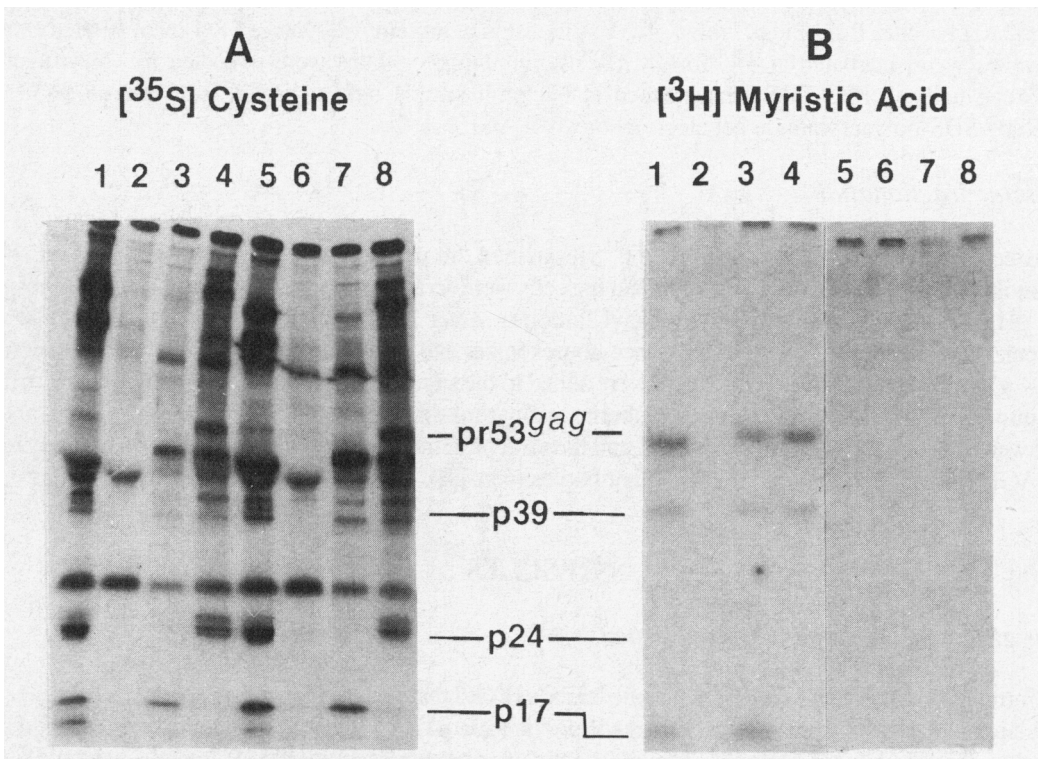


FIG. 2. HIV-1 protein profile of *cos*-1 cells transfected with pHXB2-X and pHXB2-*myr*(−). Transfected cells were labeled with [^{35}S]cysteine for 18 h (Panel A) and with [^{3}H]myristic acid for 5 h (Panel B). In each panel, lanes 1–4 represent cells transfected with pHXB2-X and lanes 5-8 with pHXB2-*myr*(−). Immunoprecipitations were performed with: an HIV-1 antibody-positive human serum (lanes 1 and 5), normal human serum (lanes 2 and 6), anti-p17 monoclonal antibody (lanes 3 and 7), and anti-p24 monoclonal antibody (lanes 4 and 8).

compartments, transfected *cos*-1 cells were labeled with [^{35}S]cysteine and fractionated into membrane and cytosolic fractions. The proteins present in each fraction were detected by immunoprecipitation with a mixture of monoclonal antibodies to gp120, p17, and p24. The *gag* protein p17 and the precursors p53 and p39 were present in both membrane and cytosolic fractions in cells transfected with wild-type DNA (Fig. 3, panel pHXB2-X). In contrast, the *gag* proteins were present only in cytoplasm of cells transfected with mutant DNA (Fig. 3, panel pHXB2-*myr*(−). However, the envelope glycoproteins gp160 and gp120 were detected in the membrane fractions of both cells. This clearly suggests that the hydrophobicity induced by myristate modification of *gag* proteins determines their localization in the intracellular membrane of virus-expressing cells.

Assembly of virus by unmyristoylated gag *protein*

It was of interest to determine whether myristoylation of *gag* proteins plays any role in assembly and budding of HIV-1 virions. To this end, cells were transfected and labeled with [^{35}S]cysteine; after labeling, the supernatants were collected, filtered, and an aliquot immunoprecipitated with a mixture of monoclonal antibodies to p17 and p24. Virus was pelleted from the remainder of the supernatants by ultracentrifugation and subjected to immunoprecipitation by the same mixture of monoclonal antibodies. As expected, p17 and p24 were immunoprecipitated from supernatants and pelleted virus when the cells were transfected with the wild-type provirus (Fig. 4, panel A). In contrast, no p17 and p24 were immunoprecipitated from the supernatant or virus pellet when the cells were transfected with the mutant DNA. The presence of infectious virus particles in the culture medium of transfected cells was determined by infecting CEM cells with these supernatants. A portion of cell-free supernatant was also pelleted, to concentrate any virus particle released in the medium and the pellet was resuspended in RPMI-1640 medium. CEM cells were infected with different

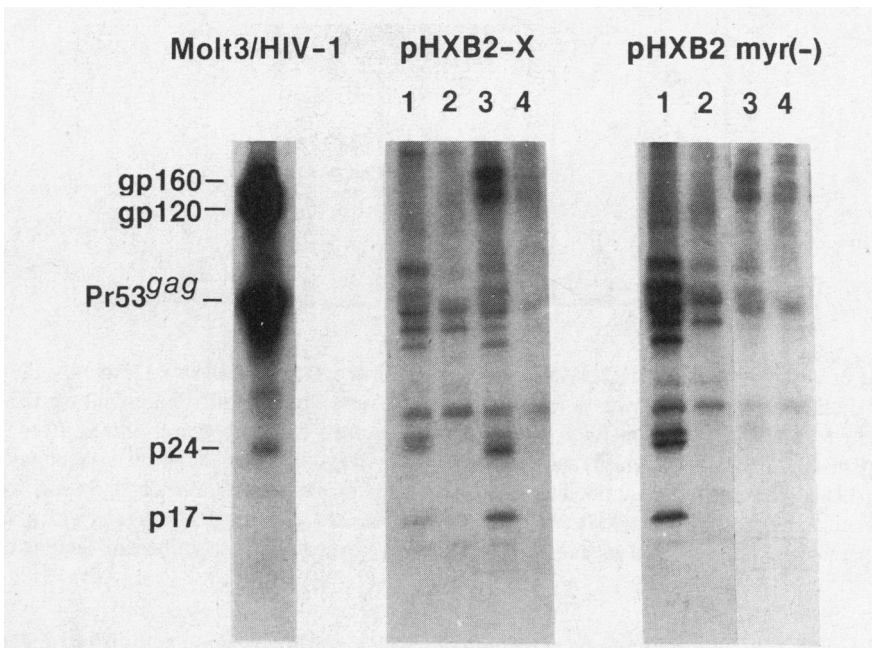
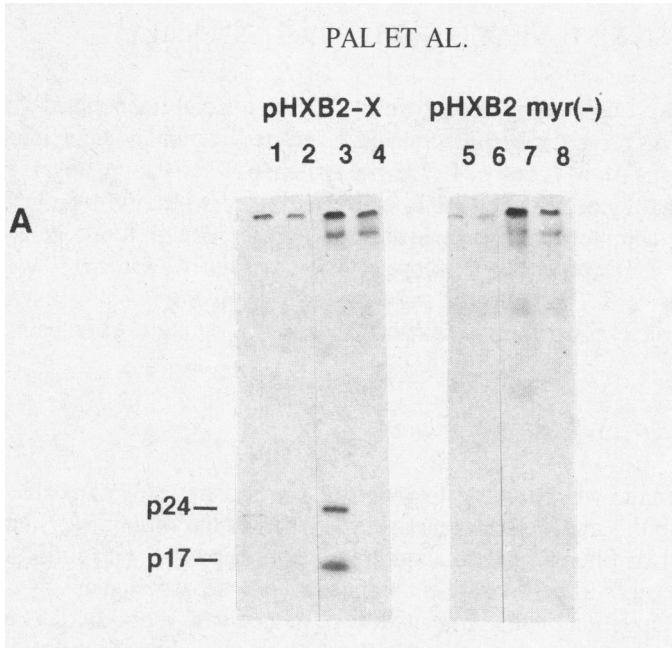


FIG. 3. Subcellular localization of HIV-1 *gag* and *env* proteins in *cos*-1 transfected cells. Cells were labeled with [^{35}S]cysteine for 7 h and fractionated as described in Materials and Methods. In both pHXB2-X and pHXB2-*myr*(−) panels, lanes 1 and 2 represent the cytosolic fraction and lanes 3 and 4 the membrane fraction. Immunoprecipitations were performed with a mixture of anti-p17, p24, and gp120 monoclonal antibodies (lanes 1 and 3) and an unrelated monoclonal antibody as negative control (lanes 2 and 4). HIV-1 proteins from chronically infected Molt3 cells, immunoprecipitated with an HIV-1 antibody-positive human serum and electrophoresed on the same gel are shown at the left of the two panels as reference.



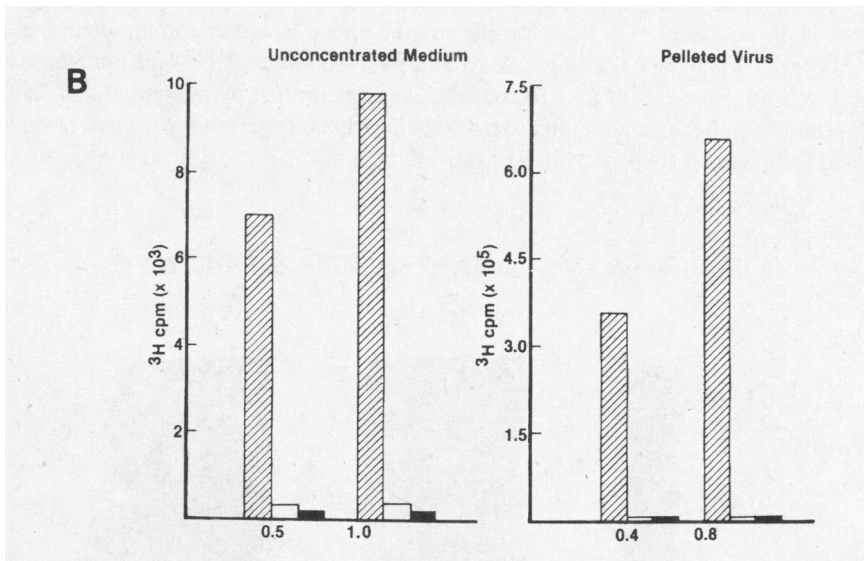


FIG. 4. Release of virus from *cos*-1 cells transfected with pHXB2-X and pHXB2-*myr*(−). Panel A, *cos*-1 cells were transfected with pHXB2-X and pHXB2-*myr*(−). After 48 h, the cells were labeled with [^{35}S]cysteine for 18 h. The culture medium was filtered through a 0.45 μm filter and a portion was then centrifuged at 150,000 g for 90 min. The uncentrifuged supernatant (lanes 1, 2, 5, and 6) and the pelleted virus (lanes 3, 4, 7, and 8) were immunoprecipitated with a mixture of anti-p17 and p24 monoclonal antibodies (lanes 1, 3, 5, and 7) or with an unrelated monoclonal antibody (lanes 2, 4, 6, and 8). Panel B, *cos*-1 cells were mock transfected (■) or transfected with pHXB2-X (▨) and with pHXB2-*myr*(−) (□). After 48 h supernatants were collected and the infectivity assay was performed as described in Materials and Methods.

dilutions of either cell-free supernatants or resuspended pellets and the reverse transcriptase activity in the medium was determined after 7 days. Supernatants and pellets from cells transfected with the pHXB2-X DNA clearly infected CEM cells, whereas no infection was observed with samples from cells transfected with pHXB2-*myr*(−) (Fig. 4, panel B). These results suggest that cells transfected with the mutant DNA do not release HIV-1.

Cells were also examined by electron microscopy for the presence of virions. No budding virus or mature virus particles were detected in *cos*-1 cells transfected with the mutant DNA, whereas budding and mature virus particles were readily observed in cells transfected with the wild-type DNA (data not shown). All these

results clearly suggest that the assembly and budding of HIV-1 is completely inhibited when *gag* polyproteins are not myristoylated.

Expression and biological activity of surface glycoproteins in cells transfected with pHXB2-myr(−)

As shown earlier, the envelope glycoproteins in cells transfected with the mutant DNA were associated only with the membrane fraction. It was of interest to determine whether the glycoproteins associated with the membrane were expressed on the cell surface. Live cell immunofluorescence assay with a monoclonal antibody to HIV-1 gp120 revealed that the glycoprotein was expressed on the surface of cells transfected with the mutant DNA (data not shown). In order to test the ability of gp120 expressed on the cell surface to form syncytia, pHXB2-*myr*(−) was transfected into CD4-expressing HeLa cells. Large syncytia were readily formed, comparable in both size and number to the ones obtained with the wild-type virus under the same experimental conditions (Fig. 5). These results suggest that the lack of myristoylation of the *gag* protein has no effect on the expression and function of *env* glycoproteins.

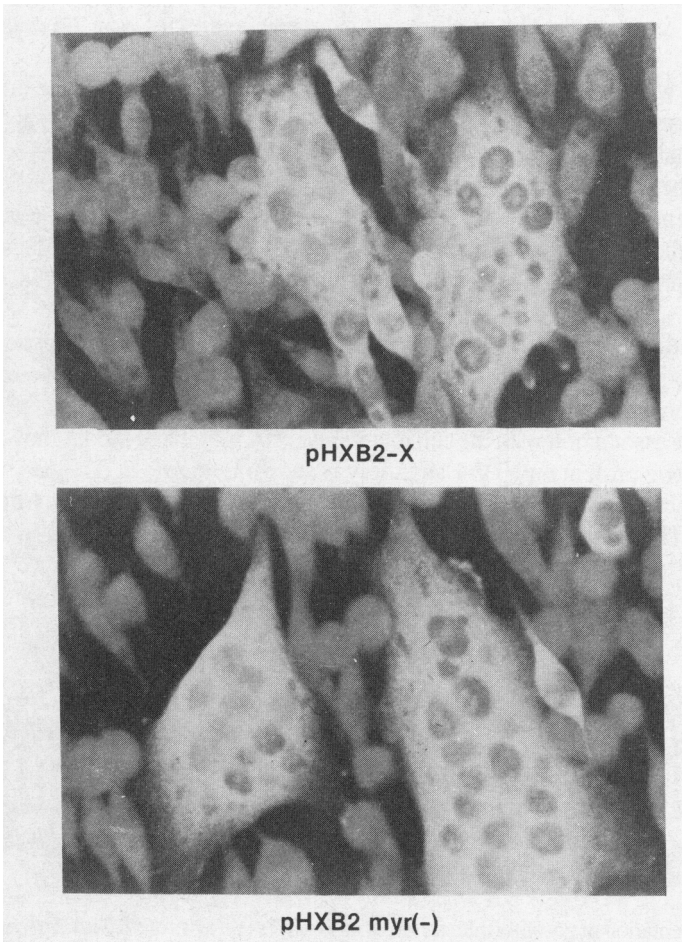


FIG. 5. Syncytia formation in HeLa CD4$^+$ cells transfected with pHXB2-X and pHXB2-*myr*(−). HeLa CD4$^+$ cells were transfected and syncytia were scored after 15 h.

DISCUSSION

We have reported on the alteration of the N-terminal glycine of HIV-1 $Pr53^{gag}$ to an alanine. This change abolished myristoylation of *gag* proteins and resulted in complete disruption of virus assembly. A very recent study by Gottlinger et al. also noted similar inhibition of HIV-1 morphogenesis when the myristoylation site was altered in the *gag* gene.[14] As shown in this study, unmyristoylated *gag* polyprotein p53 was cleaved to p17 and p24, suggesting that the proteolytic processing of *gag* gene products occurs before virus assembly. Preliminary pulse-chase studies indicated a slower rate of cleavage of *gag* proteins in cells transfected with the mutant DNA compared with the one transfected with the wild-type DNA (F. di Marzo Veronese, unpublished observations). It is possible that the myristate in the *gag* polyproteins may be important for their interactions with the intracellular membrane where p53 cleavage occurs. Failure to present precursor proteins to the viral protease in a lipid environment may result in slower cleavage. In the MuLV system, similar mutation of the *gag* gene completely abrogated cleavage of $Pr65^{gag}$.[3,5] We do not rule out the possibility that the processing of *gag* proteins in cells transfected with pHXB2-*myr*(−) DNA may be due to cellular proteases. However, it is unlikely that an efficient and specific cleavage of viral *gag* polyproteins could be mediated by cellular enzymes. Furthermore, mutation in the viral protease gene completely inhibited the processing of *gag* proteins with no evidence of an involvement of cellular proteases.[14] Thus, if we assume that the viral protease is mediating the cleavage of the unmyristoylated *gag* precursors, this would suggest that *gag* proteolytic processing can occur before virus assembly. This possibility would be hard to explain in light of the widely accepted mechanism of assembly for type C retroviruses which involves postbudding processing of *gag* precursors. However, it is possible that multiple or alternate modes of *gag* processing might exist in HIV-1-infected cells.

Cos-1 cells transfected with the mutant genome showed strong immunofluorescence on the cell surface and formed syncytia when cocultured with $CD4^+$ cells. This suggests that the envelope glycoprotein gp160 of HIV-1 is processed normally in cells transfected with the mutant genome, and the glycoproteins gp120 and gp41 are transported to the cell membrane in the absence of myristoylated *gag* proteins. It is clear from this result that the envelope and *gag* proteins of HIV-1 are transported to the cell surface in infected cells with dissimilar kinetics and these proteins mature independently of each other. Indeed, similar independent maturation of envelope proteins in the absence of *gag* gene products has been noted in murine leukemia virus.[15]

The absence of any budding virions from *cos*-1 cells transfected with the mutant genome clearly suggests that the myristic acid in *gag* proteins plays a significant role in virus assembly. It is not clear how the myristate modification of the *gag* protein can influence virus assembly. It has been shown that the myristate moiety in $pp60^{src}$ is necessary for its association with the cell membrane.[16] A schematic model showing the possible role of myristoylation of the *gag* protein on HIV-1 assembly is shown in Figure 6. The fatty acyl residue on p17 could provide a hydrophobic domain at the N-terminus, thus facilitating its interaction with the lipid bilayer on the plasma membrane. The myristate residue at the N-terminal region of *gag* could ensure that the p17

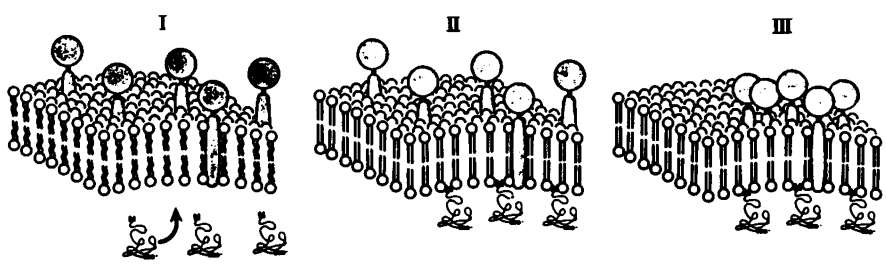


FIG. 6. Schematic representation of possible interaction of myristoylated *gag* protein with the lipid bilayer in the plasma membrane. The different stages of this interaction are: (I) transport of *gag* protein molecules to the plasma membrane, (II) interaction with the lipid bilayer leading to an increase of membrane rigidity, and (III) molecular clustering of viral glycoproteins in the bilayer.

molecules are aligned parallel to each other, with their N termini in the membrane and their carboxy termini projecting into the cytoplasm. This interaction of p17 with the lipid bilayer may decrease membrane fluidity, which in turn, may lead to molecular clustering of glycoproteins on the plasma membrane. The site on the plasma membrane where glycoprotein molecules are concentrated may become the nucleation site for virus assembly and budding. Indeed, a similar model of virus assembly was proposed for vesicular stomatitis virus where viral matrix (M) protein can perform the function of p17.[17–19] Although this study suggests a crucial role of myristic acid in the assembly of HIV-1, it should be noted that *gag* gene products of avian-type C viruses and some lentiviruses, are not myristoylated, arguing that other structural components may perform the same function as myristate.

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