An Early Stage of Mason-Pfizer Monkey Virus Budding Is Regulated by the Hydrophobicity of the Gag Matrix Domain Core

Elizabeth Stansell, Ewan Tytler,[†] Mark R. Walter, and Eric Hunter^{*}

Department of Microbiology and Center for AIDS Research, University of Alabama at Birmingham, Birmingham, Alabama 35294

Received 24 November 2003/Accepted 12 January 2004

Intracellular capsid transport and release of Mason-Pfizer monkey virus are dependent on myristylation of the Gag matrix domain (MA). A myristylated MA mutant, in which Thr41 and Thr78 are replaced with isoleucines, assembles capsids that are transported to the plasma membrane but are blocked in an early budding step. Since the nuclear magnetic resonance structure of MA showed that these Thr residues point into the hydrophobic core of the protein, it was hypothesized that the T41I/T78I mutant was defective in release of myristic acid from the more hydrophobic core. In order to further investigate whether an increase in the hydrophobicity of the MA core modulates capsid-membrane interactions and viral budding, three tyrosine residues (11, 28, and 67), oriented toward the MA core, were replaced individually or in a pair-wise combination with the more hydrophobic phenylalanine residue(s). As a control, Tyr82, oriented toward the outer surface of MA, was also replaced with phenylalanine. These Tyr-to-Phe substitutions did not alter capsid assembly compared to wild type in a capsid assembly assay. Pulse-chase, immunofluorescence, and electron microscopy studies demonstrated that single substitutions of Tyr11, Tyr28, and Tyr67 recapitulated the T41I/T78I mutant phenotype of decreased budding kinetics and accumulation of capsids at the plasma membrane. MA double mutants with a combination of these Tyr substitutions exhibited a phenotype that was even more defective in budding. In contrast, MA mutants with Tyr82 replaced by Phe resulted in a transport-defective phenotype. These results strongly support the hypothesis that myristic acid is sequestered inside MA prior to capsid-membrane interactions.

Mason-Pfizer monkey virus (M-PMV), a betaretrovirus, assembles an immature protein shell (capsid) from its Gag polypeptides in a pericentriolar region of the cytoplasm prior to plasma membrane transport and viral budding (14, 27, 32). In contrast, viruses such as human immunodeficiency virus (HIV) assemble an immature capsid as they bud at the plasma membrane (14). This means that instead of simultaneously extruding the membrane as they assemble, assembled betaretrovirus capsids must interact with the plasma membrane in a way that stimulates wrapping of the spherical structure with the lipid bilayer. The matrix (MA) domain of the M-PMV Gag polypeptide plays a critical role in this process (25), as well as in directing translating polysomes to the intracellular assembly site (32) and subsequent transport to the plasma membrane (8, 25, 27, 32). MA is cotranslationally modified with myristic acid, a 14-carbon saturated fatty acid, and while myristylation of this domain is dispensable for capsid assembly, it is required for transport and release of capsids (26, 29).

Based on a nuclear magnetic resonance (NMR) analysis of the M-PMV matrix protein, which is comprised of four helical domains arranged in two perpendicularly aligned pairs (9), and on an MA mutant (T41I/T78I) that accumulated immature capsids at the plasma membrane (25), it was previously hypothesized that the myristic acid moiety is sequestered within the

MA domain prior to plasma membrane interactions (9). T41I/T78I immature capsids are assembled and transported to the plasma membrane with wild-type kinetics but are defective at an early stage of budding (25). Because the structural analyses showed that both of the threonine residues replaced in this mutant are oriented toward the core of the matrix protein, it was reasoned that substitution with the hydrophobic isoleucine residues might prevent release of myristic acid from the more hydrophobic MA core, even after the T41I/T78I capsid interacted with the lipid bilayer (9).

Sequestration of the covalently attached myristate into the hydrophobic interior of the M-PMV matrix domain is consistent with the cytosolic conformation of a protein that exhibits a myristyl switch mechanism (1, 38). This mechanism is an induced change in the orientation of myristic acid from the hydrophobic interior of a protein to the hydrophobic interior of the plasma membrane, and it has been well described for recoverin, a myristylated cellular protein found in retinal rod cells (2, 24, 38, 41). NMR structures of myristylated recoverin have shown that the myristyl group is buried in the center of a nonfunctional helix-loop-helix, the EF hand-like motif (38). When calcium binds the two functional EF hand motifs of the protein, the myristate is extruded from the protein core and is available for insertion into the plasma membrane (2). The structural data for myristylated recoverin first defined the myristyl switch mechanism that mediates protein membrane interactions.

A second example of a myristyl switch mechanism has been described for the myristylated alanine-rich protein kinase C substrate (MARCKS), which undergoes an electrostatic myristyl switch (18, 30). In this case, myristate is sequestered within

^{*} Corresponding author. Mailing address: University of Alabama at Birmingham, 845 19th St. South, BBRB 256, Birmingham, AL 35294-2170. Phone: (205) 934-4321. Fax: (205) 934-1640. E-mail: ehunter@uab.edu.

[†] Present address: Department of Surgery, University of Alabama at Birmingham, Birmingham, AL 35294.

the protein core prior to an electrostatic association of the positively charged, basic effector domain of MARCKS with acidic phospholipid head groups on the inner leaflet of the plasma membrane (18). The N-terminal myristylated domain and the basic effector domain (residues 151 to 175) anchor the dephosphorylated MARCKS protein to the plasma membrane, bringing the substrate in close proximity to protein kinase C (4, 12, 16, 37, 39). In vitro studies support this model by demonstrating that lysine residues in the basic effector domain of MARCKS interact with the acidic head groups of phosphatidylserine and phosphatidylinositol 4,5-bisphosphate, which are concentrated in the inner leaflet of the plasma membrane (19, 23, 40).

The presence of myristic acid and a basic domain on many retroviral MA proteins raises the possibility that an electrostatic switch mechanism similar to that of the MARCKS protein occurs during Gag-membrane interactions (9, 21, 35). HIV type 1 (HIV-1) assembles capsids at the plasma membrane, where the structural Gag polypeptide must associate with the lipid bilayer. It is known that covalent attachment of myristate to the matrix domain of HIV-1 Gag is required for this interaction to occur and for capsids to assemble (6, 13). A deletion mutation that disrupts the structure of the HIV-1 matrix domain without altering myristylation redirects capsid assembly and membrane extrusion to the endoplasmic reticulum (10). Moreover, HIV-1 capsid assembly is redirected to the Golgi or post-Golgi vesicles when basic residues on the outer surface of the matrix domain are replaced with acidic residues (20) or when hydrophobic residues that face the core of the matrix domain are replaced with less hydrophobic residues (11). These biochemical data suggest the bipartite signal in the matrix domain of HIV-1 Gag directs protein association with the plasma membrane.

The molecular interactions necessary for a myristylated Gag polypeptide to associate with the plasma membrane and initiate capsid assembly and budding (C-type morphology) or membrane extrusion (B/D-type morphology) are poorly defined. M-PMV is an ideal system to specifically investigate the interaction between the Gag proteins and the plasma membrane, since capsid assembly and budding are spatially and temporally separate for this prototype D-type retrovirus. To provide support for the hypothesis that the M-PMV matrix domain undergoes a myristyl switch mechanism and to determine whether substitutions that increase the hydrophobicity of the matrix domain inner core could interfere with virus budding, we have carried out further mutagenesis of this region. To increase hydrophobicity without disrupting structure, three tyrosine residues (at positions 11, 28, and 67) that are spatially oriented toward the protein core were replaced with the more hydrophobic phenylalanine residue. A fourth tyrosine (at position 82), which is oriented towards the exterior of the molecule, was similarly replaced as a control. Single substitutions of any of the first three tyrosine residues to phenylalanine resulted in decreased budding kinetics and accumulation of immature capsids at the plasma membrane. In contrast, replacement of tyrosine 82 with phenylalanine resulted in a defective transport phenotype. These results support a model in which the matrix domain of M-PMV Gag undergoes a myristyl switch at the initial stages of membrane extrusion.

MATERIALS AND METHODS

Model of myristic acid in M-PMV MA. A conceptual three-dimensional model of the M-PMV matrix protein containing myristic acid was generated by molecular graphics. The position of myristic acid in the model was obtained by superimposing the protein coordinates of myristylated recoverin in the calcium-free state onto the nonmyristylated M-PMV structure with the program O (9, 15, 38). The molecular surface for myristic acid and the M-PMV ribbon diagram were generated with the program Ribbons (7).

Cells and antibodies. COS-1 cells were obtained from the American Type Culture Collection (Manassas, Va.) and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Sigma, St. Louis, Mo.), 10 U of penicillin G sodium/ml, and 10 µg of streptomycin sulfate/ml (Pen-Strep; GibcoBRL, Rockville, Md.). Anti-M-PMV mouse monoclonal antibody 10.10, which binds to the p12 domain of M-PMV Gag, was used at a concentration of 12 µg/ml (28). Alexa Fluor 594 goat anti-mouse immunoglobulin G was purchased from Molecular Probes, Inc. (Eugene, Oreg.). Cell nuclei were stained with bis-benzimide (Hoechst no. 33258; Sigma).

Construction of mutant proviruses. Mutant derivatives of the M-PMV proviral vector pSARM4 were constructed in the following manner. An M-PMV *gag* fragment corresponding to nucleotides 351 to 1167 was removed from pSARM4 by *NarI* and *SacI* digestion and ligated into the cloning vector, pBluescript II KS(+) (Stratagene, Cedar Creek, Tex.) that had been digested with *ClaI* and *SacI* to create pNCS. The desired codon(s) was generated in pNCS by PCR-directed mutagenesis. For each single mutant, complementary and reverse-oriented mutagenic primers with a single base pair change compared to the wild-type sequence were designed. Double mutants were generated by including two single mutagenic primer pairs in the PCR. Multiple-round PCR using *Pfu-turbo* DNA polymerase (Stratagene) incorporated the mutation from the primers into the pNCS plasmid. Following amplification, the PCR product was digested with *DpnI* to remove the methylated wild-type template, leaving the mutated pNCS vector intact. The nucleotide sequence of the mutated region of each *gag* construct was determined to confirm that only a single base pair change was introduced, and then the *EagI*-*PacI*-mutated M-PMV *gag* fragment corresponding to nucleotides 407 to 750 was reengineered into the M-PMV proviral expression vector pSARM4 (33).

Metabolic labeling and immunoprecipitation of Gag. COS-1 cells were transfected with wild-type or mutant proviral constructs by the Eugene 6 method (Roche Molecular Biochemicals, Indianapolis, Ind.). Approximately 24 h post-transfection, COS-1 cells expressing the wild-type or mutant M-PMV proviruses were starved for 10 min with methionine- and cysteine-deficient DMEM (Sigma) and then pulse-labeled in six-well plates for 15 min at 37°C with 75 µCi of [³⁵S]methionine-[³⁵S]cysteine protein labeling mix (Perkin-Elmer NEN, Boston, Mass.) in 250 µl of the same medium. The radioactive medium was removed at the end of the pulse period, and cells were chased in complete DMEM for 1, 2, or 4 h. Pulse cells were washed with cold Tris-buffered saline (TBS) and lysed in 1% Triton X-100, 1% sodium deoxycholate, 50 mM sodium chloride, and 25 mM Tris-HCl (pH 8.0) for 5 min at room temperature. Nuclei were removed from lysates by centrifuging for 10 min at 14,000 rpm (Eppendorf 5415C microcentrifuge), and then the supernatants were adjusted to a concentration of 0.1% sodium dodecyl sulfate (SDS). Chase cells were processed in the same manner as the pulse cells. The culture medium of the chase cells was filtered through a 0.45-µm-pore-size filter and then adjusted to 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS. Viral proteins were immunoprecipitated from the pulse, chase, and chase cell media with polyclonal rabbit anti-Pr78 (M-PMV Gag) sera 3492 (28) and separated by SDS-polyacrylamide gel electrophoresis (PAGE).

Quantitation of Gag polypeptide processing. The SDS-12% PAGE gels were dried, and the radiolabeled protein bands were quantitated on a Packard Cyclone system using OptiQuant software (Packard, Meriden, Conn.). For each time point, band intensities for Pr78 (Gag), Pr95 (Gag-Pro), Pr180 (Gag-Pro-Pol), and p27 (CA) were acquired for pulse-labeled cells, pulse-chase cells, and the chase culture medium. The quantitated results of individual band intensities were adjusted to reflect the number of methionine residues present in each protein, divided by the sum of the band intensities, and multiplied by 100 to calculate the percentage of each individual protein. The percent total Gag precursor is the summation of the percent Pr78 (Gag), Pr95 (Gag-Pro), and Pr180 (Gag-Pro-Pol). The percent total CA is the summation of the percent p27 associated with the pulse-chase cells and released into the culture medium. The calculations assume all labeled Gag is incorporated into mature virions and do not take into consideration labeled Gag proteins that undergo degradation. These calculations are sufficient to compare changes in the rate of Gag processing for mutant M-PMV Gag to that of the wild type.

Immature capsid assembly assay. COS-1 cells were transfected with wild-type or mutant proviral constructs as described above. Approximately 24 h posttransfection, cells were pulse-labeled as described previously and then chased in complete DMEM for 30 min. After washing in cold TBS, cells were lysed in 0.5% Triton X-100, 0.5% sodium deoxycholate, 140 mM sodium chloride, 1 mM Na₂EDTA, 100 mM sucrose, and 12.5 mM Tris-HCl (pH 8.0) for 5 min at room temperature, and nuclei were removed from the lysate as described above. Cell lysates overlaying a 35% (wt/vol) sucrose cushion were centrifuged at 100,000 × *g* for 30 min at 4°C. Following centrifugation, the unassembled soluble Gag polyprotein (supernatant) was adjusted to 0.1% SDS. The assembled Gag polyprotein (pellet) was disrupted in 50 µl of 1% SDS in phosphate-buffered saline (PBS) and then adjusted to 0.1% SDS, 1.0% Triton X-100, 0.5% sodium deoxycholate, 50 mM sodium chloride, and 25 mM Tris-HCl (pH 8.0). Viral proteins were immunoprecipitated as described previously and separated by SDS-PAGE.

Quantitation of percent Gag incorporated into capsids. Protein bands on SDS-PAGE gels were quantitated as described above, and then band intensities for Pr78 were acquired for the assembled and unassembled Gag polyprotein. The percentage of Pr78 assembled into capsid versus unassembled Pr78 was calculated for wild type and each mutant by dividing the intensity of each fraction by the total intensity of Pr78 for both assembled and unassembled Gag. Where some processing of Gag was observed, the p27 intensity associated with the cell was included in the calculation of total assembled Gag.

Immunofluorescence microscopy. COS-1 cells expressing wild-type or mutant M-PMV proviruses were grown on 22-mm glass coverslips and fixed for 5 min in freshly prepared 4% paraformaldehyde in PBS. After fixation, the remaining paraformaldehyde was quenched by addition of 10 mM NH₄Cl, and then cells were washed in PBS and permeabilized by 0.01% Triton X-100. The cells were washed in PBS and blocked for 10 min with 2.5% goat serum and 0.2% Tween 20 in PBS. Subsequently, the cells were blocked for 10 min with 0.4% fish skin gelatin and 0.2% Tween 20 dissolved in PBS. Coverslips were incubated in 80 µl of a 12-µg/ml solution of monoclonal antibody 10.10 diluted in 2.5% goat serum and 0.2% Tween 20 in PBS for 45 min at 37°C. Then, the cells were washed with 0.2% Tween 20 in PBS and blocked a second time as described above. The fluor-conjugated secondary antibody was diluted 1:100 (20 µg/ml) in 2.5% goat serum and 0.2% Tween 20 in PBS, and the coverslips were incubated in 100 µl for 30 min at 37°C. The cells were then washed with 0.2% Tween 20 in PBS, and the nuclei were stained with bis-benzimide (Hoechst 33258) diluted 1:1,000 in 0.2% Tween 20 in PBS. After three additional washes in PBS, the coverslips were mounted in 9:1 glycerol-PBS containing 0.1% *q*-phenylenediamine to prevent quenching. Images were visualized with an Olympus 1X70 fluorescence microscope. Images of optical sections (300 nm) were captured with a cooled monochrome Qimaging Retiga 1300 camera. The images were deconvoluted and analyzed using IPLab Spectrum software (Scanalytics Inc., Fairfax, Va.).

Electron microscopy. COS-1 cells expressing wild-type or mutant M-PMV were fixed in the dark in electron microscopy-grade 1% glutaraldehyde, 2% osmium tetroxide, 2 mM CaCl₂, 4 mM MgSO₄ in 0.1 M cacodylate buffer (pH 7.2) for 5 min at 37°C followed by 20 min on ice. The samples were washed three times in 0.1 M cacodylate buffer (pH 7.2) and then incubated in 2% osmium tetroxide for an additional hour at room temperature. Samples were washed and incubated in 0.5% tannic acid at room temperature for 10 min and then stained in 1% aqueous uranyl acetate and lead citrate. Cells were dehydrated in a graded ethyl alcohol series and embedded on a copper grid. Approximately 90-nm sections of the cells were analyzed with a Hitachi 7000 series electron microscope at an acceleration voltage of 75 kV.

RESULTS

Construction of M-PMV matrix mutants. To investigate whether an increase in the hydrophobicity of the internal core of matrix altered the efficiency and kinetics of viral budding, three tyrosine residues (11, 28, and 67) that are predicted to face into the interior of the matrix domain and one (residue 82) that is oriented towards the exterior (Fig. 1) were replaced individually and in a pair-wise combination with the more hydrophobic phenylalanine residue(s). Replacement of tyrosine with phenylalanine involves the loss of a single hydroxyl group and would not reduce the bulk of the amino acid, but it does increase the hydrophobicity. Following confirmation that correct mutations were introduced, the M-PMV MA mutants

Y11F, Y28F, Y67F, Y82F, Y11F/Y28F, Y11F/Y67F, Y11F/Y82F, Y28F/Y67F, Y28F/Y82F, and Y67F/Y82F as well as the previously described matrix mutant T41I/T78I were studied in the context of an infectious provirus.

Synthesis and expression of wild-type and mutant M-PMV Gag. To determine whether the mutants with an increased hydrophobic core were translated as stable proteins within the cell, a pulse-chase assay was conducted. COS-1 cells transfected with wild-type and mutant M-PMV proviruses were pulse-labeled with [³⁵S]methionine and then chased for 4 or 24 h in complete medium. Cell lysates from pulse-labeled cells and pulse-chase cells, as well as culture medium from the latter, were collected. M-PMV gag gene products were immunoprecipitated as described in Materials and Methods. Samples were subjected to SDS-PAGE and imaged using a phosphor screen.

During the pulse, M-PMV Gag polyprotein (Pr78) was synthesized for each mutant at levels similar to those for the wild type (Fig. 2). After a 4-h chase, levels of M-PMV Gag polyprotein equivalent to that of the wild type were observed for each mutant. The Gag polyprotein is proteolytically cleaved into MA (p10), pp16/18, p12, capsid (CA; p27), nucleocapsid (NC; p14) and p4 after release of virions (5, 22, 34). Therefore, release of virus was assessed by measuring the extent to which Gag was processed into capsid protein (p27) during the chase. For mutants Y11F and Y82F, wild-type levels of cell-associated and virion-associated p27 were observed (Fig. 2B and C), while mutants Y67F and Y11F/Y82F exhibited less p27 than did the wild type, suggesting that these mutants release virions less efficiently. The double mutants T41I/T78I and Y11F/Y67F released lower levels of capsid protein than the Y67F mutant. Neither processing nor release of Gag was detected after 4 h for Y28F, Y11F/Y28F, Y28F/Y67F, Y28F/Y82F, and Y67F/Y82F. In a second series of experiments, variable processing of Gag was observed for all mutants after a 24-h chase (data not shown), indicating that the lower rate of Gag processing into capsid protein is the result of a delay in the release of mature M-PMV virions.

Increasing hydrophobicity of M-PMV matrix domain hydrophobic core decreases kinetics of virus release. To better define the kinetics with which mutant Gag proteins were released and processed, a pulse-chase experiment with multiple chase times (1, 2, and 4 h) was carried out. This laboratory has previously shown that Gag precursor processing and virus release are temporally linked and that in the absence of capsid release Gag processing is not observed (25). Following analysis of samples with SDS-PAGE, OptiQuant software was used to obtain the band intensities for Gag (Pr78), Gag-Pro (p95), Gag-Pro-Pol (p180), and capsid (p27). The percentage of total Gag precursor and the percent capsid (p27) relative to total Gag were calculated for each chase time point as described in Materials and Methods. The processing half-life of total M-PMV Gag precursors in this experiment was under 3 h for the wild type (Fig. 3A). Matrix mutants Y11F (Fig. 3B) and Y82F (data not shown) were released with slower kinetics than wild type (*t*_{1/2} of 3.3 and 3.8 h, respectively). Mutants Y67F (Fig. 3C) and Y11F/Y82F (data not shown) have delayed kinetics compared to wild type, with only 20% of Gag processed after the 4-h chase. Approximately 5% of total Gag was processed for T41I/Y78I and Y11F/Y67F after 4 h (data not shown). For

FIG. 1. Location of tyrosine-to-phenylalanine substitutions in the M-PMV matrix domain. (A) Schematic diagram of the M-PMV Gag polyprotein. Myristate is shown attached to the N-terminal MA domain of Gag. The primary sequence of MA is shown, with tyrosine residues 11, 28, 67, and 82 circled. (B) Conceptual model of myristic acid in the NMR structure of M-PMV matrix protein. The myristic acid moiety from the structure of myristylated recoverin in the calcium-free state (37) was modeled into the NMR structure of the nonmyristylated M-PMV matrix protein (9). The side chains of tyrosine residues 11, 28, and 67 are oriented toward the hydrophobic interior of the matrix protein, while the side chain of tyrosine 82 is oriented away from the core.

all mutants that contained the Y28F substitution, processing of Gag was not detected after a 4-h chase (Fig. 3D).

M-PMV matrix domain mutants assemble immature capsids similar to wild type. To investigate whether the delayed processing and release of Gag is observed because fewer capsids assemble within the cell, an immature capsid assembly assay was conducted (Fig. 4). This assay measures the percentage of pulse-labeled Gag incorporated into pelletable capsids during a 30-min chase. Cells were lysed after the chase, and a sucrose cushion was used to separate Gag incorporated into assembled immature capsids from soluble, unassembled Gag proteins. Following separation of proteins by SDS-PAGE, the band intensity of Pr78 was measured for wild type and each mutant using phosphorimaging. Eighty percent of pulse-labeled Gag polyprotein was incorporated into immature capsids for wild type and A18V, a mutant that accumulates capsids in the cytoplasm (25). R55W, a mutant that assembles capsids at

the plasma membrane (27), demonstrated only 50% Gag polyprotein was incorporated into capsids after the 30-min chase. In contrast, in cells expressing the major homology domain mutant, L163H, which does not assemble immature capsids (28, 36), less than 20% of Gag was pelletable. A pattern indistinguishable from that of the wild type was observed for each of the tyrosine mutants, demonstrating that the decreased budding kinetics observed in the pulse-chase experiments is not due to assembly of fewer immature capsids.

Immunofluorescence of M-PMV-infected cells. Immunofluorescence was employed to investigate whether the mutants that have delayed virion release are blocked at an intracellular transport or membrane extrusion stage in the virus assembly pathway. The distribution of M-PMV Gag in transiently transfected COS-1 cells was probed with a monoclonal antibody directed against the p12 region of the Gag polyprotein.

Based on the distribution of anti-p12, wild-type M-PMV-

FIG. 2. Synthesis and processing of wild-type and mutant M-PMV Gag. COS-1 cells were transfected with wild-type and mutant M-PMV proviral genomes. Viral proteins were metabolically labeled with [35 S]methionine-cysteine and then immunoprecipitated from cell lysates and analyzed by SDS-PAGE. (A) Autoradiogram of proteins immunoprecipitated from pulse-labeled cell lysates. Positions of the wild-type (WT) and mutant viral precursor proteins Pr78, Pr95, and Pr180 are shown. (B) Viral proteins immunoprecipitated from lysates of cells pulse-labeled and then chased for 4 h. The positions of the precursors and major cleavage product of Gag (p27) are shown. (C) Viral proteins immunoprecipitated from the culture medium collected after the 4-h chase.

expressing cells exhibited dispersed cytoplasmic staining (Fig. 5A). In contrast, defined staining of the plasma membrane was observed in cells expressing the T41I/T78I MA mutant (Fig. 5B) in addition to cytoplasmic staining of Gag. A similar anti-p12 pattern with both cytoplasmic and plasma membrane staining was observed for two of the single mutants Y11F (data not shown) and Y28F (Fig. 5C) and two double mutants, Y11F/Y28F and Y28F/Y67F (data not shown). In contrast to cells infected with wild-type virus, the periphery of each cell expressing these mutant proteins was outlined by fluorescently stained Gag. This finding is consistent with these mutations interfering with budding of virions from the plasma membrane. In addition to the dispersed cytoplasmic Gag staining observed for wild-type virus-expressing cells, mutants Y82F and Y67F/Y82F exhibited concentrated staining in the pericentriolar region of the cell (Fig. 5D, Y67F/Y82F). Thus, these mutations appear to interfere with transport of immature capsids from the pericentriolar assembly region. Increased pericentriolar and plasma membrane staining relative to wild type was observed for mutants Y11F/Y82F (Fig. 5E), Y67F, and Y11F/Y67F, suggesting that both transport and budding might be defective in these mutants.

Electron microscopy of M-PMV-infected cells. To determine the location of assembled immature capsids at higher resolution, thin sections of M-PMV-infected cells were analyzed by electron microscopy. Cells expressing wild-type M-PMV exhibited few immature capsids in the cytoplasm (data not shown) and large numbers of mature virions released into the intracellular space (Fig. 6A, WT). Cells expressing the T41I/T78I mutant provirus exhibited an accumulation of immature capsids at the plasma membrane (Fig. 6B, T41I/T78I). A similar pattern of immature capsid distribution was observed for three

FIG. 3. Kinetics of Gag precursor processing and virion release from cells. COS-1 cells transfected with an M-PMV proviral genome were metabolically labeled and then chased for 0, 1, 2, and 4 h. Viral proteins were immunoprecipitated from cell lysates and culture medium at each time point and, following SDS-PAGE, were quantitated. Solid squares represent the percentage of the total Gag precursor at each time point. Open triangles represent percent total capsid protein. (A) Wild type; (B) Y11F; (C) Y67F; (D) Y28F.

of the single mutants (Y11F, Y28F, and Y67F) and one double mutant (Y11F/Y67F), although in general fewer capsids were present at the plasma membrane (Fig. 6C, Y11F). Moreover, the Y11F/Y28F and Y28F/Y67F double mutants recapitulated the T41I/T78I phenotype, with large numbers of immature capsids accumulated at the plasma membrane (Fig. 6D, Y11F/Y28F). All of the mutants with a substitution at position 82 appeared to be somewhat defective in intracellular transport, since immature capsids were mainly dispersed throughout the cell (Fig. 6E, Y11F/Y82F). This phenotype was not observed for the Y28F and Y11F mutants. Occasional intracellular ac-

FIG. 4. Assembly of wild-type and mutant immature capsids. COS-1 cells were metabolically labeled for 10 min and then chased at 37°C for 30 min. Cells were lysed, and Gag precursors were centrifuged through a 35% sucrose cushion. Unassembled Gag remained on top of the sucrose cushion, while assembled Gag pelleted through the cushion. M-PMV Gag polypeptides were immunoprecipitated from soluble and pellet fractions and were analyzed by SDS-PAGE. The percentages of wild-type (WT) and mutant M-PMV Gag polypeptide in soluble and pelletable fractions were calculated using quantitation of band intensities by OptiQuant phosphorimaging. The mean of three experiments ($\pm$ standard deviation) is shown. $\square$, unassembled Gag; $\blacksquare$, assembled Gag.

cumulations of immature capsids were also observed for Y67F and the Y82F double mutants (Fig. 6F, Y28F/Y82F).

DISCUSSION

In this study, the hydrophobicity of the M-PMV Gag matrix interior domain was modified to be more hydrophobic to determine whether this would interfere with budding of capsids at the plasma membrane. Substitution of three tyrosine residues that are predicted to be oriented toward the interior of the MA domain with a phenylalanine residue, singly (Y11F, Y28F, and Y67F) or in combination, reduced the rate of capsid envelopment and release without affecting the rate of capsid assembly. These data support the concept that the interaction between the N-terminal myristate moiety and the hydrophobic core of the M-PMV Gag matrix domain modulates the initial steps of membrane extrusion, or budding.

In contrast to the substitution of the three interior tyrosines, Y11, Y28, and Y67, replacement of tyrosine 82, which is predicted to be oriented toward the exterior of the MA domain, with phenylalanine delayed the intracellular transport of immature capsids. Y82F immature capsids were primarily dispersed throughout the cytoplasm, with some accumulating at the pericentriolar region of the cell, even though these mutant capsids were released with kinetics only 30% slower than the wild type. Few capsids were observed in the process of budding at the plasma membrane for this mutant. These observations suggest that the Y82F substitution alters the tertiary structure of MA in a manner similar to the previously characterized A79V transport-defective matrix mutant (25). However, the A79V MA mutant exhibits a greater defect and accumulates large numbers of immature capsids at a location in the cytoplasm (25). This difference is reflected in the rate of virion release for the two mutants, with Y82F releasing 50% of pulse-labeled Gag in approximately 4 h while the A79V mutant requires more than 12 h (25). This laboratory has recently

FIG. 5. Immunofluorescent staining of M-PMV Gag. COS-1 cells transfected with a wild-type or mutant M-PMV proviral genome were fixed 24 h later and probed with a monoclonal antibody directed against the p12 region of the Gag polypeptide. Arrowheads denote Gag stain at the plasma membrane, while arrows denote Gag stain at the pericentriolar region of the cell. Bar, 40 μ m. (A) Wild type; (B) T41I/T78I; (C) Y28F; (D) Y67F/Y82F; (E) Y11F/Y82F.

FIG. 6. Thin-section electron microscopy of COS-1 cells expressing wild-type and mutant M-PMV proviruses. COS-1 cells transfected with wild-type or mutant M-PMV proviral genomes were fixed 24 h later in 1% glutaraldehyde and 2% osmium tetroxide to preserve proteins and membranes, respectively, prior to embedding and thin sectioning. Bar, 200 nm. N, nucleus; PM, plasma membrane. (A) Wild-type; (B) T411/T78I; (C) Y11F; (D) Y11F/Y28F; (E) Y11F/Y82F; (F) Y28F/Y82F.

demonstrated that efficient transport of immature capsids is dependent on a functional endocytic pathway and on the presence of the M-PMV envelope protein (31). Thus, it is possible that tyrosine residue 82 and alanine residue 79 modulate the Gag polyprotein-envelope glycoprotein interaction.

Immature capsids for the MA mutants Y11F, Y67F, and Y28F, which would be predicted to have an increased hydrophobic interior, can be observed underlying the plasma mem-

brane by both immunofluorescence and electron microscopy, consistent with a rate-limiting delay in the early stages of membrane extrusion. It is possible that the increased hydrophobic interior of the MA domain for these mutants sequesters the myristate moiety more efficiently and interferes with the insertion of the myristate into the membrane, although the possibility that these mutations also affect interactions of the basic domain of MA with the plasma membrane cannot be entirely

ruled out. In support of our interpretation, mutations that increased the hydrophobic environment within the N terminus of HIV-1 MA have also been reported to have a detrimental effect on viral budding (20, 21). The phenotypic effects of these HIV-1 MA mutations, which also reduced Gag membrane binding, could be reversed by the substitution of polar or charged residues for conserved hydrophobic residues in the globular core of MA, consistent with effects on myristate exposure. The M-PMV Y28F MA mutant exhibited a phenotype similar to that of HIV-1 MA mutants in which a single conservative substitution increased hydrophobicity and blocked viral budding, since Y28F was essentially defective in budding when compared to wild type, with no detectable levels of processed Gag after a 4-h chase. In contrast to the HIV-1 MA mutants, however, the Y28F mutant had no significant effect on immature capsid assembly. The other two M-PMV MA mutants, Y11F and Y67F, resulted in less drastic phenotypes, even though capsids did accumulate at the plasma membrane. The kinetics of viral protein release for the Y11F mutant are only delayed 30 min compared to wild type. While the Y67F mutant releases 20% of the labeled Gag after 4 h, this delay in capsid release may reflect not only a defect in early membrane extrusion but also in transport, since immature capsids for this mutant also accumulate at the pericentriolar region of the cell. These observations suggest that myristate exposure may be affected differentially by the individual substitutions, perhaps reflecting access of the newly introduced phenylalanine residues into the hydrophobic pocket. This conclusion is supported by the more-extensive defects in virus release and accumulation of immature capsids at the plasma membrane for each of the double mutants that involved these three residues (Y11F/Y67F, Y11F/Y28F, and Y28F/Y67F) and is consistent with the greater predicted increase in the hydrophobic environment of the myristic acid.

The delay in membrane extrusion for the mutants Y11F, Y28F, Y67F, Y11F/Y28F, Y11F/Y67F, and Y28F/Y67F supports a mechanism in which myristic acid is sequestered inside the hydrophobic interior of the M-PMV Gag matrix domain prior to early stages in viral budding. Therefore, we propose that the initial steps of membrane extrusion occur when basic residues on the outer surface of the assembled capsid associate with acidic phospholipid head groups localized on the inner leaflet of the plasma membrane. This electrostatic interaction could induce a conformational change in the matrix domain of Gag in which the myristate moiety changes orientation and embeds into the hydrophobic interior of the lipid bilayer. The electrostatic interactions on one surface of the assembled capsid combined with the insertion of myristic acid in the membrane would facilitate proximal capsid-membrane interactions and provide the driving force for the spherical capsid to be wrapped by the plasma membrane.

Myristate on the N terminus of recoverin is sequestered in a structure composed of five α -helices, which is similar to the α -helical structure of HIV-1 MA and that of M-PMV MA (9, 17, 38). Amino acid substitutions in recoverin that result in a less hydrophobic myristate-binding pocket have been shown to promote the calcium-induced conformation in which the myristate is extruded from the protein (3). In the corollary of this, increasing the hydrophobic environment of the core of the matrix domain could promote sequestration of myristic acid,

thereby inhibiting its release from the core to embed into the plasma membrane. Previous NMR studies demonstrated that the membrane-proximal surface of the M-PMV MA domain was rich in basic residues; thus, it is possible that immature capsids with tyrosine substitutions are able to interact electrostatically with the acidic phospholipid head groups on the inner leaflet of the plasma membrane to induce an abortive conformational change in the matrix. Electron micrographs showing immature capsids tightly apposed to but not extruding the plasma membrane (Fig. 6) support this possibility.

Several questions remain regarding the mechanism by which the combination of basic residues and myristate orchestrates the process of membrane extrusion and virus budding. In particular, it is not clear whether a specific "receptor" molecule analogous to the phosphatidylinositol 4,5-bisphosphate for the MARCKS protein (30, 40) exists at the plasma membrane to trigger myristate exposure or whether the charge-charge interaction between MA and the phospholipid head groups is sufficient to bring the hydrophobic membrane environment in close enough proximity to induce myristate exposure.

In the results presented here, we show that increases in the hydrophobicity of the M-PMV MA core result in progressively greater defects in viral budding, which provides strong support for sequestration of the N-terminal myristic acid of Gag until interactions with the plasma membrane stimulate a myristyl switch essential to capsid envelopment.

ACKNOWLEDGMENTS

We thank Cynthia Derdeyn, Marantha Heil, and Chisu Song for their insight in critically reading the manuscript. We also thank Leigh Millican and Albert Tousson at the UAB High Resolution Imaging Facility; Susan Dubay, Tobitha McKinney, and Tshana Thomas for their technical assistance; as well as Jeff Sfakianos for help with immunofluorescence microscopy.

This work was supported by grant R01 CA-27834 from the National Institutes of Health. E. Stansell was supported by an NIH Institutional NRS A T32-CA09467.

REFERENCES

1. Aderem, A. 1992. The MARCKS brothers: a family of protein kinase C substrates. *Cell* **71**:713–716.
2. Ames, J. B., R. Ishima, T. Tanaka, J. I. Gordon, L. Stryer, and M. Ikura. 1997. Molecular mechanics of calcium-myristoyl switches. *Nature* **389**:198–202.
3. Baldwin, A. N., and J. B. Ames. 1998. Core mutations that promote the calcium-induced allosteric transition of bovine recoverin. *Biochemistry* **37**:17408–17419.
4. Blackshear, P. J. 1993. The MARCKS family of cellular protein kinase C substrates. *J. Biol. Chem.* **268**:1501–1504.
5. Bradac, J., and E. Hunter. 1984. Polypeptides of Mason-Pfizer monkey virus. I. Synthesis and processing of the gag-gene products. *Virology* **138**:260–275.
6. Bryant, M., and L. Ratner. 1990. Myristoylation-dependent replication and assembly of human immunodeficiency virus 1. *Proc. Natl. Acad. Sci. USA* **87**:523–527.
7. Carson, M. 1997. Ribbons. *Methods Enzymol.* **277**:493–505.
8. Choi, G., S. Park, B. Choi, S. Hong, J. Lee, E. Hunter, and S. S. Rhee. 1999. Identification of a cytoplasmic targeting/retention signal in a retroviral Gag polyprotein. *J. Virol.* **73**:5431–5437.
9. Conte, M. R., M. Kikova, E. Hunter, T. Ruml, and S. Matthews. 1997. The three-dimensional solution structure of the matrix protein from the type D retrovirus, the Mason-Pfizer monkey virus, and implications for the morphology of retroviral assembly. *EMBO J.* **16**:5819–5826.
10. 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.
11. Freed, E. O., J. M. Orenstein, A. J. Buckler-White, and M. A. Martin. 1994. Single amino acid changes in the human immunodeficiency virus type 1 matrix protein block virus particle production. *J. Virol.* **68**:5311–5320.

12. George, D. J., and P. J. Blackshear. 1992. Membrane association of the myristoylated alanine-rich C kinase substrate (MARCKS) protein appears to involve myristate-dependent binding in the absence of a myristoyl protein receptor. *J. Biol. Chem.* **267**:24879–24885.
13. Gottlinger, H. G., J. G. Sodroski, and W. A. Haseltine. 1989. Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA* **86**:5781–5785.
14. Hunter, E., J. Casey, B. Hahn, M. Hayami, B. Korber, R. Kurth, J. Neil, A. Rethwilm, P. Sonigo, and J. Stoye. 2000. *Retroviridae*, p. 369–387. In M. H. V. van Regenmortel, C. M. Fauquet, D. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, J. Maniloff, M. A. Mayo, D. J. McGeoch, C. R. Pringle, and R. B. Wickner (ed.), *Virus taxonomy: classification and nomenclature of viruses*. Seventh report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego, Calif.
15. Jones, T. A., J. Y. Zou, S. W. Cowan, and Kjeldgaard. 1991. Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crystallogr. A* **47**:110–119.
16. Kim, J., T. Shishido, X. Jiang, A. Aderem, and S. McLaughlin. 1994. Phosphorylation, high ionic strength, and calmodulin reverse the binding of MARCKS to phospholipid vesicles. *J. Biol. Chem.* **269**:28214–28219.
17. Massiah, M. A., M. R. Starich, C. Paschall, M. F. Summers, A. M. Christensen, and W. I. Sundquist. 1994. Three-dimensional structure of the human immunodeficiency virus type 1 matrix protein. *J. Mol. Biol.* **244**:198–223.
18. McLaughlin, S., and A. Aderem. 1995. The myristoyl-electrostatic switch: a modulator of reversible protein-membrane interactions. *Trends Biochem. Sci.* **20**:272–276.
19. Nakaoka, T., N. Kojima, T. Ogita, and S. Tsuji. 1995. Characterization of the phosphatidylserine-binding region of rat MARCKS (myristoylated, alanine-rich protein kinase C substrate). Its regulation through phosphorylation of serine 152. *J. Biol. Chem.* **270**:12147–12151.
20. Ono, A., J. M. Orenstein, and E. O. Freed. 2000. Role of the Gag matrix domain in targeting human immunodeficiency virus type 1 assembly. *J. Virol.* **74**:2855–2866.
21. Paillart, J. C., and H. G. Gottlinger. 1999. Opposing effects of human immunodeficiency virus type 1 matrix mutations support a myristyl switch model of Gag membrane targeting. *J. Virol.* **73**:2604–2612.
22. Parker, S. D., and E. Hunter. 2001. Activation of the Mason-Pfizer monkey virus protease within immature capsids in vitro. *Proc. Natl. Acad. Sci. USA* **98**:14631–14636.
23. Rauch, M. E., C. G. Ferguson, G. D. Prestwich, and D. S. Cafiso. 2002. Myristoylated alanine-rich C kinase substrate (MARCKS) sequesters spin-labeled phosphatidylinositol 4,5-bisphosphate in lipid bilayers. *J. Biol. Chem.* **277**:14068–14076.
24. Ray, S., S. Zozulya, G. A. Niemi, K. M. Flaherty, D. Brolley, A. M. Dizhoor, D. B. McKay, J. Hurley, and L. Stryer. 1992. Cloning, expression, and crystallization of recoverin, a calcium sensor in vision. *Proc. Natl. Acad. Sci. USA* **89**:5705–5709.
25. Rhee, S. S., 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.
26. Rhee, S. S., and E. Hunter. 1987. Myristylation is required for intracellular transport but not for assembly of D-type retrovirus capsids. *J. Virol.* **61**:1045–1053.
27. Rhee, S. S., 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.
28. Sakalian, M., S. D. Parker, R. A. Weldon, Jr., and E. Hunter. 1996. Synthesis and assembly of retrovirus Gag precursors into immature capsids in vitro. *J. Virol.* **70**:3706–3715.
29. Schultz, A. M., and S. Oroszlan. 1983. In vivo modification of retroviral gag gene-encoded polypeptides by myristic acid. *J. Virol.* **46**:355–361.
30. Seykora, J. T., M. M. Myat, L. A. Allen, J. V. Ravetch, and A. Aderem. 1996. Molecular determinants of the myristoyl-electrostatic switch of MARCKS. *J. Biol. Chem.* **271**:18797–18802.
31. Sfakianos, J. N., and E. Hunter. 2003. M-PMV capsid transport is mediated by Env/Gag interactions at the pericentriolar recycling endosome. *Traffic* **4**:671–680.
32. Sfakianos, J. N., R. A. LaCasse, and E. Hunter. 2003. The M-PMV cytoplasmic targeting-retention signal directs nascent Gag polypeptides to a pericentriolar region of the cell. *Traffic* **4**:660–670.
33. Song, C., and E. Hunter. 2003. Variable sensitivity to substitutions in the N-terminal heptad repeat of Mason-Pfizer monkey virus transmembrane protein. *J. Virol.* **77**:7779–7785.
34. Sonigo, P., C. Barker, E. Hunter, and S. Wain-Hobson. 1986. Nucleotide sequence of Mason-Pfizer monkey virus: an immunosuppressive D-type retrovirus. *Cell* **45**:375–385.
35. Spearman, P., R. Horton, L. Ratner, and I. Kuli-Zade. 1997. Membrane binding of human immunodeficiency virus type 1 matrix protein in vivo supports a conformational myristyl switch mechanism. *J. Virol.* **71**:6582–6592.
36. Strambio-de-Castillia, C., and E. Hunter. 1992. Mutational analysis of the major homology region of Mason-Pfizer monkey virus by use of saturation mutagenesis. *J. Virol.* **66**:7021–7032.
37. Stumpo, D. J., J. M. Graff, K. A. Albert, P. Greengard, and P. J. Blackshear. 1989. Molecular cloning, characterization, and expression of a cDNA encoding the “80- to 87-kDa” myristoylated alanine-rich C kinase substrate: a major cellular substrate for protein kinase C. *Proc. Natl. Acad. Sci. USA* **86**:4012–4016.
38. Tanaka, T., J. B. Ames, T. S. Harvey, L. Stryer, and M. Ikura. 1995. Sequestration of the membrane-targeting myristoyl group of recoverin in the calcium-free state. *Nature* **376**:444–447.
39. Taniguchi, H., and S. Manenti. 1993. Interaction of myristoylated alanine-rich protein kinase C substrate (MARCKS) with membrane phospholipids. *J. Biol. Chem.* **268**:9960–9963.
40. Wang, J., A. Arbuzova, G. Hangyas-Mihalyne, and S. McLaughlin. 2001. The effector domain of myristoylated alanine-rich C kinase substrate binds strongly to phosphatidylinositol 4,5-bisphosphate. *J. Biol. Chem.* **276**:5012–5019.
41. Zozulya, S., and L. Stryer. 1992. Calcium-myristoyl protein switch. *Proc. Natl. Acad. Sci. USA* **89**:11569–11573.