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Capsid -membrane interactions in Mason -Pfizer monkey virus budding.

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CAPSID-MEMBRANE INTERACTIONS IN MASON-PFIZER MONKEY VIRUS BUDDING

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ABSTRACT

Retroviral capsid assembly involves the accumulation of the structural Gag polyprotein in association with genomic RNA at a specific location within an infected cell. Mason-Pfizer monkey virus (M-PMV), the prototypic D-type retrovirus, assembles capsids at the pericentriolar region of the cell. Capsids are transported to the plasma membrane, where M-PMV catalyzes the membrane envelopment of the spherical structure during viral release. The assembly unit of a capsid is the myristylated Gag polyprotein, which is cleaved into six proteins upon maturation of the enveloped virion (p10MA, pp16/18, p12, p27CA, p14NC, and p4). Myristylation of the Gag matrix domain (MA) is essential for transport and release but not assembly of M-PMV. A MA mutant in which threonine residues at positions 41 and 78 were replaced with isoleucine residues assembled capsids that are transported to the plasma membrane but are delayed in an early stage of budding. Since the nuclear magnetic resonance structure of MA showed that these threonine residues are oriented into the hydrophobic core of the protein, it was reasoned that the myristate moiety is sequestered within MA and that this mutant was defective in release of myristic acid from the more hydrophobic core. Analysis of the M-PMV MA structure also identified a positive-charge density on the membrane-proximal surface of MA which is postulated to facilitate the association of MA with acidic phospholipid head groups at the plasma membrane. In this dissertation a genetic approach was used to test the hypothesis that basic amino acids in MA interact with acidic phospholipid head groups on the inner leaflet of the plasma membrane to

trigger the release of myristic acid from the hydrophobic core of MA into the hydrophobic environment of the membrane, anchoring the immature capsid and facilitating viral release. It is reported here that the hydrophobicity of the MA core can modulate M-PMV release and that basic amino acids in M-PMV MA define both the cellular location and the efficiency of virus release. These findings support a model in which the sequestration of myristic acid in MA is regulated by the positive charge of this domain, likely through specific electrostatic interactions.

DEDICATION

I dedicate this thesis to my namesakes, Heather Dawn Tugmon and Felisha Elizabeth Rowlands. May all of your dreams come true!

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LIST OF ABBREVIATIONS

293T	human kidney epithelial cell line
AIDS	acquired immunodeficiency syndrome
AIP1	apoptosis-linked gene-2 interacting protein
AP-2	clathrin-associated protein 2
AP-3	clathrin-associated protein 3
Arf6	ADP ribosylation factor 6
ATP	adenosine triphosphate
BLV	bovine leukemia virus
bp	base pair
CA	capsid domain or capsid protein
capsid	protein shell of a virion
CCD	charge-coupled device
CD63	protein of the tetraspanin transmembrane 4 superfamily
CHR	C-terminal heptad repeat
COS-1	African green monkey kidney fibroblast-like cells
Crm1	chromosomal region maintenance
CT	cytoplasmic tail
CTE	constitutive transport element
CTRS	cytoplasmic targeting/retention signal
Cys	cysteine

DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
DU	dUTP pyrophosphatase
dUTPase	dUTP pyrophosphatase
E1	ubiquitin or ubiquitin-like activating enzyme
E2	ubiquitin or ubiquitin-like conjugating enzyme
E3	ubiquitin or ubiquitin-like protein ligase
EF-hand motif	helix-loop-helix protein structure
ENEN	amino acid sequence denoted in the one letter symbol
enJS56A1	endogenous variant of jaagseike sheep retrovirus
env	envelope gene
Env	Envelope glycoprotein
ESCRT	endosomal sorting complexes required for transport
F-actin	filamentous actin
FITC	fluorescein isothiocyanate
FP	fusion peptide
gag	group specific antigen gene
Gag	group specific antigen polyprotein
GFP	green fluorescent protein
GST	glutathione S-transferase
HA	hemagglutinin
His	histidine

HIV	human immunodeficiency virus
HIV-1	HIV type 1
HTLV	human T-cell leukemia virus
I-domain	interaction domain
IN	integrase protein
KKPKR	amino acid sequence denoted in the one letter symbol
KKRR	amino acid sequence denoted in the one letter symbol
kV	kilovolt
L-domain	late domain
LTR	long terminal repeat
MA	matrix domain or matrix protein
MARCKS	myristylated alanine-rich protein kinase C substrate
MDa	megadaltons
μm	micrometer
MMTV	mouse mammary tumor virus
M-PMV	Mason-Pfizer monkey virus
mRNA	messenger RNA
MTOC	microtubule organizing center
MuLV	Moloney murine leukemia virus
MVB	multi-vesicular body
NC	nucleocapsid domain or nucleocapsid protein

Nedd4	neural precursor-cell expressed, developmentally down-regulated gene 4
NHR	N-terminal heptad repeat
nm	nanometer
NMR	nuclear magnetic resonance
nt	nucleotide
p27	M-PMV capsid protein
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pH	potential of hydrogen
Phe	phenylalanine
PIC	preintegration complex
$PI(3,5)P_2$	phosphatidylinositol 3,5-bisphosphate
PO-1-Lu	endogenous type D retrovirus of langur monkeys
pol	polymerase gene
Pol	polymerase protein domain
poly(A)tail	200-300 adenine nucleotides attached to messenger RNA
pp	phosphoprotein
PPPI	late domain amino acid sequence (one letter symbol)
PPT	polypurine tract
PPXY	late domain amino acid sequence (one letter symbol)
PPXYEPTAP	late domain amino acid sequence (one letter symbol)

PR	protease protein
Pr78	M-PMV Gag polyprotein
Pr95	M-PMV Gag-Pro polyprotein
Pr180	M-PMV Gag-Pro-Pol polyprotein
pro	protease gene
Pro	protease protein domain
pSARM4	M-PMV proviral vector
PT/SAP	late domain amino acid sequence (one letter symbol)
R	repeated sequences
RDR	the RD114/mammalian type D retrovirus receptor
retrovirus	RNA tumor virus
Rev	regulator of expression of viral proteins
RNA	ribonucleic acid
RNAPII	RNA polymerase II
RNase H	ribonuclease H
RRE	Rev response element
RSV	Rous sarcoma virus
RT	reverse transcriptase
SAIDS	simian acquired immunodeficiency syndrome
SAIDS-D	type D retrovirus that causes SAIDS
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SIV	simian immunodeficiency virus
SRV	SAIDS retrovirus
SRV-1	SRV serogroup 1
SRV-2	SRV serogroup 2
SRV-3	SRV serogroup 3/M-PMV
SRV-4	SRV serogroup 4
SRV-5	SRV serogroup 5
SRV-6	SRV serogroup 6
SRV/D	type D retrovirus that causes SAIDS
SRV/D-T	SRV/D isolate from the Tsukuba primate center
SU	surface domain or surface protein
SUMO	small ubiquitin like molecule
TBS	Tris-buffered saline
TCP-1	t-complex peptide 1
Thr	threonine
TM	transmembrane domain or transmembrane protein
TRiC	TCP-1 ring complex
tRNA	transfer RNA
Tsg101	tumor susceptibility gene 101
Tyr	tyrosine
U3	unique 3' end
U5	unique 5' end

Ubc9	ubiquitin conjugating enzyme 9
Vps4	AAA ATPase
WT	wild type
$YP(X)_NL$	late domain amino acid sequence (one letter symbol)
YXXΦ	tyrosine-based endocytosis motif

INTRODUCTION

Retroviridae. The Retroviridae, Coronaviridae, Arteriviridae, Flaviviridae, and Togaviridae comprise the five families of enveloped positive-sense, single stranded ribonucleic acid (RNA) viruses which infect vertebrates (184). Retrovirus particles are spherical structures 80-120 nanometers in diameter and contain a dimer of the 7,000-12,000 ribonucleotide (RNA) genome. A distinguishing feature of the Retroviridae family is the replication of an RNA genome through a deoxyribonucleic acid (DNA) intermediate, a process known as reverse transcription. The concept of reverse transcription was defined through studies on Rous sarcoma virus (RSV), an RNA tumor virus discovered to be the etiological agent of solid tumors in chickens. In 1911, Peyton Rous injected filtered tumor extracts into chickens and observed the cell-free transmission of a sarcoma (312). This work eventually led to the identification of RSV (63, 78). Rous's experimental transfer of RSV was the first evidence that retroviruses could be transmitted horizontally as infectious agents of vertebrates, a common feature of all exogenous retroviruses, although the understanding that this was a virus was not forthcoming for 25 years. In 1936, John Bittner found that mammary carcinoma in mice was caused by the milk-transmitted, filterable agent now known as mouse mammary tumor virus (MMTV) (36). At the time of Bittner's discovery, the idea that a virus could transform normal cells was not widely accepted; this idea would continue to be scrutinized until 1951, when Ludwik Gross showed that leukemia could be transmitted to infant mice by inoculating an extract from a mouse that had leukemia, an effect of murine leukemia virus

(153). The transforming properties of retroviruses would be further confirmed in 1958 when Howard Temin and Harry Rubin showed that a single virus particle could induce discrete foci of transformed cells in cell culture (368). It was through efforts to understand how a single virus could transform a cell and its progeny that Howard Temin pushed for the genetic concept that RNA tumor viruses transferred genetic material into its more stable DNA form in order to transform cells. This idea was supported by homology between the RNA isolated from RSV virions and the DNA in RSV-infected cells (366). Six years later, Temin and Mizutani (367) and, independently, David Baltimore (18) isolated the RNA dependent DNA polymerase, reverse transcriptase (RT), which catalyzes the transcription of RNA to DNA. This seminal discovery that the genetic information of retroviruses flows from RNA to DNA before the DNA is transcribed into RNA and is then translated into protein revolutionized the understanding of transfer of genetic information for molecular biologists. Prior to this finding it was understood that genetic information moves unidirectionally from DNA to RNA to protein (97, 98). After the discovery of reverse transcriptase, the RNA tumor viruses were classified as retroviruses (93).

The existence of endogenous retroviral genomes was first implied when a defective strain of RSV (159) spontaneously released infectious virions after infection of certain chicken cells, indicating the presence and expression of retroviral genetic material from the cellular DNA (112, 160, 381, 395). Endogenous retroviruses occur as expressed or silent forms (1, 3, 220), are archived in cells as complete or partially deleted genomic information (6, 28, 29, 113, 139, 167, 179, 274, 371), and are passed from parent to offspring by Mendelian genetics (2).

Historically, viruses within the Retroviridae were grouped based on the morphology of particle assembly determined by electron microscopic analysis (33, 34). Each newly discovered phenotype was given a letter of the alphabet. Type A retroviruses, which were first described in a plasma-cell tumor of the mouse, formed electron dense intracisternal particles that could be seen within the cell. Type B retroviruses assemble type A particles (capsids) within the cell; these particles are transported to and released from the cell by a process of budding. The released type B retrovirus virions have a morphology in which protein spikes are prominent. Type C retroviruses assemble electron dense protein patches at the plasma membrane where capsids assemble and virions are released. Retroviruses classified as type D are similar to the type B in that they assemble intracellular capsids that are transported to and released from the plasma membrane; however, released virions lack prominent spikes and in this regard are similar to the type C retroviruses (90). These morphologic types of viruses were previously grouped into three subfamilies (Oncovirinae, Lentivirinae, and Spumavirinae); however, with this scheme of classification retroviruses grouped within the Oncovirinae subfamily were not necessarily more related to other viruses grouped within the same subfamily as compared to those grouped in the Lentivirinae or Spumavirinae subfamilies. A taxonomic classification based on the similarity of sequences in the reverse transcriptase gene led to the current seven genera of the Retroviridae family: Alpharetroviruses (avian type C), Betaretroviruses (types B and D), Gammaretroviruses (mammalian type C), Deltaretroviruses (human T-cell leukemia virus (HTLV)), Epsilonretroviruses (fish retroviruses), Lentiviruses (human/simian immunodeficiency virus (HIV/SIV)), and Spumaviruses (foamy viruses) (184).

History of Mason-Pfizer monkey virus. Mason-Pfizer monkey virus (M-PMV) was first isolated in 1970 from a breast carcinoma in a rhesus macaque (90). At the time of isolation, M-PMV was found to be morphologically distinct from previously characterized RNA tumor viruses (retroviruses); thus, it is the original type D retrovirus (90, 199). In the 1970s, following the isolation of M-PMV, viruses morphologically and immunologically similar to M-PMV were isolated from rhesus monkey and human cell lines and tissues (5, 6, 14, 24, 140, 167, 176, 244, 274, 371, 415, 428, 429). Type D endogenous retroviruses of a langur monkey (PO-1-Lu, determined to be antigenically and genetically similar to M-PMV) (371), and of a squirrel monkey were also discovered (167). These findings were the first support that M-PMV was representative of a larger group of viruses. Classification of M-PMV based on taxonomy groups this virus within the betaretrovirus genus, which includes exogenous and endogenous retroviruses of the mouse (type B), sheep, and primates (type D) (184).

Since M-PMV has many similarities with other RNA tumor viruses and was isolated from a tumor, it was originally thought that M-PMV was an oncogenic retrovirus (90, 129, 190). However, experimental transmission of M-PMV into juvenile macaques did not produce tumors but rather resulted in lymphadenopathy, thymic atrophy, weight loss, and death (64, 128). This original inoculation of M-PMV into juvenile macaques was the first evidence that M-PMV can cause an immuosuppressive disease in monkeys; however, at the time the significance of this finding was not appreciated (128). In the early 1980s, it was realized that acquired immune deficiency syndrome (AIDS) in humans had a similar pathology to the disease that had been observed in colonies of macaque monkeys in the United States and that was at that time termed simian acquired

immunodeficiency syndrome (SAIDS) (171, 183, 210, 374). It was shown that SAIDS could be passed to healthy macaques via a filterable agent present in tissue and plasma of sick monkeys, suggesting there was an infectious agent passed to the macaques that developed SAIDS (143, 150, 183, 209, 218, 231). In 1984, an exogenous type D retrovirus that could cause SAIDS in Asian macaques was isolated at the New England primate center (100) and a second was isolated at the California regional primate center (231). The virus isolated at both centers had identical restriction enzyme patterns that were different from M-PMV. The newly isolated virus showed a close antigenic relationship to the core protein of M-PMV in radioimmunoassays but was distinct antigenically from the M-PMV surface protein, supporting the conclusion that this virus is different from the prototype D retrovirus (231). This virus was therefore named the SAIDS retrovirus (SRV) (231). An exogenous type D virus was also isolated from a macaque with SAIDS at the Washington primate center (355) and from a Celebes black macaque at the Oregon center (230). In addition to inducing SAIDS, this virus was found to be associated with retroperitoneal fibromatosis, proliferation of fibrous tissue in the abdominal cavity (143, 230, 355, 373, 374). This new type D retrovirus was found to be antigenically different from M-PMV by radioimmunoassay and nucleic acid hybridization (355). Later, these strains were differentiated serologically (230, 355) and it was determined that the SRV isolated at the New England and the California primate centers (SRV-1) is distinct from the SRV isolated at the Washington and Oregon primate centers (SRV-2). Cross-neutralization data showed that each of these strains was serologically distinct from M-PMV, which was designated SRV-3, with antibodies directed toward SRV-1 not reacting with SRV-2 but partially recognizing M-PMV (57, 230). Each of these strains; SRV-1,

SRV-2, and SRV-3; has been molecularly cloned and the sequences have been determined (20, 291, 347, 369). In the mid 1980s, a fourth serologically distinct isolate (SRV-4) was acquired from a cynomolgus macaque at the University of California, Davis. This strain was not characterized fully and it is not known if it is genetically distinct from the other SRV strains (229). SRV-5 was isolated at the Oregon Primate Research Center from a rhesus macaque that had been imported from the People's Republic of China, and partial sequence data have been reported (212). Reports of sequences of SRV-6, obtained from an Indian spectacled langur, have been shown to be genotypically distinct from SRV serogroups 1, 2, 3, 4, and 5 (252, 253) and genotypically similar to PO-1-Lu (338). Most recently, the full sequence of a type D retrovirus isolated from cynomolgus macaques showing clinical signs of SRV/D infection at the Tsukuba primate center has been reported with sequence comparison to SRV-1, SRV-2, and SRV-3 (161). It is not known if this virus is different serologically from the previous six strains and this strain is currently denoted as SRV/D-T until further classification studies are conducted (161).

Analysis of the SRV species led to the understanding that these retroviruses are genetically unrelated to HIV (65), the virus isolated from patients with AIDS (22, 288), which targets T-lymphoid cells to overcome the human immune system. The SAIDS retroviruses target and deplete both B- and T-lymphoid cells (64, 66, 374), are less restricted in cell tropism (151, 236), and quickly elicit a pathology closely resembling that of terminal AIDS (64, 170, 210, 231, 235, 374). While the SAIDS retroviruses were not an applicable animal model for the study of the human immunodeficiency disease, the simian immunodeficiency virus was characterized in 1985 as being genetically

similar to HIV (101, 193), as well as eliciting a disease progression similar to that of HIV. SIV is now the best animal model for understanding the pathogenesis of HIV (99). After the discovery of SIV, the SRV strains were no longer referred to as the SAIDS retroviruses but are either denoted as SRV-1 to SRV-6 or referred to as the SAIDS-D or type D simian retroviruses.

Since SRV strains can cause fatal immunodeficiency, it is necessary to protect the captive monkey population from SAIDS-D disease. Vaccines using recombinant protein or killed virus have been shown to protect monkeys from SRV-1, SRV-2 and SRV-3 (57, 177, 232) and have facilitated protection of the captive monkey population (12, 201). This population is still screened for the presence of SRVs, first by an enzyme-linked immunosorbent assay or a western blot (88, 208, 219, 277, 350), and more recently by a rapid polymerase chain reaction (PCR)-based assay (207, 326, 392, 408).

In addition to eliciting type D retroviral immunosuppression (38, 64), a topic that has been insufficiently studied for years (229), M-PMV undergoes a process of morphogenesis that facilitates studies of the molecular interactions necessary to assemble and release infectious virions. A striking characteristic of M-PMV replication is the intracytoplasmic assembly of the capsid shell, which occurs in the proximity of the nucleus and the centrioles. The assembled capsids are trafficked through the cytoplasm to the plasma membrane, where the capsid associates with the glycoprotein-containing lipid bilayer to extrude the membrane and pinch off from the cell. The temporal and spatial separation of M-PMV capsid assembly, intracellular trafficking, and membrane envelopment facilitates the demarcation of the assembly processes of a betaretrovirus.

The mature virion. M-PMV, like all retroviruses, forms a spherical, enveloped virion with an electron dense rim and an electron dense core. The diameter of a virion is heterogeneous and ranges from 80 to 110 nm, and the weight of any single retrovirus ranges from 164 to 174 MDa as determined by scanning transmission electron microscopy (273). The electron dense core of a retroviral virion is often cylindrical (M-PMV) or conical (HIV) in shape and centered within the virion; however, for the prototype betaretrovirus, MMTV, the core is spherical and located eccentrically (Fig. 1). At the center of the condensed core are two copies of the single-stranded, positive sense genomic RNA which are noncovalently linked together (184, 382). For M-PMV, each single strand of RNA is approximately 8,000 bases long (20, 21, 347), which is in the range, 7,000 to 12,000 bases, of genomic RNA for members of the Retroviridae (184, 382). The genome of M-MPV contains four open reading frames or genes common to all retroviruses (Fig. 1) (206, 347, 382). These are *gag*, the group specific antigen in which the major structural proteins matrix (MA), capsid (CA), and nucleocapsid (NC) are encoded; *pro*, which for M-PMV encodes the enzymes dUTP pyrophosphatase (dUTPase; DU) and protease (PR); *pol*, in which the RT and the integrase (IN) enzymes are encoded; and *env*, which encodes the surface (SU) and transmembrane (TM) viral envelope proteins. For simple retroviruses like M-PMV, these common genes encode all of the viral components necessary to generate mature infectious virions. Some retroviruses (for example, HTLV, HIV, SIV, and the Epsilonretroviruses) encode accessory genes in addition to these four common viral genes and these viruses are known as complex retroviruses (184). In addition to containing the coding sequences, the viral genomic RNA contains sequences necessary for replication of the genome as well as

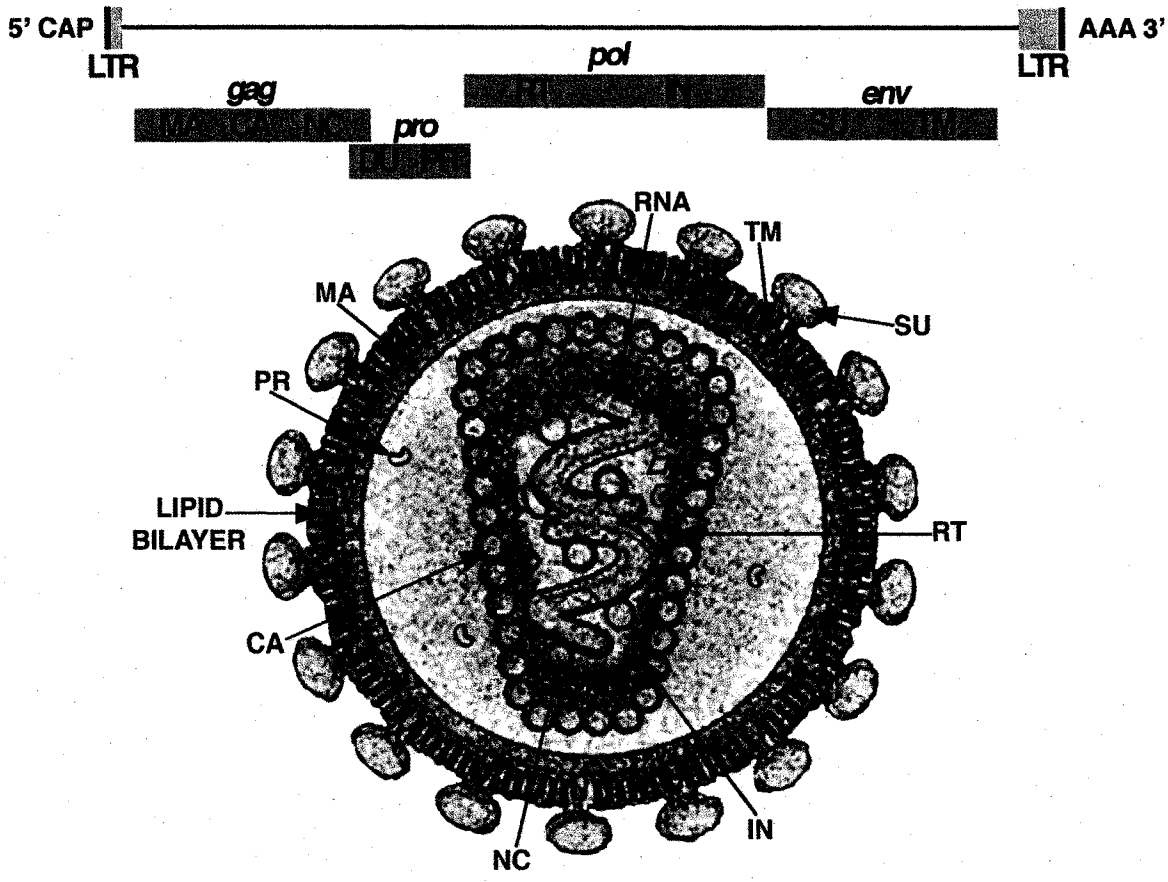

FIG. 1. Schematic of M-PMV genomic RNA and model of a mature virion. Top: M-PMV genomic RNA showing the open reading frames of the genes common to all retroviruses (*gag*, *pro*, *pol*, and *env*) and the proteins encoded in each gene. MA, matrix; CA, capsid; NC, nucleocapsid; DU, dUTPase; PR, protease; RT, reverse transcriptase; IN, integrase; SU, surface; TM, transmembrane. Bottom: The spatial orientation of the major proteins common to all retroviruses in a mature virion with a conical core. The mature virion picture is adapted from Wikipedia, the Free Encyclopedia (http://en.wikipedia.org/w/index.php?title=HIV&oldid=38170848, 403). The AIDS virus image is a work of a United States Department of Energy employee, taken or made during the course of an employee's official duties. As a work of the U.S. federal government, the image is in the public domain.

sequences critical for the retroviral life cycle that regulate gene expression. In association with the viral genomic RNA is the nucleocapsid protein (Fig. 1) (31, 102, 426). A subset of retrovirus virions, including M-PMV and MMTV, contain two forms of NC that bind the genomic RNA, the NC and a fusion protein of NC-dUTPase (19, 30). The NC:RNA complex and the viral enzymes reverse transcriptase, integrase, dUTPase, and protease are contained in a shell of the capsid protein (CA) which together comprise the core of the mature virion. Two of these proteins, the protease and capsid proteins, are not exclusively contained within the core of the virion (54, 202, 399). At the outer rim of the virion, the MA protein is associated with the membrane and forms a dense layer that can be visualized by electron microscopy (Fig. 1) (382). The matrix protein was shown to be in close proximity of radioactively labeled phospholipid in cross-linking studies for the avian sarcoma and leukemia virus (279-281). In cryo-electron microscopy studies of HIV virus-like particles lacking the MA, the protein density under the membrane was not seen and a gap between the membrane and the internal density was observed (407). In cryo-electron microscopy studies of the mature HIV virion, a similar protein density under the membrane is observed and attributed to MA (55). On the outer surface of the virion is a lipid bilayer which is acquired as the capsid is released from a membrane of the cell. The lipid bilayer of a virion contains phospholipids; these lipids are enriched in sphingomyelin and cholesterol as compared to the plasma membrane of a cell in the case of HIV and RSV (9, 10, 284, 294, 295). For M-PMV, it is not known if the lipid of a virion is enriched or depleted in cellular components as compared to the host cell plasma membrane; however, in Moloney murine leukemia virus (MuLV) cellular proteins appear to be nonspecifically incorporated into the virus envelope (158).

Spanning the lipid of an infectious virion are trimers of the viral envelope (Env) glycoprotein, which is composed of six subunits, three surface (SU) and three transmembrane (TM). The Env TM protein crosses through the membrane with a cytoplasmic tail extending into the virion, where, as suggested by mutational analyses, this protein interacts with the matrix protein (58, 59, 132, 134, 248, 343, 345). The ectodomain of the TM is noncovalently associated with the SU domain in most retroviruses, and this portion of Env extends from the virion as spikes (83, 325, 382).

Retroviral life cycle. Retroviruses enter a cell when the SU domain of Env binds a specific cellular receptor on the plasma membrane (Fig. 2). Through interference assays the receptor for M-PMV and all strains of simian immunosuppressive type D retroviruses has been determined to be the cellular protein RDR, which normally functions in the cell as a neutral amino acid transporter (298, 341). Further support that RDR is the receptor for the SRVs comes from a study that defines the amino acids within the Env SU of SRV-1 and SRV-2 that interact with RDR. This study also showed that polyclonal and monoclonal antibodies directed toward the binding epitope in the Env SU domain of each strain neutralize only the respective viral serotype (12). After the SU of Env binds to the specific receptor, conformational changes occur in the ectodomain of the TM region that mediate fusion of the lipid bilayer on the virion with the cellular plasma membrane. The mechanism of viral fusion was first structurally described for the hemagglutinin (HA) protein of influenza virus. For HA, a dramatic pH-dependent conformational change occurs in the protein subunit anchored to the viral membrane in which a heptad repeat motif transitions from a loop to a helix structure, creating an extended triple-stranded, coiled coil that relocates the fusion peptide closer to the target

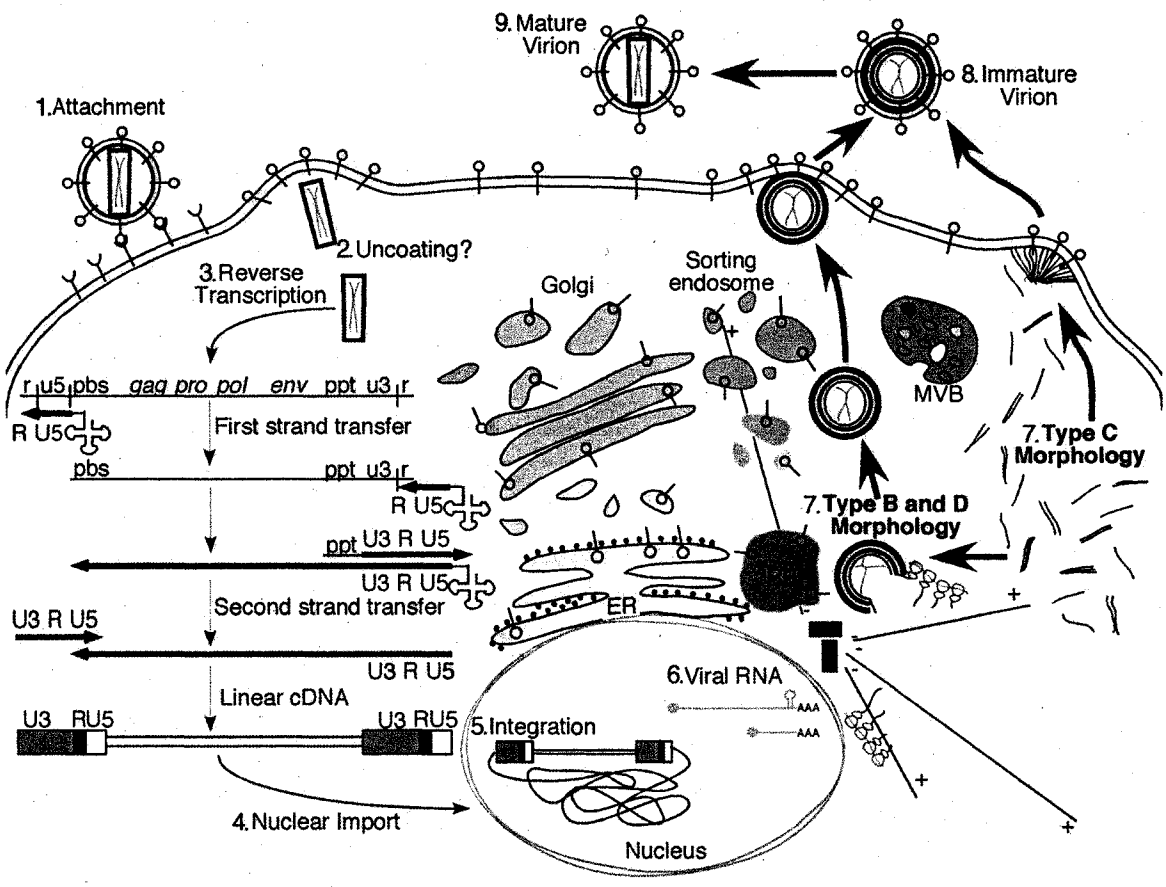

FIG. 2. Retroviral life cycle. From left to right: Virions attach via protein receptors and fuse with the cell membrane following partial uncoating. The viral RT converts the positive strand RNA to double-stranded, linear DNA with LTRs. The PIC is transported to the nucleus and actively enters the nucleus, most likely through the nuclear pore (HIV) or following mitosis and nuclear disruption (MuLV). Host RNAPII transcribes the viral RNA, which is transported out of the nucleus for translation. A subset of the unspliced genomic RNA is translated on polyribosomes in the cytoplasm into the Gag polyproteins. The Gag polyproteins multimerize along with viral genomic RNA to form protein shells either within the cytoplasm (types B and D morphology) or at the plasma membrane (type C morphology). Env is translated from spliced mRNA on ribosomes associated with the ER. Env is trafficked through the secretory pathway of the cell and then endocytosed into the endosomal compartments. At the plasma membrane the Gag protein shell is enveloped by a lipid bilayer that contains the Env proteins and an immature virion is released. The viral protease is activated, resulting in the cleavage of the Gag polyprotein, and the CA collapses on the RNA to form a dense core which generates a mature virus particle.

cell membrane (77). While there are differences between the triggering of the conformational change for influenza virus (pH) and retroviruses (SU binding receptor), the ectodomain of the retroviral transmembrane Env protein contains a fusion peptide and two heptad repeat motifs that have been shown to have the capacity to form a helical, coiled-coil structure similar to that of HA (Fig. 3) (70, 79, 80, 127); these regions have also been shown to influence retroviral fusion and infectivity (86, 87, 114, 344, 400, 405, 406). The heptad repeat regions of retroviral Env have been shown to form a six-helix bundle in which the N-terminal heptad repeats form a central triple-stranded coiled coil and the C-terminal heptad repeat region packs antiparallel into the hydrophobic grooves on the surface of the N-terminal heptad repeat coiled coil (Fig. 3) (80, 358, 396). The order of events of the fusion process with regard to six-helix bundle formation and formation of a fusion pore is still debated in the retrovirus field. Recently, new technology that fluorescently follows the fusion of a single avian sarcoma and leukosis virion with a cell revealed that Env folding into a six-helix bundle catalyzes fusion pore opening and enlargement (239), which is in agreement with that reported on the fusion events of HA (43, 85). The formation of a fusion pore allows the core of the virion to enter the cytoplasm of a host cell, although the exact mechanism by which this occurs is not well understood. It is known that in the cytoplasm the core encounters host restriction factors such as APOBEC3G and the TRIM family of proteins. The process by which members of the TRIM family restrict retroviruses remains obscure, although it appears to be at a stage prior to completion of reverse transcription (258). Retroviral restriction by APOBEC3G appears to occur during the process of reverse transcription (162). APOBEC3G deaminates cytosine bases to generate uracil in the newly synthesized

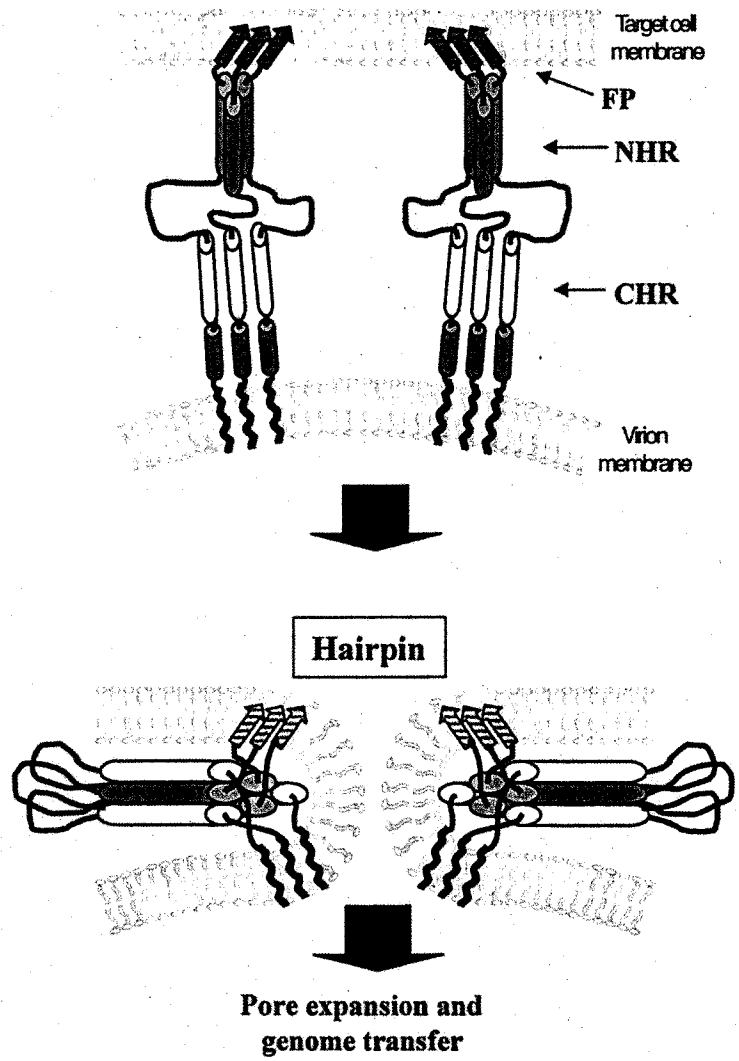

FIG. 3. Model of HIV envelope mediated membrane fusion. Following receptor binding, the SU is predicted to be released and the TM functions to bring the viral and target membranes together. Top is the model of the extended coiled-coil conformation of the TM with the fusion peptide interacting with the target cell membrane. Bottom: Six-helix bundle of the N-terminal and C-terminal heptad repeat packed together as described in the text. FP, fusion peptide; NHR, N-terminal heptad repeat; CHR, C-terminal heptad repeat. Adapted from S. Shnaper, K. Sackett, S.A. Gallo, R. Blumenthal, and Y. Shai, J Biol Chem. **279**:18526-18534, 2004 (336). Reprinted with permission from the American Society for Biochemistry and Molecular Biology, © 2006 by the American Society for Biochemistry and Molecular Biology.

DNA, ultimately resulting in G to A hypermutations that generate stop codons throughout the retroviral genome (162, 163, 204, 224, 424). HIV encodes an accessory protein, Vif, that initiates the degradation of APOBEC3G to protect the viral genome (225, 351, 386, 416). It is not understood how simple retroviruses such as M-PMV counteract the effects of APOBEC3G.

Reverse transcription occurs in the cytoplasm of the host cell, where nucleotides necessary for DNA synthesis are available (Fig. 2) (365). Reverse transcriptase, the viral protein required for reverse transcription, has enzymatic properties of a DNA polymerase that can synthesize DNA from either an RNA or DNA template and properties of a nuclease specific for RNA, ribonuclease H (RNase H). The process of retroviral reverse transcription begins when a tRNA molecule bound at the primer binding site of the RNA serves as a primer for negative strand DNA synthesis. A short (100-150 bp) negative strand DNA (minus-strand strong stop DNA) is generated that contains complementary sequences to the RNA from the primer binding site to the 5' end of the genomic RNA. The RNA:DNA duplex that is formed during DNA synthesis provides a substrate for the RNase H property of RT, which degrades the 100-150 nt RNA portion as the strong stop DNA is synthesized. The single stranded negative-strand strong stop DNA hybridizes to or is transferred to the 3' end of the genomic RNA, where repeat sequences (R) that are common to both the 5' and 3' ends of the genome facilitate the process. The 3' hydroxyl group of the negative-strand strong stop DNA then serves as a primer for continued negative strand DNA synthesis, which continues to the primer binding site, which is now the 5' end of the RNA. While the negative strand DNA is synthesized, the RNA template is degraded by RNase H. However, the polypurine tract

(PPT) segment of the template RNA is resistant to RNase H degradation and serves as the initial primer for positive strand DNA synthesis. A short positive strand of DNA (plus-strand strong stop) is generated from the PPT sequences to a modified residue within the tRNA still covalently attached to the end of the negative strand DNA. A second strand transfer occurs in which the primer binding site is the sequence in common between the negative strand DNA and the plus-strand strong stop DNA. The latter can now serve as the primer for plus strand DNA synthesis. DNA synthesis continues with the plus and minus strands each serving as the template to complete the other strand, resulting in a blunt end linear double-stranded viral DNA, or provirus (365).

The proviral DNA is transported into the nucleus in the context of viral and cellular proteins known as the preintegration complex (PIC) (45), which contains at least integrase, matrix, reverse transcriptase and nucleic acids (69). The preintegration complex is best described for HIV, where the integrase protein (44, 126), the matrix protein (67, 69, 385), and an overlap of plus-strand DNA (422) have been reported to facilitate the entry of the newly synthesized DNA into the nucleus. The PIC of HIV can actively enter the nucleus in both dividing (44, 195) and non-dividing (68, 133, 323, 394) cells. For MuLV inhibitors of mitosis delay integration, supporting the concept that the PIC for this retrovirus (and most non-lentiviruses) enters the nucleus when the membrane is disassembled during cell division (13, 211, 311). The proviral DNA is incorporated into the host genome through an integration process that is catalyzed by the viral enzyme integrase (Fig. 4). First, integrase hydrolyses a phosphodiester bond, resulting in the loss of 2 nucleotides at both the 3' and 5' long terminal repeats (LTRs) of the viral DNA. Then, the 3'-hydroxyl group of each viral DNA strand attacks a strand of the host DNA

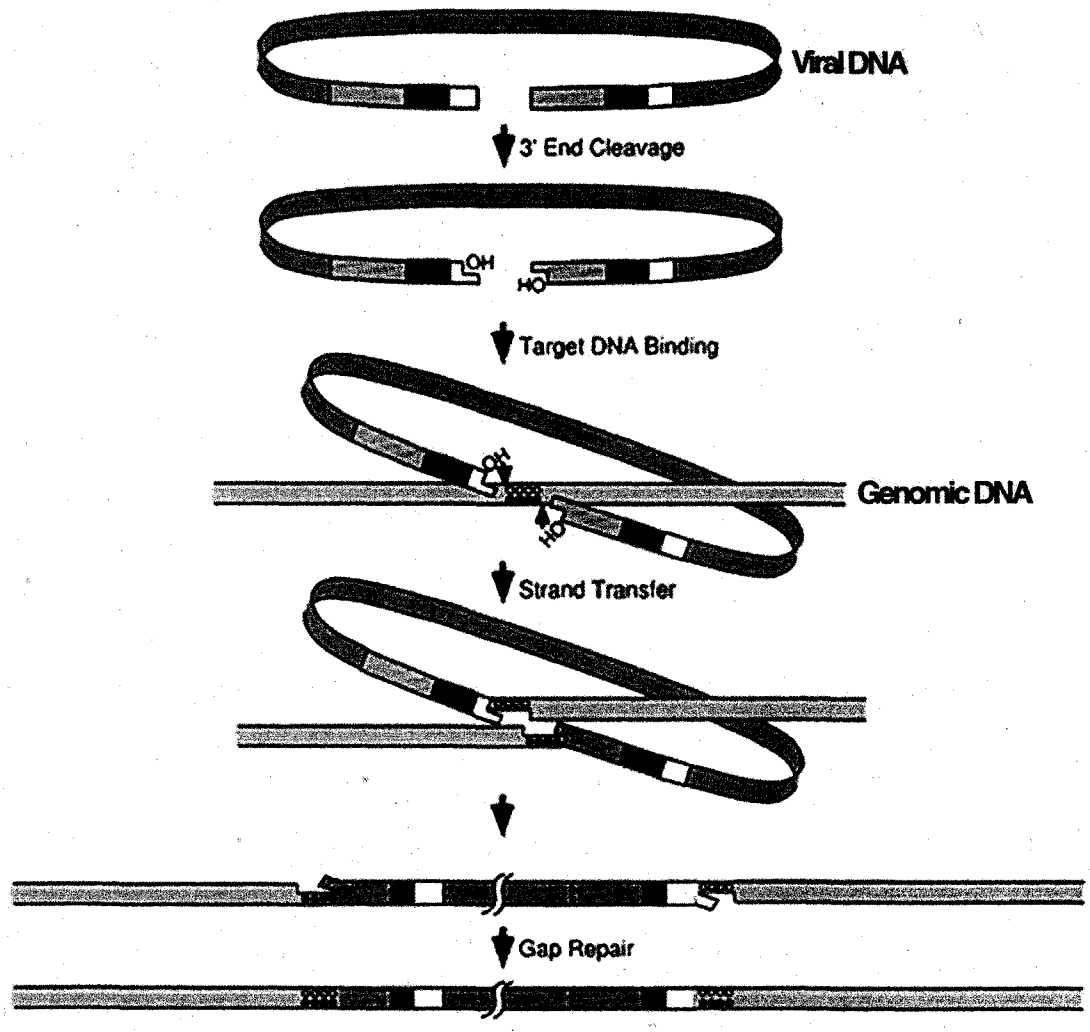

FIG. 4. Schematic outlining the principal steps in retroviral DNA integration. The viral integrase enzyme cleaves each LTR, resulting in the loss of 2 nucleotides and free 3' hydroxyl groups. The 3'-hydroxyl group of each LTR attacks the host DNA, and then DNA synthesis extending the free 3' end of the target DNA results in repairing the gaps and a fully integrated proviral DNA sequence. Adapted from P.O. Brown, Integration, p. 161-204, 1997. In J.M. Coffin, S.H. Hughes, and H.E. Varmus (ed.), Retroviruses. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (62). Reprinted with permission from the Cold Spring Harbor Laboratory Press, © 1997 by the Cold Spring Harbor Laboratory Press.

and is joined by transesterification (124, 142, 196). Recently, the M-PMV integrase enzyme has been characterized (337). It was reported that the integration site of M-PMV sequences was not completely random and that this process is most likely dependent on sequence or structure of the DNA. M-PMV integrase preferred a more relaxed sequence requirement as compared to the organized DNA structure preferred by HIV integrase for the strand transfer that joins the viral DNA to the host DNA (304, 337).

Following integration, the proviral DNA is transcribed by the cellular RNA polymerase II (RNAPII) under transcription control of the LTR. The LTR of retroviruses includes the unique 3' end (U3), repeated (R) and unique 5' end (U5). Within the LTR the U3 contains sequences that direct the RNAPII to the DNA template. Approximately 25 nucleotides upstream of the retroviral transcription start site is a TATA box consensus sequence to which RNA polymerase II is recruited via interactions with a cellular protein complex. Transcription of the retroviral sequences generates a full length genomic RNA message that includes the R and U5 regions from the left LTR, the coding regions, and the U3 and R regions from the right LTR; these transcripts are subject to cellular processing events, capping, polyadenylation, and splicing, which generate a mature messenger RNA (mRNA) transcript. The pre-mRNA viral transcript is capped with a 7-methyl guanosine triphosphate at the 5' end by cellular posttranscriptional machinery. At the 3' end, the viral RNA is cleaved and a poly(A) approximately 200-300 adenine nucleotides in length is added. The poly(A)tail promotes the stability of the RNA and facilitates the exit of the transcript from the nucleus. Following capping and polyadenylation, a fraction of the viral transcripts are spliced by the cellular spliceosome complex, which functions to remove non-coding sequences from mRNA. All retroviral

genomic length transcripts contain signals recognized by the cellular spliceosome to remove at least one sequence for the generation of a subgenomic viral mRNA (296).

For expression of the retroviral genes and incorporation of genomic RNA into progeny virus, both the spliced and unspliced RNA transcripts need to be exported from the nucleus. The spliced RNA associates with ribonucleoprotein complexes during transcription and RNA processing that aid in nuclear export. Unspliced cellular RNA is known to be actively retained in the nucleus (81, 205). To export unspliced RNA, a retrovirus contains a posttranscriptional regulatory mechanism, one of which was first described for HIV. HIV encodes the viral protein Rev, regulator of expression of viral proteins, and a cis-acting RNA sequence, the Rev response element (RRE). Rev interacts with the structured RRE to allow nuclear export of singly spliced and unspliced RNA molecules (81, 92, 156, 166, 222, 421). Simple retroviruses do not encode a trans-activating protein such as Rev; however, these retroviruses also export unspliced genomic RNA from the nucleus. For M-PMV a small, 219 nucleotide element from the genome, which is located between the *env* gene and the LTR, is capable of substituting for Rev and the RRE in transporting pre-mRNA from the nucleus (51). This element, the constitutive transport element (CTE), which acts in cis to mediate RNA export, blocked export of cellular mRNA from the nucleus when it was microinjected at saturating levels (275). This was the first evidence that the RNA export pathway accessed by the CTE was most likely a general mRNA export pathway. The CTE has been shown to directly bind the TAP/NXF1 protein and microinjection of TAP can rescue mRNA export from the nucleus in cells that were previously expressing saturating levels of the CTE (154 , 174). The identification of this mRNA export pathway has been reported for

at least Xenopus, yeast, and Caenorhabditis elegans (194, 275, 359). Like M-PMV, a similar cis-acting element has been found in the SRV-1 (357) and the SRV-2 (213). Microinjections of the SRV-1 CTE element also inhibit export of cellular mRNAs (314), suggesting that the SRV-1 CTE also utilizes the TAP/NXF1 cellular export pathway.

Unspliced genomic RNA exported from the nucleus is either translated into viral proteins or packaged into progeny virions. In the cytoplasm of an infected cell, some of the unspliced viral RNA is recognized by a ribosome. Then, translation, the process of converting the nucleotide sequence into amino acids, of the viral Gag polyprotein commences. Ribosomal profile analysis demonstrated that the RU5 region of the M-PMV LTR directs the recognition of the unspliced viral RNA by ribosomes such that the RNA template is associated with polyribosomes (182). Translation of the M-PMV gag gene results in polyprotein which has six protein domains. Tryptic peptide mapping identified the p27, pp16, p14, p12, and p10 proteins (48), and the p4 was identified when M-PMV viral proteins were eluted from a reversed-phase, high-pressure liquid chromatography column (169). The order of the six domains within the Gag polyprotein was determined by pactamycin mapping and sequencing of the M-PMV/6A provirus to be NH_2-p10(MA)-pp24-p12-p27CA-p14NC-p4-COOH (Fig. 5) (50, 347).

During the tryptic peptide mapping analysis of the M-PMV Gag polyprotein (Pr78), a Gag related protein that was larger (Pr95) than the Gag polyprotein was present in infected cells at levels approximately 10% of those of the Gag polyprotein (48). Screening human cell lines for replication defective mutants revealed a *pol* deficient mutant containing a 160 kilodalton protein rather than a 180 kilodalton viral protein (82). These data, along with the predicted protein sizes from the sequence of the

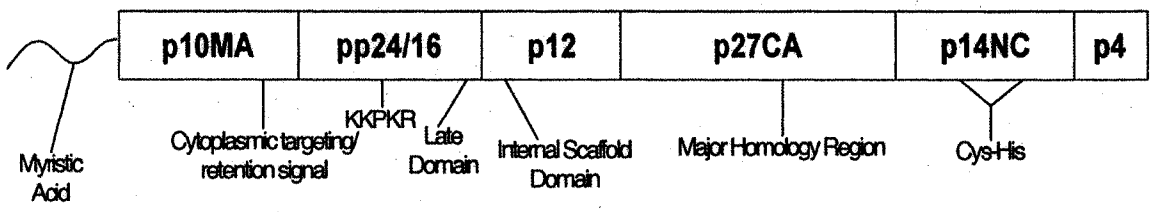

FIG. 5. Schematic of the Mason-Pfizer monkey virus Gag polyprotein. The major proteolytic products of the Gag polyprotein are indicated from the N terminus to the C terminus as follows: p10MA, matrix; pp24/16, phosphoprotein; p12; p27CA, capsid; p14NC, nucleocapsid; and p4. Within these domains discrete subdomains are denoted for which specific morphologic functions have been predicted or defined. Myristic acid is attached to the N-terminal matrix domain.

M-PMV provirus (347), supported the concept that M-PMV undergoes a ribosomal frameshift to translate the proteins encoded in the *pro* and *pol* genes. Ribosomal frameshifting, the switching of the translating ribosome from one reading frame to another reading frame, was demonstrated in a cell-free mammalian translation system in which the starting material was a single RNA species synthesized from a cloned DNA with a bacteriophage RNA polymerase for the Gag-Pol polyprotein of RSV. Under these conditions translation of the Gag and Gag-Pol proteins occurred at a ratio (20:1) previously observed in infected cells (188). Retroviral ribosomal frameshifting occurs when the ribosome shifts backward one nucleotide into an overlapping portion of the *pro-pol* reading frame. Sequence analysis of the protein products generated as a result of frameshifting demonstrated that the ribosomal frameshift occurs at a specific codon, the frameshift codon (173, 185, 186). A frameshift mechanism, in which the translating ribosome slips into a new reading frame when the ribosome encounters a seven-nucleotide slippery region just prior to the frameshift codon as well as a pseudoknot structure in the RNA just downstream of the frameshift codon, was deduced from mutational analysis of the RNA nucleotide sequences upstream and downstream of the frameshift codon (52, 185). In RSV and HIV ribosomes undergo a single frameshift to generate the viral enzymes from a Gag-Pol fusion protein (186, 188). Betaretroviruses were found to encode two frameshifting sites, one at the end of *gag* and one at the end of *pro* that generate the Gag-Pro and Gag-Pro-Pol fusion proteins, respectively (187, 347).

The translated Gag, Gag-Pro, and Gag-Pro-Pol precursor proteins of M-PMV in conjunction with the genomic RNA come together in the cytoplasm of the cell to form an immature viral capsid. Transmission electron micrographs of infected cells revealed

two main capsid assembly pathways for members of the Retroviridae. The Betaretroviruses, types B (MMTV) and D (M-PMV), and Spumaviruses assemble a capsid from the Gag precursor proteins in the cytoplasm of the cell. The assembled immature capsid, which has an electron dense ring and a translucent center, can readily be seen in the cytoplasm. Fully assembled capsids can also be visualized in the process of membrane extrusion. For Betaretroviruses capsids are released from the plasma membrane, while capsids of the Spumaviruses are released into membrane vesicles within the cell. In contrast, type C retroviruses, which represent the majority of retroviruses, for the most part assemble capsids at the plasma membrane and extrude the membrane simultaneously. In electron micrographs of type-C virus infected cells, a characteristic, frequently crescent-shaped, electron dense patch of protein at the inner leaflet of the plasma membrane can be visualized. The morphologic determinants of capsid assembly for a retrovirus are contained within the Gag polyprotein, since expression of the Gag polyprotein is all that is necessary for virus-like particles to be released from a cell (109).

The process of capsid assembly begins during or shortly after translation of the Gag polyprotein. The first domain of Gag to be translated is the matrix domain (MA), which contains the determinants of assembly morphology. The MA of M-PMV Gag contains an 18 amino acid sequence, the cytoplasmic targeting/retention signal (CTRS), which is necessary for capsid assembly to occur within the cytoplasm, since a single change in an amino acid within the CTRS resulted in the prototypic type D retrovirus efficiently assembling capsids at the plasma membrane with a morphology likened to that of a type C retrovirus (89, 305, 412). Capsid assembly is directed to the plasma membrane for type C retroviruses through a bipartite signal within MA of myristic acid

and positively charged amino acids (135, 172, 430). Myristylation, covalent attachment of myristic acid to a glycine residue at position 2 (372, 404), of the retroviral Gag MA is a common modification and has been reported for type B (MMTV) and type D (M-PMV), as well as murine (MuLV), feline (Rickard strain of feline leukemia virus), bovine (bovine leukemia virus (BLV)), non-human primate (baboon endogenous virus), and human primate (HIV) type C retroviruses (168, 243, 327). The second signal for capsid assembly to occur at the plasma membrane, a density of positive charge within the MA domain, has been identified in structural studies of the MA proteins of the type D (M-PMV) and type C (BLV, SIV and HIV) retroviruses (94, 172, 234, 297, 361). While both type D and type C retroviral MAs contain signals to direct capsid assembly to the plasma membrane, type C retroviruses lack sequences homologous to the CTRS which acts as a dominant signal to direct capsid assembly to a specific site within the cytoplasm.

The CTRS of M-PMV has recently been shown in indirect immunofluorescent studies to direct Gag to a pericentriolar region where Gag accumulated near the centriole marker, γ tubulin, in cells expressing wild-type M-PMV, but not in cells expressing a CTRS mutant (334). CTRS-dependent accumulation of Gag near the centriole, a component of the cellular microtubule organizing center (MTOC), which contributes to the cellular morphology and structure by connecting large arrays of microtubules, suggested that the CTRS might interact with a cellular component to bring Gag to this location. Active transport of cellular cargo to the MTOC is dependent on the dynein motor protein complex and the presence of microtubules (377). When cells were treated with nocodazole, a microtubule depolymerizing agent (104), or when the dynein motor complex was

disturbed by expression of a dominant-negative subunit (119), accumulation of Gag stain at the pericentriolar region was inhibited, supporting the concept that the dynein motor interacts with the CTRS to target Gag to a region near the MTOC. Electron micrographs of the peri-centriolar region, 200-1,000 nanometers from the centriole, of multiple infected cells showed partially assembled capsids in microdomains heavily populated with ribosomes; however, assembled capsids within this region were in areas devoid of ribosomes. This was the first evidence that the CTRS might be cotranslationally relocating polysomes to the pericentriolar region of the cell. To determine if the pericentriolar region is the site of Gag protein synthesis, indirect immunofluorescence studies were conducted such that Gag was blocked in an accumulated state at the MTOC and then released from this block in the presence or absence of cycloheximide, an inhibitor of translation. After the pericentriolar block was released, Gag was dispersed throughout the cytoplasm with no accumulation of Gag at the pericentriolar region for cells treated with cycloheximide. In contrast, the Gag stain for untreated cells or cells treated with nocodazole was both dispersed in the cytoplasm and accumulated at a pericentriolar region. These results argue that synthesis of the Gag polyprotein occurs at a pericentriolar region (334).

The perinuclear region of the cell is rich in the cytoplasmic chaperone molecules heat shock protein 73 and t complex peptide 1 (TCP-1) (60, 61). The TCP-1 ring complex, TRiC, interacts with a small subset of newly synthesized proteins of 30 to 60 kilodaltons in size (370). In a yeast-two hybrid assay, the TCP-1-γ subunit of TRiC interacted with the pp24/16-12 and p4 domains of M-PMV Gag. The complex between TRiC and the p4 domain could be coimmunoprecipitated from cellular lysates. Incubat-

ing the cellular lysates with increasing amounts of ATP before coimmunoprecipitation disrupted this interaction, showing that M-PMV Gag depends on ATP hydrolysis to be released from the chaperone. The TRiC-p4 interaction facilitates capsid assembly since deletion mutants that lack the p4 region inefficiently assemble into capsids in an *in vitro* capsid assembly assay. These data provide evidence that TRiC promotes proper protein folding of M-PMV Gag, which is necessary for the capsid assembly process (175).

M-PMV capsids assembling at the pericentriolar region must incorporate RNA molecules prior to the closure of the protein sphere. A combined approach of indirect immunofluorescence and in situ hybridization demonstrated that HIV Gag interacts with genomic RNA at the pericentriolar region (286). This region is in close proximity to the nucleus and there is growing evidence in the field that at least a subset of retroviral Gag molecules come in contact with the nucleus during the assembly process. Nuclear cycling of RSV Gag has been reported to occur by way of a nuclear localization signal, which in general is a sequence containing positively charged amino acids, within MA and NC that interacts with the cellular proteins to facilitate the nuclear entry of Gag (72, 321). RSV Gag exits the nucleus via the Crm-1 export pathway since treatment of cells with the Crm-1 inhibitor leptomycin B results in accumulation of wild-type RSV Gag in the nucleus. For RSV basic amino acid MA mutants treatment of cells with leptomycin B did not result in nuclear Gag accumulation, suggesting that these amino acids facilitate nuclear entry (74, 321). Evidence that M-PMV Gag interacts with the nucleus comes from yeast-two hybrid and GST-pull down assays which demonstrated a specific interaction between M-PMV Gag and the ubiquitin conjugating enzyme 9 (Ubc9) protein (397). Ubc9 is an E2 small ubiquitin-like (SUMO) conjugating enzyme which functions in the

sumoylation pathway (Fig. 6) and has been shown by immunogold labeling to localize to the cytoplasmic and nuclear cytoplasmic filaments of the nuclear pore complex (423). While there is no evidence that M-PMV Gag is conjugated with SUMO, interactions of Gag and the nuclear pore as demonstrated by indirect immunofluorescence have been reported to be modulated by a sequence in the pp24 phosphoprotein domain. Furthermore, a deletion of a KKPKR sequence within the pp24 domain decreased incorporation of RNA without affecting capsid assembly and release, particle maturation, or viral glycoprotein incorporation (42).

Based on *in vitro* and *in vivo* studies of type C retroviruses, a model for capsid assembly suggests that the multivalent interactions of capsid assembly initiate when the Gag NC domain binds the viral genomic RNA (8, 71, 91, 103, 191, 221, 249, 250, 425, 427). The NC domain of Gag has a high content of positively charged amino acids and contains at least one Cys-His zinc binding motif. Positively charged amino acids in NC have been found to affect the general interaction with RNA (91, 287, 322), while alterations in the zinc binding motifs can cause loss of RNA packaging or incorporation of futile viral or cellular mRNAs (31, 115, 116, 123, 240-242, 426). The NC of RSV, HIV and MuLV contains an interaction domain (I-domain) which has been shown to mediate Gag-Gag interactions intracellularly and at the plasma membrane (27, 47, 107, 249, 271, 318, 319, 398). In conjunction with the NC, the Gag CA domain contains sequences that mediate Gag-Gag interactions, which are critical for the correct morphology of a retroviral particle. Saturation mutagenesis of the M-PMV CA major homology region resulted in mutants that did not assemble intracellular capsids or in mutants that were defective in early stages of replication (353). Both the CA and NC domains of M-PMV were

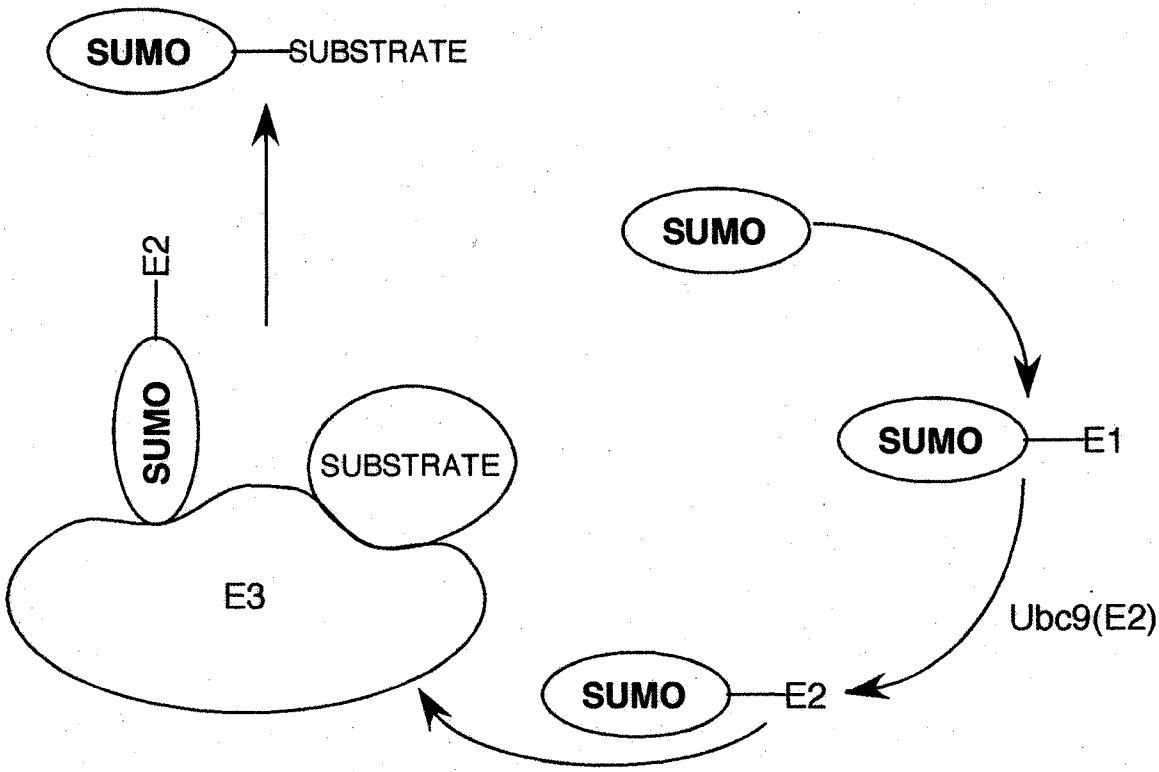

FIG. 6. Sumoylation pathway. The small ubiquitin-like molecule, SUMO, is covalently ligated to a variety of target proteins through the action of a ubiquitin-like activating enzyme (E1), ubiquitin-like conjugating enzyme (E2) and ubiquitin-like protein ligase (E3). Substrate, target protein.

necessary for assembly of macromolecular protein arrays in bacteria, while sequences N-terminal to CA directed spherical particle formation. Interestingly, a single proline residue N-terminal to the CA of M-PMV was the critical shape determinant, since loss of this residue resulted in the formation of a spherical particle (254, 313). In addition to the CA and NC domains, the M-PMV Gag p12 contains an internal scaffold domain which promotes the membrane independent cytoplasmic capsid assembly process (313, 315, 316, 340).

Completion of capsid assembly results in an immature particle at an intracellular (types B and D retroviruses) or membrane associated location (type C retroviruses). In electron microscopy studies, immature particles appear to have radial arrays of the Gag polyprotein with the matrix domain at the outer surface of the protein shell and the Gag molecule extending into the center of the sphere. Negative stain, freeze-fracture and cryo-electron microscopy studies of HIV and M-PMV immature capsids have resulted in reports that Gag is well ordered in hexagonal or fullerene-like arrays; this ordering most likely reflects the order of lateral molecular interactions (53, 215, 254-257, 273). The spherical morphology of a particle, along with a variation of mass and size, argues against this organization continuing at a three-dimensional level (53, 136, 273, 384, 407, 414). The mass of assembled M-PMV capsids isolated from the cytoplasm of insect cells varied (164-174 MDa). Through a combination of scanning-transmission electron microscopy and matrix-assisted laser desorption ionization-time of flight mass spectroscopy, it was determined that approximately 2,000 copies of the Gag and/or GagPro molecules were assembled into an M-PMV capsid (273).

While the structural Gag polyprotein contains the elements necessary to assemble a protein shell, production of an entry-competent virion requires incorporation of the viral envelope protein (Env). Env is synthesized from singly spliced viral mRNAs and is cotranslationally directed to the rough endoplasmic reticulum by an amino terminal signal peptide of 25 amino acids (49, 347). In the endoplasmic reticulum, the Env precursor protein, NH_2-SU-TM-COOH, oligomerizes into trimers through interactions of the TM domain (117, 118, 120-122) and shortly thereafter is transported to the Golgi, where Env is terminally glycosylated (49, 83, 84). In the late Golgi compartment, most likely the trans-Golgi network, the oligomerized precursor is cleaved into the SU and TM proteins by a cellular furin-like protease (49, 84, 331, 347). After cleavage, the SU and the ectodomain of the M-PMV TM are noncovalently associated in a protein complex contained within the lumen of a trans-Golgi compartment (49, 56, 347). Hydrophobic residues in the membrane-spanning domain function to anchor the protein in the lipid bilayer. In addition, the C-terminal domain of TM, which for M-PMV is 38 amino acids long, extends into the cytoplasm of the cell (49, 285, 308, 347, 401). The cleaved Env protein complex is then trafficked from the trans-Golgi network to the plasma membrane.

The viral envelope protein is incorporated into the virus particle when the capsid extrudes the plasma membrane. Traditionally, it has been thought that the first interaction between Env and Gag occurs at the surface of the cell. An interaction between the cytoplasmic tail (CT) of the Env TM protein and the Gag MA has been shown *in vitro* for HIV in an assay that used GST-EnvCT proteins purified from bacteria to pull down expressed MA from COS-1 cell lysates. Neither MA mutants in which a leucine at

position 12 is substituted with an acidic amino acid (132, 134) nor MA mutants that have small deletions (111, 417), which have been shown to incorporate reduced levels of Env into budding virions, interacted with the GST-Env protein *in vitro* (95). In cells expressing both the SIV Env and Gag, these proteins could be coimmunoprecipitated (380), and the MA and Env for RSV could be chemically cross-linked (138). The interaction between Env and Gag is apparently not entirely essential for glycoprotein incorporation, since removal of the RSV cytoplasmic tail did not reduce capsid assembly or infectivity (282). MuLV Env incorporation is not perturbed in a majority of the deletion mutations within the CT or for any of 21 mutations introduced into the MA (189, 342). The HIV MA mutants that did not incorporate Env could be reverted when coexpressed with HIV Envs with short cytoplasmic tails or when coexpressed with a heterologous Env protein (132, 134, 223). In the case of mutants where the envelope glycoprotein and Gag are not predicted to interact within the cell, it is thought that the Env expressed on the surface of the cell is passively incorporated into virions as the capsid extrudes the membrane.

While glycoprotein incorporation can occur either actively through Env-Gag interactions or passively during the budding process, there is growing evidence that Gag and Env interact intracellularly at locations determined by cellular trafficking signals inherent in each protein. The Env CT of many retroviruses, including M-PMV, contains a tyrosine-based YXXΦ endocytosis motif in which X is any amino acid and Φ is a hydrophobic amino acid (46, 108, 259, 320, 343, 402). The membrane-proximal tyrosine motif of HIV CT has been shown to interact with the clathrin-adaptor protein, AP2, to direct the Env glycoprotein complexes from the plasma membrane into intracellular compartments (32, 41, 261). In polarized epithelial cells, the tyrosine-based endocytosis

motif of the Env CT is critical for basolateral targeting of viral budding, since, in the absence of Env or when the tyrosine of the dominant endocytosis signal of the CT is mutated, Gag is released equally from both the apical and basolateral cell surfaces. In contrast, 94% of Gag is located to the basolateral surface when the wild-type Env is expressed (217, 269). In non-polarized cells, alteration of the membrane-proximal tyrosine residue of HIV had no effect on Env incorporation (402). The M-PMV Env CT has two potential tyrosine-based endocytosis motifs. Mutations to the membrane proximal tyrosine-based motif, in which a non-aromatic residue replaced the tyrosine, altered internalization of Env in antibody uptake assays and resulted in less radiolabeled Env incorporated into released virions. Surface biotinylation assays detected similar levels of Env expressed on the cell surface for these mutants as compared to wild-type. Mutants that incorporated less glycoprotein also released fewer virions as determined by CA levels in the supernatant (343). Pulse-chase experiments in which the kinetics of virion release for cells expressing Gag and Env were compared to that of cells expressing only Gag demonstrated that capsid release is seven times more efficient when the viral Env glycoprotein is expressed in the cell (333). In the absence of Env, Gag stain was accumulated at the centriolar region of the cell in 80% of transfected cells, validating that Env is important in the trafficking of Gag from the site of capsid assembly (334). Furthermore, Env and Gag colocalize at the pericentriolar region of the cell in indirect immunofluorescence assays (333).

The pericentriolar region is rich in cytoskeletal tracks composed of tubulin or actin that intersect at the MTOC. A reversible block to cellular trafficking of these networks, which are known to facilitate intracellular transport and vesicular sorting, can be

induced when cells are incubated at 20 degrees Celsius (233, 293). In experiments where vesicular transport was blocked, Gag accumulated at the pericentriolar region of the cell, supporting that an interaction of Gag with endosomes is required for the transport of assembled capsids from the pericentriolar region to the plasma membrane. Furthermore, a GTPase protein, Rab11, which regulates trafficking through the recycling endosome (375), colocalized with wild-type M-PMV Gag; in addition a dominant negative mutant of Rab11 delayed the release of M-PMV (333).

The data for M-PMV support a model in which Env is endocytosed into the endosomal compartments of the cell and trafficked to the pericentriolar region, where an interaction between Env and Gag occurs intracellularly to facilitate both the transport of capsids to the plasma membrane and the incorporation of Env into the released virion. It is not known if the Env mediated transport of Gag is a direct interaction between the viral proteins, independent interactions of Gag and Env with the same endosomal vesicle, or if transport involves multiple interactions involving both the interactions between Gag and Env as well as interactions between these two proteins and the endosomal compartments. Recently, the clathrin associated adaptor complexes AP-3 and AP-2, which have previously been reported to interact with HIV Env (32, 41, 261), were also reported to interact with HIV Gag. In GST pull-down assays and coimmunoprecipitations, HIV Gag directly interacted with AP-3. Disruption of this interaction via expression of a dominant negative N-terminal fragment of the AP-3 δ subunit prevented Gag from reaching the multi-vesicular body component of the endocytic pathway (110). Expression of a dominant negative clathrin-associated adaptor complex AP-2 was shown by immunofluorescence to alter the localization of HIV Gag in cells and virions released

exhibited reduced infectivity (23), also supporting an interaction between Gag and cellular proteins that traffic through the endosomal compartments of the cell (25, 105, 155).

Retroviral Gag proteins have been found to interact with components of protein complexes that are associated with endosomes and that function in the invagination and fission of cellular membranes. Retroviral Gag polyproteins contain one or more cis-acting tetra-peptide sequences, PT/SAP, PPXY, and $YP(X)_nL$ (in which X is any amino acid), that have been termed "late domains" and which when mutated result in capsids that are arrested at a late stage of budding and that in electron micrographs extend from the cell surface at the end of a membrane stalk (145, 147, 178, 411). Electron micrographs of a murine leukemia virus late domain mutant showed some particles extended at the end of a membrane stalk and the extension of tube-like structures from the plasma membrane (418). In biochemical assays, retroviral late domains mutants in which the tetra-peptide, proline-rich late domain sequence was either deleted or disrupted by amino acid substitutions resulted in a decrease of capsid released from the cell for HIV (PTAP and $YP(X)_nL$) (145, 178, 226, 352), RSV (PPXY) (409, 410), M-PMV (PPXY and PSAP) (147, 411), MuLV (PTAP and PPXY) (419), HTLV-1 (PPXYEPTAP) (203), equine infectious anemia virus ($YP(X)_nL$) (292, 352), and foamy virus (PTAP and PPPI) (Fig. 7A) (276). The late domain function of mediating viral release was found to be redundant since the late domain of one virus, for example RSV or HIV, could restore particle release in an MuLV L-domain mutant (418). Virion release was partially or fully restored for L-domain mutants in which a functional L-domain sequence was introduced into the Gag polyprotein, and this phenomenon was independent of the

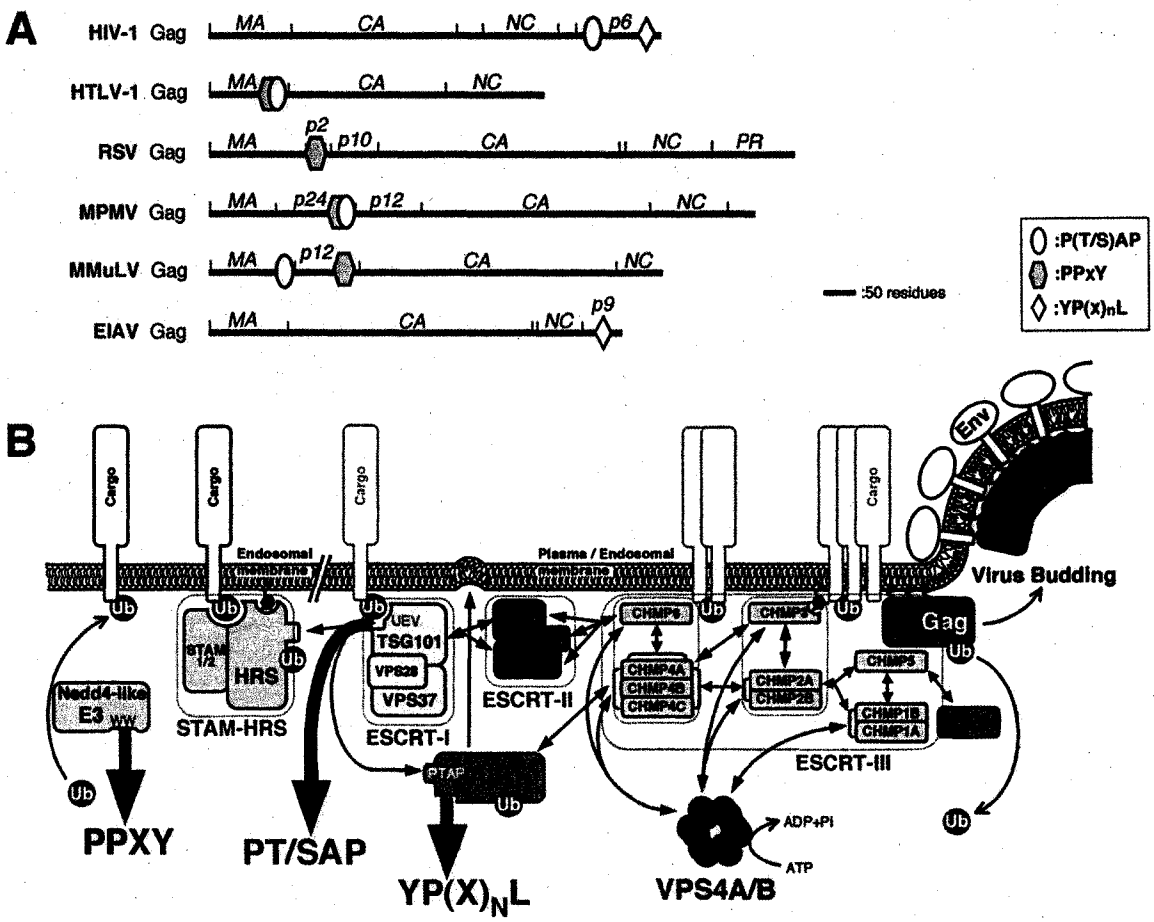

FIG. 7. Retroviral late domains and the ESCRT complexes. (A) Late domain motifs in the structural proteins of retroviruses. (B) Model showing the interaction network of the ESCRT complexes. Nedd4-like E3 proteins with WW domains interact with Gag proteins containing the PPXY late domain. Tsg101 interacts with PT/SAP containing Gag proteins. AIP1 binds Gag with $YP(X)_NL$ late domain motif. Adapted from Morita and Sundquist, 2004 (245). Reprinted, with permission, from the *Annual Review of Cell and Developmental Biology,* Volume 20, © 2004 by Annual Reviews www.annualreviews.org.

position within the Gag polyprotein at which the functional L-domain was inserted (214, 238, 271). The overlap in function of the three different retroviral L-domains is most likely a result of all three L-domains interacting with proteins that are a component of the same cellular pathway, which functions in the biogenesis of the multi-vesicular body (MVB) and involves at least three complexes of proteins called the endosomal sorting complexes required for transport (ESCRT). The protein complexes ESCRT-I, ESCRT-II, and ESCRT-III, for which 27 proteins have been identified, come together sequentially with ESCRT-III being recruited by proteins in either the ESCRT-I or the ESCRT-II complex (Fig. 7B) (16, 35). Retroviral Gag proteins that contain a PT/SAP motif have been shown to interact with Tsg101, a protein in the ESCRT-1 complex, and this interaction has been biochemically and structurally characterized (106, 137, 289, 290). A Tsg101 interacting protein, AIP1 (227, 388), has been identified to interact with the $YP(X)_nL$ late domain motif in both equine infectious anemia virus and HIV (352). It is less clear how Gag proteins which contain the PPXY motif enter the pathway. Proteins that contain a PPXY motif have been shown to interact with the cellular protein Nedd4 or a Nedd4-like protein (317, 378, 413). The Nedd4 family of proteins are ubiquitin E3 ligases which are involved in the covalent attachment of ubiquitin to a membrane associated protein (39). Ubiquitin is a 76 amino acid protein that is covalently attached to proteins via a pathway similar to sumoylation (Fig. 6), and mono-ubiquitination has been shown to direct the modified protein toward the MVB (246). The ubiquitination of multiple Gag proteins has been reported to be important in viral release which likely functions to direct the PPXY L-domain proteins to the ESCRT machinery (39, 148, 245, 267, 268, 349, 383). Additional support that all three late domains interact with the

ESCRT-III complex is provided by findings that expression of an AAA ATPase-defective Vps4, the ATPase that interacts with ESCRT-III to complete membrane fission (15, 16, 35, 329, 330), inhibits virion release for retroviruses which are dependent on either a functional PTAP or a functional PPXY L-domain (137, 147). The interaction between retroviral Gag L-domains and the ESCRT complexes is not entirely delineated and new protein-protein interactions are likely to be found. It also remains to be determined where in the cell the protein components of the ESCRT machinery first interact with retroviral Gag proteins. It is clear that retroviruses are not the only virus family that interacts with this cellular pathway. Late domain motifs that facilitate release of virions have been identified in the structural proteins of Ebola virus (Filovirus) (216, 228), vesicular stomatitis virus (Rhabdovirus) (96, 165), Lassa virus (Arenavirus) (283, 354), an influenza virus (Orthomyxovirus) (180, 181), and parainfluenza virus 5 (Paramyxovirus) (324), supporting a common mechanism for enveloped RNA virus budding.

Release of a retroviral capsid from the cell activates the viral protease, which cleaves the Gag polyprotein to generate an infectious mature virion. The activation of protease is regulated by an unknown mechanism. Initial insights into conditions that might activate protease *in vivo* come from *in vitro* studies of M-PMV capsids isolated from the cell cytoplasm that demonstrate that M-PMV protease can be activated by a reducing agent (272). Activation of protease and proteolysis of Gag yields at least the MA, CA, and NC proteins. Cleavage of CA from the polyprotein releases constraints to allow both inter- and intra-molecular interactions between capsid molecules (202, 387). The change in CA-CA interactions is essential for the CA protein to collapse and encase the NC:dimeric RNA complex which generates the mature core, an essential component

of the infectious virion (130, 144, 146, 200, 266, 353, 362, 363, 387, 391). The viral protease also cleaves the M-PMV and MuLV Env glycoprotein cytoplasmic tail to enhance the fusion capability of the glycoprotein (40, 58, 152, 303, 339, 346).

Protein-membrane interactions. At the plasma membrane, M-PMV capsids that have assembled intracellularly must catalyze the membrane envelopment of a 90 nm spherical particle. The Gag MA domain, on the surface of the capsid, is critical for membrane association of an assembled capsid (306). The M-PMV MA is cotranslationally modified with myristic acid (327) and contains a dense positive charge on the membrane-proximal surface of the protein as determined by the position of amino acids in a nuclear magnetic resonance structure (94). The hydrophobic and electrostatic interactions conferred from the myristylation and positive-charge region of a protein have been reported to facilitate the interaction of the cellular myristylated alanine-rich C kinase substrate (MARCKS) (4), Src (192), and Ras (73) as well as the retroviral Gag matrix domain of MuLV, HIV (172, 361), SIV (297), M-PMV (94), and BLV (234) with the plasma membrane.

The specific membrane targeting of myristylated cellular proteins led to the idea that a linked myristyl group could act as a dynamic partner in protein trafficking within the cell. This idea was substantiated by the observation that the viral Src protein is oncogenic when myristate, a 14-carbon saturated fatty acid, is covalently attached to the N-terminus of the protein, but does not have transforming properties in the absence of the hydrophobic moiety (192). The dynamics of a myristylated protein were not fully understood until the crystal structure of the unmyristylated recoverin, a Ca^{2+} sensor found in retinal rod cells (300), was solved (131). This structure revealed that recoverin

was comprised of two helix-loop-helix, EF-hand motifs (17, 335) which could bind calcium and two EF-hand motifs that were defective for calcium binding. The N-terminal seven amino acids were disordered, supporting flexibility of this region; also, a hydrophobic crevice between two of the EF-hand motifs was potentially a site where myristate might be sequestered within the protein (131). The nuclear magnetic resonance structure of myristylated recoverin in the absence of calcium determined that the myristyl group is buried in the center of the nonfunctional helix-loop-helix, EF-hand-like motif (360). 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 (11). The structural data for myristylated recoverin first defined that a myristyl-switch mechanism, an induced change in the orientation of myristic acid from the hydrophobic interior of a protein to the hydrophobic interior of the plasma membrane, mediates protein-membrane interactions.

A similar but distinct myristyl-switch mechanism has been described for the myristylated alanine-rich protein kinase C substrate (MARCKS) protein. The MARCKS protein is an unstructured, rod-like protein as confirmed by high axial ratio and circular dichroic spectral analysis (7, 37, 164). In the case of MARCKS, myristate is thought to be sequestered within the protein prior to an electrostatic association of the positively charged, basic effector domain (residues 151-175) of MARCKS with acidic phospholipid head groups on the inner leaflet of the plasma membrane (237). This model is from data in which substitution of the N-terminal glycine residue with alanine prevents myristylation and abrogates the MARCKS protein capacity to bind membrane (141, 149, 332, 356). Experiments with artificial membranes and the purified MARCKS proteins

suggest that both hydrophobic insertion of myristate and electrostatic interactions mediate the protein-membrane association (198, 364, 379). A MARCKS deletion mutant which lacks residues 6-140, which would remove a potential sequestration site for myristate and bring the myristate moiety closer to the positively charged effector domain, binds tightly to cellular membranes and is not cycled into the cytoplasm as determined in subcellular fractionation studies (332). *In vitro* studies support the electrostatic myristyl-switch 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 (251, 299, 390).

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 (94, 270, 327, 348, 361). Support for a common assembly unit and membrane-binding mechanism for retroviruses is present in the α-helical similarity of the matrix protein structures. The HIV and SIV matrix protein consists of five α-helices capped by a three-stranded mixed β-sheet (172, 297, 361). The BLV and M-PMV matrix proteins are comprised of four α-helices arranged in two perpendicularly aligned pairs (94, 234). In addition to the similarity of the first four helices, these matrix proteins each contain a structurally analogous localized position of basic amino acids (94, 172, 234, 297, 361).

Covalent attachment of myristate to the matrix domain of type C retroviruses (HIV and MuLV) is required for the Gag polyprotein to multimerize and assemble capsids at the plasma membrane (146, 302, 328, 389). A deletion mutation that disrupts

the structure of the HIV matrix domain without altering myristylation redirects capsid assembly and membrane extrusion to the endoplasmic reticulum (125). Moreover, HIV capsid assembly is redirected to the MVB (265), or capsids are released into intracellular vesicles (76), when basic residues on the outer surface of the matrix domain are substituted with acidic residues. An MuLV MA charge mutant in which four tándem basic amino acids (KKRR) were substituted with alternating acidic amino acids (ENEN) redirected capsid assembly to intracellular vesicles and some assembled intracellular capsids could be detected in electron micrographs (342). HIV MA mutants that replace hydrophobic residues oriented toward the core of the matrix domain with less hydrophobic residues also result in redirection of capsid assembly to the MVB (135, 263). Furthermore, when cellular phosphatidylinositol (4,5) bisphosphate was depleted from the plasma membrane by over-expression of a specific cellular phosphatase or when a constitutively active form of Arf6 was expressed, HIV Gag was redirected to late endosomes (262). These biochemical data support the hypothesis that electrostatic interactions between negatively charged phospholipid head groups and the bipartite signal within MA facilitate Gag association with the plasma membrane.

Recently, the nuclear magnetic resonance structure of myristylated HIV matrix protein demonstrated that myristate is partially buried within the protein monomer and exposed when either the matrix protein is in a trimer or when C-terminal subdomains of Gag that promote self association are present (MA-CA protein) (361).

For type D retroviruses, covalent attachment of myristate to the matrix domain is not required for capsids to assemble. Electron micrographs showed that an M-PMV MA mutant in which glycine at position 2 is replaced with valine to abrogate myristylation

assembles capsids that accumulated at a perinuclear location of the cytoplasm, providing support that myristylation is critical in intracellular transport of assembled capsids (307). In addition to transport, protein-lipid interactions that involve the MA domain of M-PMV Gag are required for the preassembled capsid to catalyze membrane extrusion. Introduction of random point mutations within MA by sodium bisulphate mutagenesis generated a mutant in which the threonine amino acids at positions 41 and 78 were replaced with the more hydrophobic isoleucine residues (T41I/T78I) (94). Release of capsids from infected cells was delayed for the T41I/T78I mutant in pulse-chase assays and electron micrographs showed assembled capsids accumulated at the plasma membrane in an early stage of membrane extrusion. Since the threonines at positions 41 and 78 were oriented toward the core of the matrix protein in the nuclear magnetic resonance structure, it was reasoned that M-PMV MA likely undergoes a myristyl-switch mechanism to initiate the budding process and that the defect of this MA mutant is in release of myristic acid from the more hydrophobic MA core (94, 306).

The studies presented in this thesis test genetically the hypothesis that the M-PMV MA domain sequesters the myristate moiety prior to a phospholipid-triggered exposure of myristate into the hydrophobic environment of the plasma membrane (Fig. 8). If M-PMV MA undergoes a specific electrostatic myristyl-switch mechanism, the hydrophobicity of the MA core and the positive charge on the outer surface of M-PMV MA would be expected to modulate the interaction of capsids with the plasma membrane.

The first article in this thesis describes a study in which the hydrophobicity of the MA core was increased to investigate whether the hydrophobic nature of the proposed

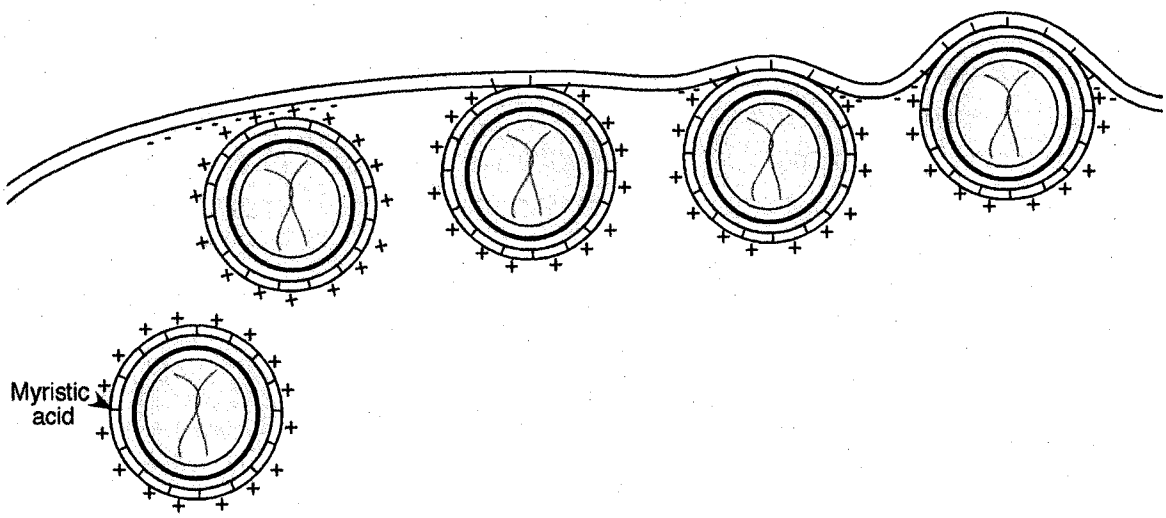

FIG. 8. Electrostatic myristyl-switch hypothesis. The proposed events that ensure assembled M-PMV capsids are released at the plasma membrane. The hypothesis states that myristic acid is sequestered within the matrix domain of the assembled capsid. Upon capsid transport to the plasma membrane, positively charged amino acids on the surface of MA interact with specific negatively charged phospholipid head groups to trigger the release of myristate into the hydrophobic environment of the lipid bilayer.

myristic acid binding site modulates capsid-membrane interactions and viral budding. Three tyrosine residues oriented toward the core of the protein in the NMR structure of MA were substituted individually or in a pair-wise combination with the more hydrophobic phenylalanine residue(s). As a control, a tyrosine oriented toward the outer surface of MA was also substituted with phenylalanine. These tyrosine to phenylalanine substitutions did not alter capsid assembly as compared to wild type in a capsid assembly assay. Pulse-chase, immunofluorescence and electron microscopy studies demonstrated that single substitutions of tyrosine residues oriented toward the core of MA recapitulated the mutant phenotype of decreased budding kinetics and accumulation of capsids at the plasma membrane. MA double mutants with a combination of these tyrosine substitutions exhibited a phenotype that is even more defective in budding. In contrast, MA mutants that incorporated the replacement of tyrosine with a phenylalanine that was oriented toward the surface of MA resulted in a transport defective phenotype. These results strongly support the hypothesis that myristic acid is sequestered inside MA prior to capsid-membrane interactions.

The second article in this thesis details a study in which the positive charge cluster, predicted to be on the membrane-proximal surface of the M-PMV MA based on the nuclear magnetic resonance structure (94), was altered to determine if basic amino acids in MA alter capsid-membrane interactions. MA mutants in the context of the provirus were constructed in which lysine and arginine residues potentially involved in these interactions (R10, K16, K20, R22, K25, K27, K33 and K39) were substituted singly and in pairs with alanine. With the exception of K16A, which was released rapidly from cells, a majority of the charge substitution mutants were released with kinetics that were

slower than that of wild type. Electron and immunofluorescent microscopy of mutant Gag expressing cells revealed distinct phenotypes. MA mutants in which a basic charge close in proximity to the proposed myristic acid binding site was eliminated (K16A and K20A) resulted in immature capsids that accumulated on and budded into intracellular vesicles. These capsids colocalized with the endosomal markers for the pericentriolar recycling endosome, the sorting endosome, and the MVB. For K33A and K39A, immature capsids accumulated at the inner leaflet of the plasma membrane in an early stage of membrane extrusion, much like the phenotype observed for the MA hydrophobic core mutants; the defective step of these mutants may be in triggering the release of myristate from the core of MA. Mutant capsids accumulated below (R22A) or were traversing through the filamentous actin (R10A and K27A), indicating a role for actin in M-PMV capsid transport. These results support the hypothesis that basic residues in the M-PMV MA define both cellular location and efficiency of virus budding through the regulation of myristate exposure and membrane association.

AN EARLY STAGE OF MASON-PFIZER MONKEY VIRUS BUDDING IS REGULATED BY THE HYDROPHOBICITY OF THE GAG MATRIX DOMAIN CORE

by

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ABSTRACT

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.

INTRODUCTION

Mason-Pfizer monkey virus (M-PMV), a betaretrovirus, assembles an immature protein shell (capsid) from its Gag polyproteins in a pericentriolar region of the cytoplasm prior to plasma membrane transport and viral budding (14, 25, 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 polyprotein plays a critical role in this process (26), as well as in directing translating polysomes to the intracellular assembly site (32) and subsequent transport to the plasma membrane (8, 25, 26, 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 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 polyprotein 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 polyprotein 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 non-myristylated 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 Fugene 6 method (Roche Molecular Biochemicals, Indianapolis, Ind.). Approximately 24 h posttransfection, 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 [^{35}S]methionine-[^{35}S]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 polyprotein 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 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 Na_2EDTA, 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 NH_4Cl, 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% gluteraldehyde, 2% osmium tetroxide, 2 mM $CaCl_2$, 4 mM $MgSO_4$ 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 [^{35}S]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

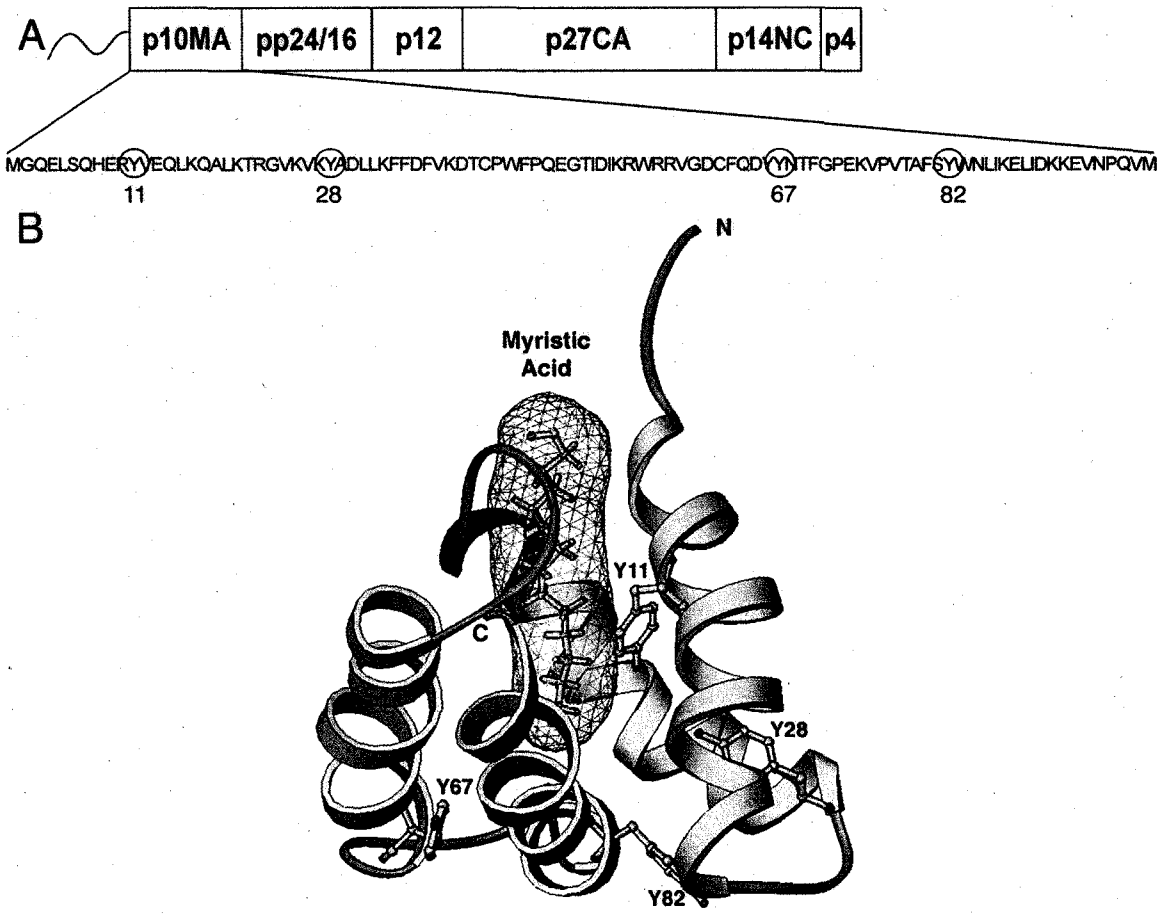

FIG. 1. Location of tyrosine-to-phenylalanine substitutions in the M-PMV matrix domain. (A) Schematic diagram of the M-PMV Gag polyprotein. Myristate is shown attached to the N-terminal MA domain of Gag. The primary sequence of MA is shown, with tyrosine residues 11, 28, 67, and 82 circled. (B) Conceptual 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.

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 slower 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).

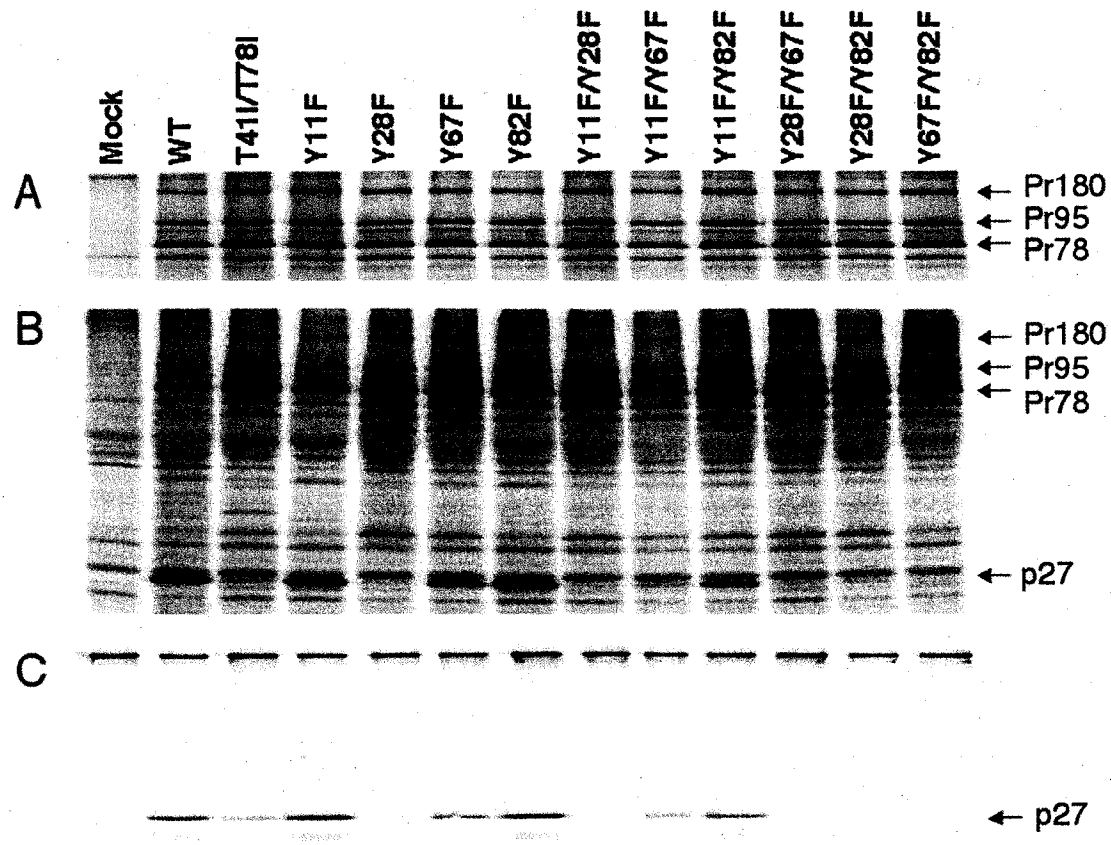

FIG. 2. Synthesis and processing of wild-type and mutant M-PMV Gag. COS-1 cells were transfected with wild-type and mutant M-PMV proviral genomes. Viral proteins were metabolically labeled with [^{35}S]methionine-cysteine and then immunoprecipitated from cell lysates and analyzed by SDS-PAGE. (A) Autoradiogram 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.

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 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,

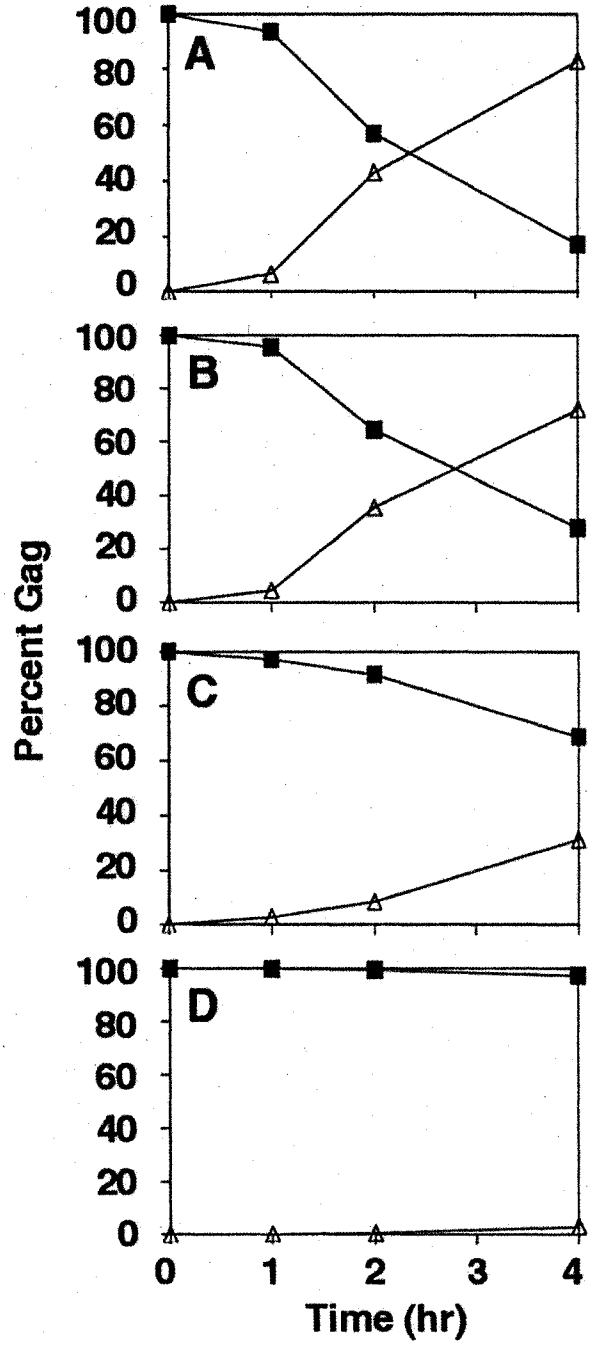

FIG. 3. Kinetics of Gag precursor processing and virion release from cells. COS-1 cells transfected with an M-PMV proviral genome were metabolically labeled and then chased for 0, 1, 2, and 4 h. Viral proteins were immunoprecipitated from cell lysates and culture medium at each time point and, following 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.

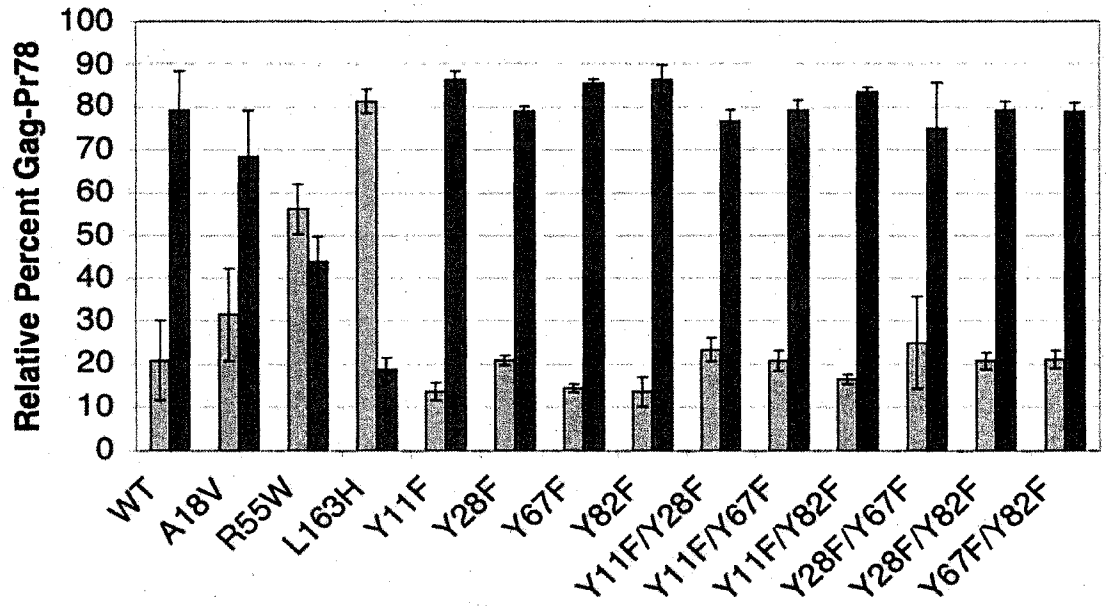

FIG. 4. Assembly of wild-type and mutant immature capsids. COS-1 cells were metabolically labeled for 10 min and then chased at 37°C for 30 min. Cells were lysed, and Gag precursors were centrifuged through a 35% sucrose cushion. Unassembled Gag remained on top of the sucrose cushion, while assembled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

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

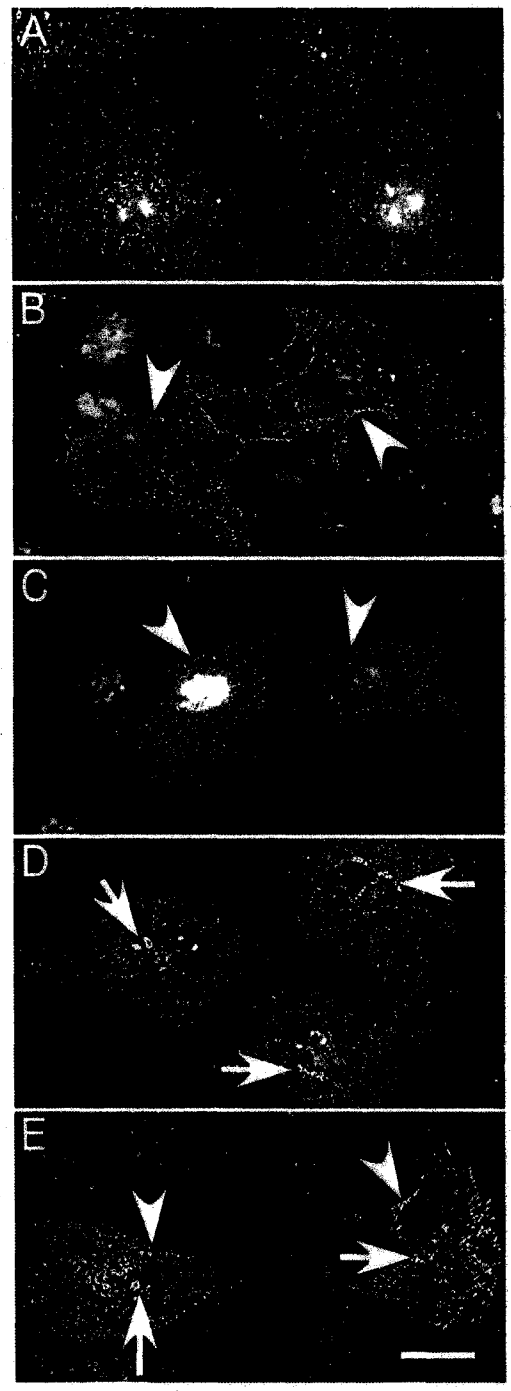

FIG. 5. Immunofluorescent staining of M-PMV Gag. COS-1 cells transfected with a wild-type or mutant M-PMV proviral genome were fixed 24 h later and probed with a monoclonal antibody directed against the p12 region of the Gag polyprotein. Arrowheads denote Gag stain at the plasma membrane, while arrows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

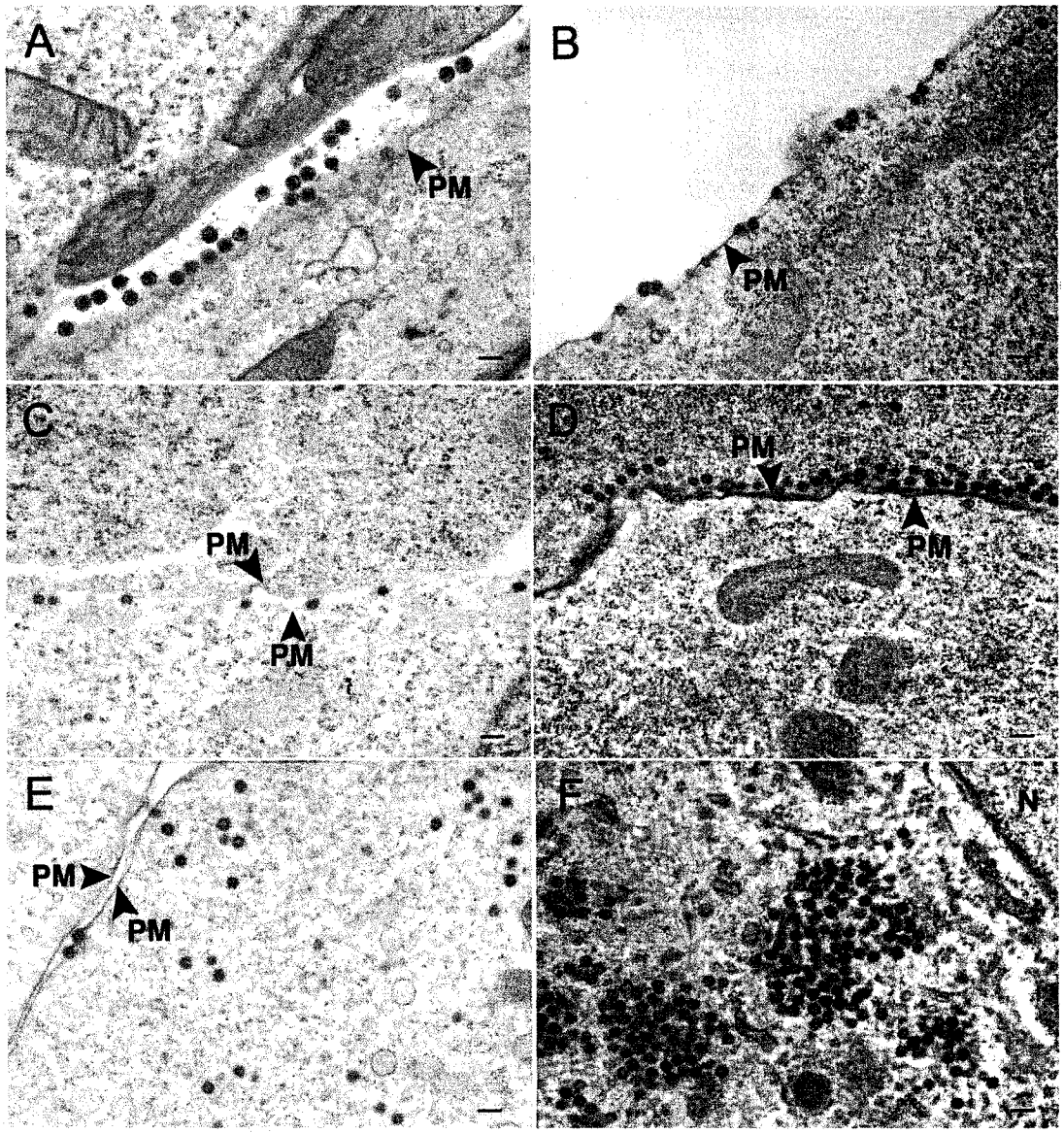

FIG. 6. Thin-section electron microscopy of COS-1 cells expressing wild-type and mutant M-PMV proviruses. COS-1 cells transfected with wild-type or mutant M-PMV proviral genomes were fixed 24 h later in 1% glutaraldehyde and 2% osmium tetroxide to preserve proteins and membranes, respectively, prior to embedding and thin sectioning. Bar, 200 nm. N, nucleus. PM, plasma membrane. (A) Wild-type; (B) T41I/T78I; (C) Y11F; (D) Y11F/Y28F; (E) Y11F/Y82F; (F) 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 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 membrane 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 has 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.

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BASIC RESIDUES IN THE MASON-PFIZER MONKEY VIRUS GAG MATRIX DOMAIN REGULATE CAPSID-MEMBRANE INTERACTIONS.

by

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ABSTRACT

Mason-Pfizer monkey virus (M-PMV) capsids that have assembled in the cytoplasm must associate with the plasma membrane and be enveloped by a lipid bilayer during viral release. We recently described mutants that increased the hydrophobicity of the matrix (MA) domain core and blocked M-PMV release at an early stage of budding, arguing that a myristyl-switch mechanism facilitates envelopment (E. Stansell, E. Tytler, M. R. Walter, and E. Hunter, J. Virol. 78:5023-5031, 2004). Structural studies have identified a positive-charge density on the membrane-proximal surface of MA. To investigate if basic residues in MA play a role in triggering the release of myristic acid from the hydrophobic core, mutants were constructed in which lysine and arginine residues potentially involved in these interactions (R10, K16, K20, R22, K25, K27, K33, and K39) were substituted singly and in pairs with alanine. With the exception of K16A, which was released rapidly from cells, a majority of the charge substitution mutants were released with kinetics that were slower than that of wild type. Electron and immunofluorescent microscopy of mutant Gag expressing cells revealed three distinct phenotypes. For K16A and K20A, immature capsids accumulated on and budded into intracellular vesicles and colocalized with the endosomal markers Rab11, Rab5, and CD63. For R10A, R22A, K27A, K33A, and K39A, immature capsids accumulated near or at the inner surface of the plasma membrane, while K25A immature capsids appeared to be defective in intracellular transport. We postulate that, through regulation of myristate exposure and membrane association, basic amino acids in the M-PMV MA define both cellular location and efficiency of virus budding.

INTRODUCTION

Mason-Pfizer monkey virus (M-PMV), an immunosuppressive betaretrovirus, catalyzes the membrane envelopment of a preassembled spherical protein shell (capsid) to release infectious virions. In contrast, viruses such as human immunodeficiency virus (HIV) simultaneously assemble an immature capsid as they extrude the plasma membrane (19). The presence of myristic acid and a basic domain on many retroviral matrix (MA) proteins has led to the hypothesis that a bipartite signal initiates the molecular interactions necessary for a myristylated Gag polyprotein to associate with the plasma membrane and instigate the processes of capsid assembly and budding (C-type morphology) or membrane extrusion (B/D-type morphology) (4, 62, 63).

M-PMV assembles capsids from its Gag polyproteins in a pericentriolar region of the cytoplasm prior to plasma membrane transport and viral budding (46). Transport of capsids from the assembly site to the plasma membrane is dependent on a functional endosomal pathway, and release of capsids is seven times more efficient in the presence of the viral envelope glycoprotein (45). The envelope glycoprotein must enter the endosomal pathway, following cleavage into the surface (gp70) and transmembrane (gp22) subunits in the Golgi, in order to be incorporated into capsids (47, 49).

Myristylation of MA is also required for transport and release of capsids. Myristic acid, a 14-carbon saturated fatty acid, is covalently attached to a glycine residue at position 2 of MA during translation of the Gag polyprotein, and a glycine to valine mutant that is defective for myristic acid attachment assembles capsids that remain at a perinuclear region of the cell (39). MA mutants that have a more hydrophobic core are myristylated and transported to the plasma membrane but are defective at an early stage

of budding. This supports the hypothesis that the myristic acid moiety is sequestered within the MA domain upon capsid assembly, that this conformation is important for plasma membrane transport, and that it reorients into the plasma membrane during envelopment via a myristyl-switch mechanism (38, 53).

The concept of a myristyl switch was first defined from NMR analyses of recoverin, a myristylated cellular protein found in retinal rod cells (35). These studies showed that the N-terminal myristate moiety was buried within the protein in the absence of calcium and extruded from the protein when calcium was bound (1, 55, 64). A similar but distinct myristyl-switch mechanism has been described for the myristylated alanine-rich protein kinase C substrate (MARCKS) protein (21, 44). In this case, myristate is sequestered within the protein core prior to an electrostatic interaction of the positively charged, basic effector domain (residues 151-175) of MARCKS with acidic phospholipid head groups on the inner leaflet of the plasma membrane (12, 34, 54, 57, 59).

The hypothesis that myristic acid is also sequestered inside the matrix domain of M-PMV prior to plasma membrane interactions was first proposed upon NMR analysis of the M-PMV matrix protein. The M-PMV MA is comprised of four helical domains arranged in two perpendicularly aligned pairs, with two distinct positively charged regions located on opposite sides of the molecule (6). The positively charged region which contains basic side chains of amino acids from helices A and B is analogous to the N-terminal basic region seen in the structures of HIV (18, 56) and SIV (33) MA. 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 (6, 28, 32, 33, 52, 56). Covalent attach-

ment of myristate to the matrix domain of HIV Gag is required for the Gag polyprotein to multimerize and assemble capsids at the plasma membrane (4, 13). A deletion mutation that disrupts the structure of the HIV matrix domain without altering myristylation redirects capsid assembly and membrane extrusion to the endoplasmic reticulum (9). Moreover, HIV capsid assembly is redirected to multi-vesicular bodies when basic residues on the outer surface of the matrix domain are substituted with acidic residues (27) or when hydrophobic residues that face the core of the matrix domain are substituted with less hydrophobic residues (10, 25). These biochemical data suggest that myristylation and positive charge residues in the matrix domain of HIV Gag direct protein association with the plasma membrane.

M-PMV is an ideal system to use to specifically investigate the interaction between the Gag proteins and the plasma membrane since capsid assembly and budding are spatially and temporally separate. To provide support for the hypothesis that the M-PMV MA undergoes a myristyl-switch mechanism and to determine whether substitutions that decrease the positive charge of the matrix domain could interfere with virus budding, we have replaced arginine and lysine residues in helices A and B with the neutral amino acid alanine. Single substitutions of the MA arginine residues to alanine as well as lysine at position 27 to alanine resulted in decreased release of virus and an accumulation of immature capsids at the peripheral filamentous actin network that underlies the plasma membrane. In contrast, replacement of lysine residues at positions 16 and 20 with alanine resulted in an increased release of virus for the former with capsids accumulating on and budding into intracellular endosomal vesicles. Substitution of lysine at position 25 resulted in a defective transport phenotype. Lysine residues at

positions 33 and 39 when replaced with alanine resulted in decreased release of virus, and capsids could be found lining the inner leaflet of the plasma membrane. These results support the hypothesis that basic amino acid residues in M-PMV MA facilitate electrostatic capsid-membrane interactions which initiate membrane envelopment via a myristyl-switch mechanism.

MATERIALS AND METHODS

Cells and antibodies. COS-1 and 293T 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 (Hyclone Laboratories, Logan, Utah), 10 U/ml penicillin G sodium, and 10 μg/ml streptomycin sulfate (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 20 μg/ml (41). Polyclonal rabbit anti-Pr78 (M-PMV Gag) serum 3493 (41) was diluted 1:500. The mouse monoclonal anti-CD63 antibody (clone H5C6) was used at a concentration of 1 μg/ml (BD Biosciences, San Jose, Calif.). FITC-Goat anti-rabbit Immunoglobulin G was purchased from Zymed Laboratories (San Francisco, Calif.). Alexa Fluor 594 goat anti-mouse Immunoglobulin G was purchased from Molecular Probes Inc. (Eugene, Ore.).

Construction of mutant proviruses. Mutant derivatives of the M-PMV proviral vector were constructed using pNCS (53), a plasmid containing a *gag* fragment corresponding to nucleotides 351 to 1167 of pSARM4 (48). The desired codon(s) was generated in pNCS by PCR-directed mutagenesis. For each single mutant, complementary and reverse-oriented mutagenic primers with a two base pair change compared to the wild-type sequence were designed. The sequences of the mutagenic oligonucleotides used were as follows: R10A, 5'-GCAGGCTTTAGCGACAGGGG-GAG; K16A, 5'-GAACAATTGGCGCAGGCTTTAAAG; K20A, 5'-GCAGGCTTTAGCGACACG-GGGAG; R22A, 5'-GGCTTTAAAGACAGCGGGAGTAAAGG; K25A, 5'-GACA-CGGGGAGTAGCGGTTAAATATGC; K27A, 5'-GGGAGTAAAGGTTCATATG-

CTGATC, K33A, 5'-GCTGATCTTTTGGCATTTTTTTGATTTTGTGAAGG, and K39A, 5'-GATTTTGTGGCGGATACTTGTC. Double mutants, with the exception of K20A/R22A (5'-GGCTTTAGCGACAGCGGGAGTAAAGG), R22A/K25A (5'-GA-CAGCGGGAGTAGCGGTTAAATATGC), and K25A/K27A (5'-GACACGGGGAGT-AGCGGTTGCATATGCTG), were generated by including two single mutagenic primer pairs in the PCR. Multiple-round PCR using *Pfu-turbo* DNA polymerase (Stratagene, La Jolla, Calif.) incorporated the mutation from the primers into the pNCS plasmid. Follow-ing 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 the desired base pair changes were introduced, and then the EagI to PacI mutated M-PMV *gag* fragment corresponding to nucleotides 407 to 750 was reengineered into the M-PMV proviral expression vector pSARM4.

Metabolic labeling and immunoprecipitation of Gag. 293T cells were transfected with wild-type or mutant proviral constructs by the Fugene 6 method (Roche Molecular Biochemicals, Indianapolis, Ind.). Approximately 24 h posttransfection, 293T cells expressing the wild-type or mutant M-PMV viral proteins were starved for 10 min with methionine and cysteine-deficient DMEM (Gibco) and then pulse-labeled for 15 min at 37°C with 100 μCi of [^{35}S]methionine-[^{35}S]cysteine protein-labeling mix (Perkin-Elmer NEN, Boston, Mass.). The radioactive medium was removed at the end of the pulse period; cells were then chased in complete DMEM for 1, 2, 4, or 24 h. Pulse cells were washed with cold tris-buffered saline and lysed in 200 μl of 1% sodium dodecyl sulfate (SDS) in phosphate-buffered saline (PBS) for 5 min at room tempera-

ture. Cell lysates were then scraped from the well, transferred to eppendorf tubes, and boiled for 5 min. The volume of each cell lysate was then adjusted to 1 ml by adding 800 μl of lysis buffer A (1% Triton X-100, 1% sodium deoxycholate, 0.05 M NaCl, 0.025 M Tris, pH 8.0). RNase-free DNase (10U; Stratagene) was added to each lysate prior to a 1 h incubation at room temperature. 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) serum 3493 (41) and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Quantitation of Gag polyprotein processing. The SDS-12% PAGE gels were dried, and the radiolabeled protein bands were quantitated on a Packard Cyclone system using OptiQuant software (Perkin Elmer, Shelton, 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; the number was divided by the sum of the adjusted band intensities and then 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 that all labeled Gag is incorporated into mature virions and does not take into consideration labeled Gag proteins that undergo degrada-

tion. These calculations are sufficient to compare changes in the rate of Gag processing for mutant M-PMV Gag to that of wild type.

Electron microscopy. COS-1 cells transiently expressing the wild-type or mutant M-PMV proviral constructs were grown as a monolayer on 13-mm thermonox plastic coverslips (Nunc, Rochester, N.Y.) and fixed for 4 h in freshly prepared 2.5% glutaraldehyde in 0.1 M cacodylate buffer. The cells were postfixed in 1% osmium tetroxide, dehydrated in an ethanol series (30, 50, 70, 80, 90, and 100%) and embedded in fresh EMBED 812 resin in a labeled Beem capsule. Ultrathin sections (90-100 nm) of COS-1 cells expressing wild-type and mutant M-PMV were cut with a diamond knife on an RMC MT 7000 Ultramicrotome and picked up on 200-mesh copper grids. Several sections were collected onto multiple grids for each sample. A JEOL JEM-1210 analytical transmission electron microscope operated at 90 kV was used to view the sections. For each sample 3-7 transfected cells were identified and images of M-PMV capsids with the cell were captured on film. The developed negatives were then scanned with an AGFA Duoscan T2500 scanner into a computer to generate high-resolution image files. Adobe Photoshop CS was then used to adjust the black, white, and gray tonal ranges of the images for better visualization of M-PMV capsids within the cell.

Immunofluorescence microscopy. Indirect immunofluorescent staining was carried out as described previously (53) with the following modifications. To look at the distribution of wild-type and mutant Gag proteins in conjunction with cellular Rab-green fluorescent protein (GFP) fusion proteins, COS-1 cells were transfected with wild-type or mutant M-PMV constructs and Rab11-GFP, Rab4-GFP, Rab5-GFP, or Rab7-GFP (51). The cells were grown on No. 1.5 12-mm glass coverslips (Fisher, Pittsburgh, Pa.)

for 24 h and then fixed at room temperature for 15 min in freshly prepared 4% formaldehyde (Tousimis, Rockville, Md.) in PBS. For primary antibody staining, coverslips were incubated in 100 μl of 20 μg/ml (anti-M-PMV Gag) monoclonal antibody 10.10 diluted in PBS containing 2.5% goat serum (Abcam, Cambridge, Mass.) and 0.2% Tween 20 (Sigma) for 45 min at 37°C. Since the GFP fluorescence from the Rab-GFP fusion proteins was not quenched in these experiments, a primary anti-GFP antibody was not used. The coverslips were mounted in Prolong antifade reagent (Invitrogen, Carlsbad, Calif.) to prevent quenching of the fluorescence signal. The distribution of wild-type and mutant Gag and CD63, a member of the tetraspanin transmembrane 4 superfamily found on multi-vesicular bodies (8, 11), was examined as follows: COS-1 cells expressing wild-type or mutant M-PMV proviral constructs were fixed as above and then permeabilized and blocked with 5% goat serum in PBS containing 0.2% Tween 20. Coverslips were incubated in a primary antibody mixture (100 μl) containing a 1:500 dilution of a rabbit polyclonal anti-M-PMV Gag antibody and a 1 μg/ml anti-CD63 monoclonal antibody diluted in PBS containing 5% goat serum and 0.2% Tween 20. For secondary antibody staining, coverslips were incubated in 100 μl of a 20 μg/ml Alexa 594 goat anti-mouse Immunoglobulin G antibody and a 6 μg/ml FITC-labeled goat anti-rabbit Immunoglobulin G antibody diluted in PBS containing 5% goat serum in 0.2% Tween 20. Coverslips were mounted in the Prolong antifade reagent. Images were visualized with a multiple-wavelength, wide-field, three-dimensional microscopy system (Intelligent Imaging Innovations, Denver, Colo.) which is based on a Zeiss 200 M inverted microscope (Carl Zeiss, Thorn-wood, N.Y.). Immunofluorescent samples were visualized using a 63x oil objective with a numerical aperture of 1.4. Images of succes-

sive 0.2 μm optical sections were captured with a cooled Coolsnap HQ CCD camera which contained an Orca-ER chip. A standard Sedat filter set (Chroma Technology, Rockingham, Utah) was used to eliminate cross talk of fluorescent emission. The images were deconvoluted using the constrained iterative algorithm (43) and analyzed using Slidebook software (Intelligent Imaging Innovations).

RESULTS

Construction of mutants for arginine and lysine residues in M-PMV Gag matrix domain. To investigate whether positively charged amino acids in the matrix domain of M-PMV Gag are critical for capsid-membrane interactions, two arginine residues (R10 and R22) and six lysine residues (K16, K20, K25, K27, K33 and K39) were replaced individually and in a pair-wise combination with the smaller, non-polar alanine residue (Fig. 1A). The Van der Waals electrostatic potential map generated in Swiss-PDB viewer (http://www.expasy.org/spdbv/, 15) predicts a large positive-charge density on helices A and B of the matrix protein (6) (Fig. 1B) in which the side chains of R10 and R22 are oriented to the outermost surface of helix A, side chains of K16 and K20 are on the opposing side of helix A from R10 and R22, lysine residues at positions 25 and 27 are in the loop between helices A and B, and K33 and K39 are located in helix B (Fig. 1C). The confirmed M-PMV MA mutants R10A, K16A, K20A, R22A, K25A, K27A, K33A, K39A, R10A/K16A, K16A/K20A, K20A/R22A, R22A/K25A, K25A/K27A, K27A/K33A, K33A/K39A as well as the previously described matrix mutant R55W (37) and the wild type were studied in the context of an infectious provirus.

Synthesis and expression of wild-type and mutant M-PMV Gag. The synthesis and stability of wild-type and mutant Gag polyproteins with a decreased positive charge in the MA domain were assayed by a pulse-chase experiment. 293T cells transfected with wild-type and mutant proviral constructs were pulse-labeled with [^{35}S]methionine and then chased for 2 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

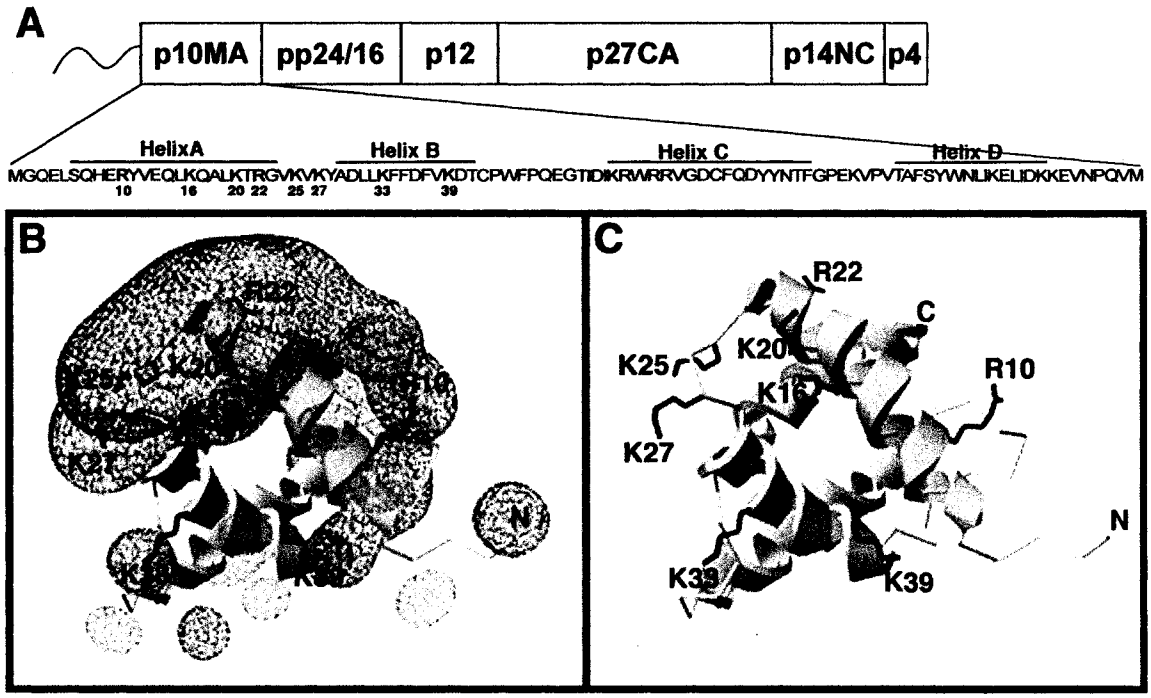

FIG. 1. Location of arginine and lysine residues in the M-PMV matrix domain. (A) Schematic diagram of the M-PMV Gag polyprotein. Myristate is shown attached to the N-terminal matrix domain (MA) of Gag. The primary sequence of MA is shown with arginine and lysine residues in bold and numbered. The lines denote the four helices of M-PMV MA. (B) The Van der Waals electrostatic potential map generated in Swiss-PDB viewer (http://www.expasy.org/spdbv/, 15). A large positive potential is depicted surrounding the arginine and lysine residues in helices A and B. (C) Positions of arginine and lysine residues in the context of the M-PMV matrix protein NMR structure.

collected. M-PMV *gag* gene products were immunoprecipitated as described in Materials and Methods and then subjected to SDS-PAGE and imaged using a Cyclone phosphorimager. During the pulse, M-PMV Gag polyprotein (Pr78) was synthesized for each mutant at levels similar to those of the wild type (data not shown). The mutant Gag polyprotein precursors displayed stability equivalent to that of wild type after a 2-h chase; however, the extent to which they were processed into mature products varied (Fig. 2A and 2B). The Gag polyprotein is proteolytically cleaved into MA (p10), pp24/16, p12, capsid (p27CA), nucleocapsid (p14NC), and p4 only after release of virions (3, 29, 50), since we have shown previously that even mutants blocked in a late stage of budding do not cleave Gag to its mature products (38, 61). Release of virus was assessed by measuring the percentage of Gag precursor proteins that were cleaved into capsid protein (p27) during the chase. Similar levels of cell-associated and virion-associated p27 were observed for the wild type and MA mutants K20A, K33A, K39A, R10A/K16A, and K16A/K20A (Fig. 2A and 2B). In contrast, increased levels of released p27 were observed for the R55W and K16A mutants, compared to that of wild type, suggesting that capsids are released more efficiently; also, M-PMV MA mutants K25A, K27A, and K33A/K39A processed less Gag precursor as determined by the lower amount of cell-associated and virion-associated p27. After a 2-h chase, cell-associated p27 could not be detected for R10A and only trace amounts of virion-associated p27 were detected, suggesting that this mutant is impaired in capsid release. Neither processing nor release of Gag was detected after a 2-h chase for R22A, K20A/R22A, R22A/K25A, K25A/K27A, and K27A/K33A.

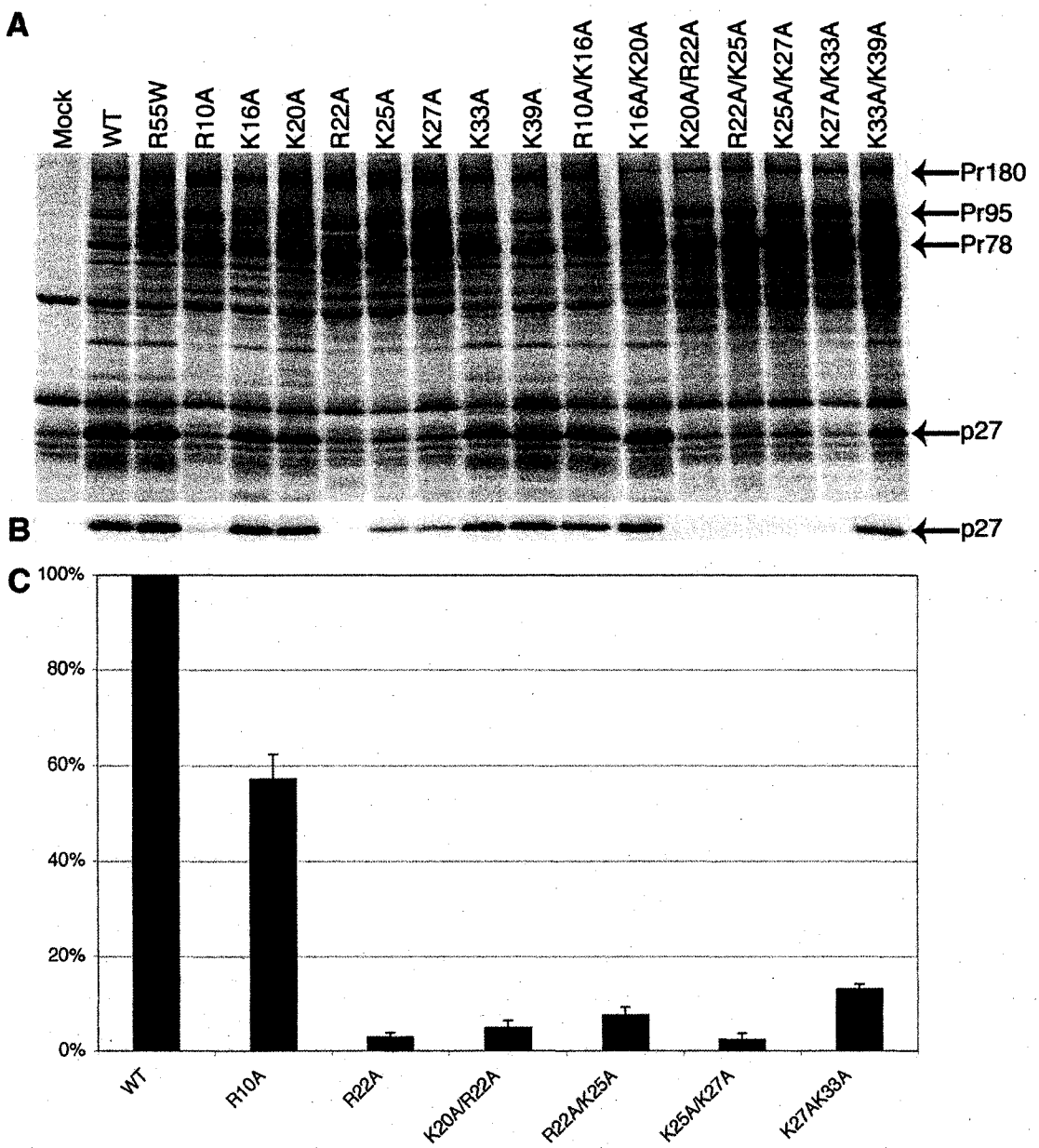

FIG. 2. Synthesis and processing of wild-type and mutant M-PMV Gag. 293T cells were transfected with wild-type and mutant M-PMV proviral genomes. Viral proteins were metabolically labeled with [^{35}S]methionine-cysteine and then immunoprecipitated from cell lysates and analyzed by SDS-PAGE as described in Materials and Methods. (A) Viral proteins immunoprecipitated from lysates of cells pulse-labeled and then chased for 2 h. Positions of the wild-type and mutant viral precursor proteins Pr78, Pr95, and Pr180 and of the major cleavage product of Gag (p27) are shown. (B) M-PMV p27CA immunoprecipitated from the culture medium collected after the 2-h chase. (C) Percentage of p27CA released after a 24-h chase.

In a separate experiment we assessed Gag processing and virus release after 24 h for the wild type, R10A, and those mutants for which processing of Gag was not detected at the 2 h time point (Fig. 2C). In these experiments, R10A released nearly 60% wild-type amounts of p27, while the R22A mutant remained more than 30-fold reduced. Of the double mutants, only K27A/K33A released more than 10% of the wild-type virus after 24 h.

Substitutions of arginine and lysine residues in helices A and B of the MA domain resulted in decreased release of virus with the exception of mutants with a single substitution of lysine at position 16. The kinetics of processing and release for wild-type and mutant Gag precursor proteins was investigated by a pulse-chase experiment in which cell-associated and virion-associated proteins were analyzed after a 1, 2, or 4-h chase. Cultures of 293T cells transfected with wild-type and mutant proviral constructs were pulse-labeled with [^{35}S]methionine and then chased for 1, 2, or 4 h in complete medium. Cell lysates from pulse-labeled cells and pulse chase cells, as well as culture medium from the latter, were collected for each time point. 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. OptiQuant software was used to obtain the band intensities for Gag (Pr78), Gag-Pro (Pr95), Gag-Pro-Pol (Pr180) and capsid (p27CA). The percentage of Gag precursor molecules and the percent capsid (CA) molecules relative to total Gag were calculated for each chase time point as described in Materials and Methods. The half-life of Gag precursor molecules processed to CA for wild-type M-PMV was 1.5 h (Fig. 3A). In contrast, accelerated kinetics of Gag precursor processing (half-life less than 1 h) was observed for MA

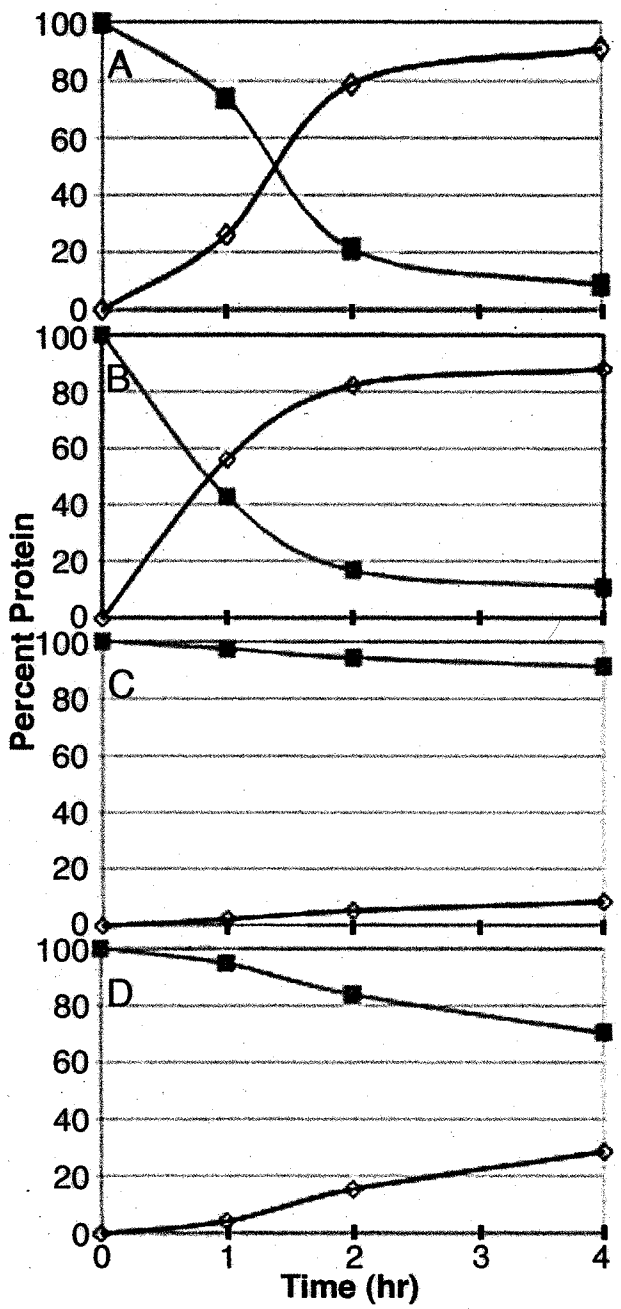

FIG. 3. Kinetics of Gag precursor processing and virion release from cells. 293T cells transfected with an M-PMV proviral genome were metabolically labeled with [^{35}S]methionine and then chased 0, 1, 2, and 4 h. Viral proteins were immunoprecipitated from cell lysates and culture medium at each time point and following SDS-PAGE were quantitated as described in Materials and Methods. The graphs show the results from a representative experiment. Solid squares, percentage of the total Gag precursor at each time point. Open diamonds, percent total capsid protein. (A) Wild type; (B) K16A; (C) R10A; (D) K27A.

mutants K16A (Fig. 3B) and R55W (data not shown). The double mutant K16A/K20A (data not shown) was processed 15 min faster than wild type, while R10A/K16A was released with kinetics similar to that of wild type (data not shown). The kinetics of Gag processing for K20A, K33A, and K39A was delayed approximately 15 min with a half-life of 1.75 h (data not shown), while mutants R10A (Fig. 3C) and K27A/K33A (data not shown) were released with substantially slower kinetics than that of wild type with only 5% of Gag processed after 4 h. Only 25% of Gag was processed at 4 h for the matrix mutants K25A (data not shown), K27A (Fig. 3D), and K33A/K39A (data not shown); for mutants that contain the R22A substitution, as well as the K25A/K27A MA mutant, no processing of Gag was detected 4 h after the pulse label (data not shown).

MA basic charge mutants accumulate at multiple intracellular locations as determined by Transmission Electron Microscopy. Positively charged amino acids in the N-terminal domain of the Gag protein appear to direct intracellular targeting and assembly of HIV (62, 63) and Rous sarcoma virus (RSV) (2, 5). Substitution of charged amino acid residues in M-PMV MA might therefore be predicted to alter the specificity of capsid-membrane interactions. To determine at high resolution the location of MA basic charge mutant capsids in COS-1 cells, thin sections of transfected cells expressing wild-type or mutant M-PMV provirus were analyzed by transmission electron microscopy. Cells expressing wild-type M-PMV exhibited few immature capsids in the cytoplasm and a large number of capsids in the process of budding or already released into the intracellular space (Fig. 4A, WT). As reported previously (37), cells expressing the R55W matrix mutant provirus exhibited a C-type assembly pattern with dense patches of protein accumulated at the plasma membrane (Fig. 4B, R55W).

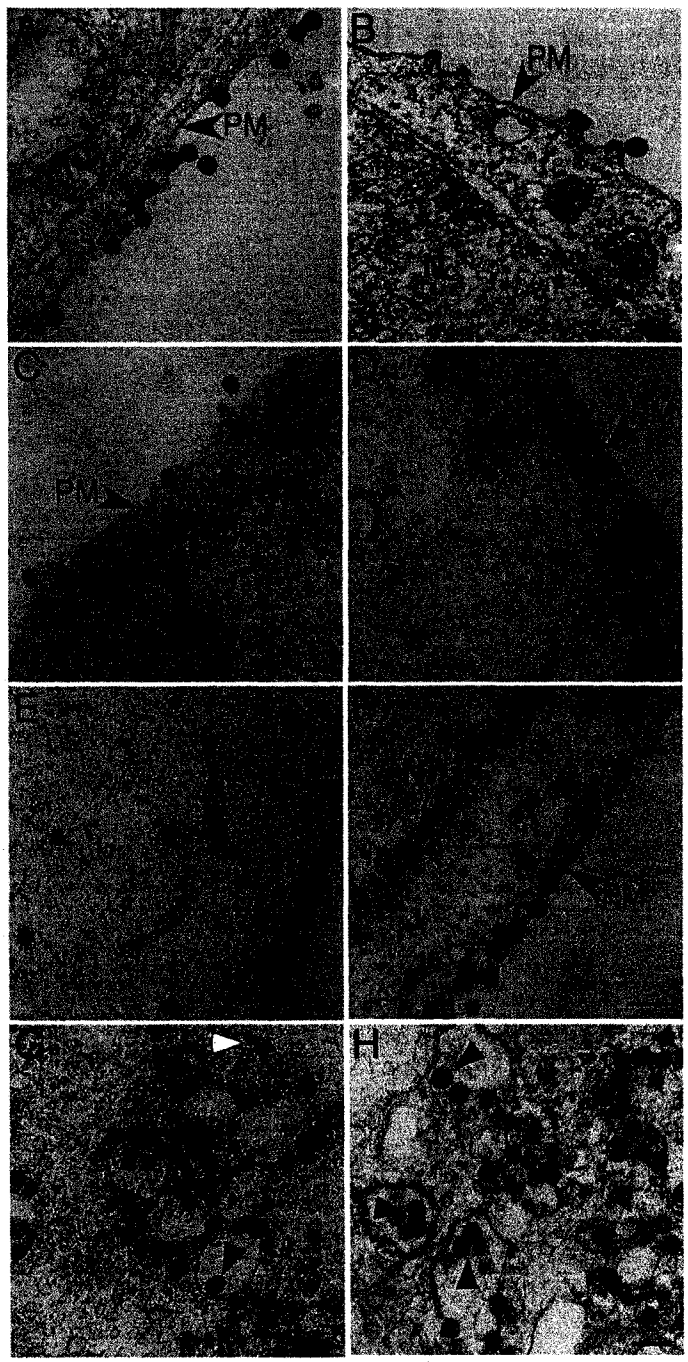

FIG. 4. Transmission electron microscopy of COS-1 cells expressing wild-type and mutant M-PMV. COS-1 cells expressing wild-type or mutant M-PMV proviral genomes were fixed 24 h posttransfection in 2.5% glutaraldehyde and postfixed in 1% osmium tetroxide to preserve proteins and membranes, respectively, prior to embedding and thin sectioning. Bar, 200 nm. N, nucleus. PM, plasma membrane. White arrow, C-type assembly. Black arrows, particles released into intracellular vesicles. (A)Wild type; (B) R55W; (C) R10A; (D) R22A; (E) K25A; (F) K39A; (G) K16A; (H) K16A/K20A.

Capsids that contain the R10A and K27A substitution were found adjacent to or traversing through what appears to be cortical actin, the dense filamentous actin (F-actin) network at the cell periphery (16, 40, 60) (Fig. 4C, R10A). This phenotype was more pronounced for R22A, K20/R22A, and R22A/K25A, with capsids accumulating in the cytoplasm under the cortical actin (Fig. 4D, R22A). A transport defective phenotype with capsids scattered throughout the cytoplasm or in small clusters in the cytoplasm near the plasma membrane was observed for the K25A mutant (Fig. 4E, K25A). This phenotype was also observed for the K25A/K27A mutant; however, in general, fewer capsids were scattered in the cytoplasm and larger accumulations close to the plasma membrane were observed. Mutants K33A, K39A, and K33A/K39A had a phenotype of immature capsid accumulation at the plasma membrane (Fig. 4F, K39A). A dramatically different phenotype in which immature capsids were found accumulating around intracellular vesicles was observed when lysine at position 16 or 20 was replaced with alanine (Fig. 4G, K16A). Both D-type and C-type assembly was detected in cells expressing K16A. Mutant K20A capsids that accumulated intracellularly were all of the D-type morphology. In cells expressing each of these single mutants, capsids were seen budding into or within intracellular vesicles (Fig. 4G, K16A). This aberrant intracellular budding was augmented for capsids that contained the double substitution K16A/K20A (Fig. 4H, K16A/K20A), and C-type assembly on intracellular vesicles was also observed for this double mutant.

K16A and K20A associate with Rab11, Rab5, and CD63 positive vesicles. This laboratory has shown previously that wild-type M-PMV Gag colocalizes with Rab11 at the pericentriolar recycling endosome and that capsid transport from the

pericentriolar region to the plasma membrane is dependent on a functional endosomal pathway (45). To investigate if the intracellular membranes around which K16A, K20A, and K16A/K20A mutant capsids cluster are of the endosomal pathway, the distribution of immunostained wild-type and mutant M-PMV Gag was compared to that of Rab11-GFP (recycling endosome), Rab4-GFP (recycling endosome), Rab5-GFP (sorting endosome), Rab7-GFP (lysosome) (51), and immunostained CD63 protein (multi-vesicular bodies) (8, 11).

COS-1 cells expressing wild-type M-PMV and Rab11-GFP had a distribution of anti-p12 Gag stain that colocalized with the Rab11 GFP signal at primarily a perinuclear location (Fig. 5A). A section of the cell was deconvoluted to remove out of focus light, and intensities from Rab11-GFP (green channel) and anti-p12 stain of M-PMV Gag (red channel) were associated in a maximum intensity projection image (yellow pixels in merged channel denoted by arrows). A similar association of K16A, K20A, and K16A/K20A mutant Gag and Rab11-GFP was seen. In contrast, neither wild-type nor mutant Gag proteins appeared to be associated with either the Rab4-GFP or Rab7-GFP signal. However, we did observe colocalization of Rab5-GFP on sorting endosomes with K16A (Fig. 5B), K20A, and K16A/K20A but not wild-type Gag. Association of the Gag stain for these mutants with the Rab5-GFP signal could also be detected in a deconvoluted maximum intensity projection image for the boxed area of the cell.

For studies of M-PMV Gag association with CD63, we utilized an anti-Pr78 polyclonal antibody to detect M-PMV Gag and a monoclonal antibody to CD63. The wild-type Gag appeared to moderately associate with CD63 in the context of whole cell sections; however, when the selected area of the cell was deconvoluted, this association

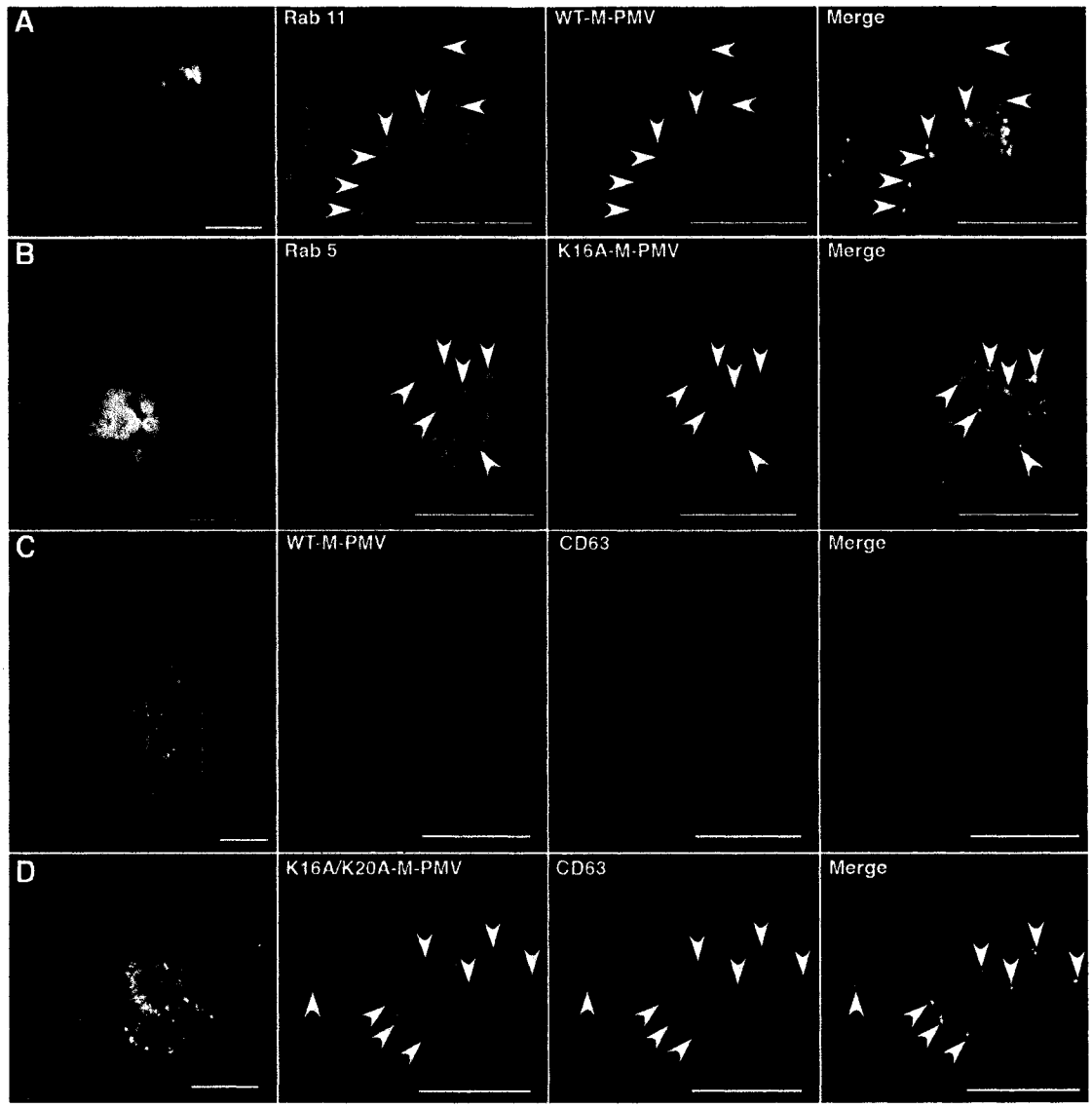

FIG. 5. M-PMV Gag associates with intracellular vesicles. COS-1 cells were either cotransfected with a wild-type (WT) or mutant M-PMV proviral genome and a Rab-GFP construct or transfected with only WT or mutant provirus. In both cases, cells were fixed 24 h later. Cotransfected cells were immunostained for Gag with an anti-p12 monoclonal antibody. Cells expressing only WT or mutant proviruses were immunostained for both Gag (anti-Pr78 polyclonal antibody) and CD63 (anti-CD63 monoclonal antibody). A single optical section of a cell is shown in the leftmost panels from which the area within the square was deconvoluted using a constrained iterative algorithm with the Slidebook software. A maximum intensity projection image of the deconvoluted sections was obtained and each channel (red and green) is shown separately and then merged. White arrows, staining that contributes to a signal of M-PMV associated with a cellular marker in the merged image (yellow pixels). Bars, 10 μm. (A) WT-Rab11; (B) K16A-Rab5; (C) WT-CD63; (D) K16A/K20A-CD63.

as determined by yellow pixels was minimal (Fig. 5C) and suggests that wild-type capsids do not associate with CD63 (Fig. 5C). In contrast, staining of the K16A, K20A, and K16A/K20A mutant Gags did overlap with that of CD63; this distribution is seen in a deconvoluted section of the cell, strongly supporting the association of these mutant capsids with CD63 positive vesicles (Fig. 5D).

DISCUSSION

In this study, the positive charge of the N-terminal M-PMV Gag matrix (MA) domain was modified to determine whether basic amino acid residues modulate release of capsids from the plasma membrane. Previous studies in HIV suggested that a combination of positive charges and the N-terminal myristic acid on the MA of Gag formed a bipartite signal for transport and attachment of Gag to the plasma membrane (10, 18, 63). Since HIV assembles at the plasma membrane, attachment to the plasma membrane through the bipartite signal of MA may effectively increase the concentration of Gag at a specific site in the membrane to facilitate capsid assembly. In contrast, the myristate and positive charge on M-PMV MA is incorporated into assembled capsids at the pericentriolar region of the cell. These capsids are transported through the cytoplasm and are specifically released at the plasma membrane, so it is likely that myristate is sequestered in M-PMV MA with the positive charge on the outer surface of the assembled capsid. At the plasma membrane, M-PMV must associate with the membrane and wrap the lipid bilayer around a spherical capsid. The studies here test the hypothesis that the positive charges exposed on the surface of M-PMV MA facilitate the intimate association of Gag with the membrane and likely trigger the exposure of myristate that is necessary for membrane envelopment.

The MA mutant with a substitution of alanine for lysine at position 25, located in the loop between helices A and B of M-PMV MA (6), delayed intracellular transport of immature capsids so that only 25% of the pulse-labeled Gag precursors were cleaved after a 4-h chase. This is in contrast to wild-type kinetics, in which 50% of Gag was cleaved at 1.5 h. Because even capsids blocked at a late stage of budding do not initiate

cleavage of Gag (38, 61), this indicates that release of K25A capsids was significantly slower than that of wild-type. Within the cell, K25A immature capsids were primarily dispersed throughout the cytoplasm; this phenotype that is similar to the previously characterized Y82F transport-defective MA mutant (53). In contrast to Y82F, however, the K25A mutant capsids did not accumulate in the pericentriolar region of the cell, suggesting that capsids can initiate intracellular transport but are inefficiently transferred to the plasma membrane. The transport defect appeared to be enhanced when K25A was combined with other mutations (R22A or K27A). In cells expressing these double mutants, capsids accumulated at a perinuclear region and in groups within the cytoplasm with no release of capsids during the 4-h chase. The K25A single mutant exhibits a greater defect in particle release than does Y82F, for which the half-life of Gag processing was delayed only 1 h (53). This greater delay in capsid release for K25A may reflect not only defective transport but also a delay in early membrane extrusion since the few capsids that reach the plasma membrane for this mutant appear to be blocked at an initial stage of budding (data not shown).

Immature capsids accumulating in the initial stages of budding were characteristic of MA mutants with substitutions of lysine residues at positions 33 and 39 that are located in helix B. For these mutants, 50% of the Gag precursor was cleaved with kinetics similar to that of wild type; however, 30% of the K33A precursor and 15% of the K39A precursor remained uncleaved after 4 h (data not shown). This residual uncleaved Gag may thus represent the capsid population accumulated at the plasma membrane. Since few capsids were observed in the cytoplasm of transfected cells, it is likely that the efficiency of intracellular transport was similar to that of wild type. For the

double mutant K33A/K39A only 20% of the Gag precursor proteins were cleaved at 4 h, a finding consistent with an additive block to release.

The accumulation of capsids at the plasma membrane in an early stage of membrane extrusion suggests that they may be defective in an initial electrostatic interaction with the negatively charged inner leaflet of the plasma membrane. Positively charged amino acids in HIV MA have been shown to facilitate Gag-plasma membrane interactions (62, 63), and the basic cluster from the HIV MA can be introduced into the Rous sarcoma virus matrix domain of Gag and restore budding of membranebinding domain mutants (2). Similarly, loss of positive charge residues in RSV MA resulted in defects in Gag association with the plasma membrane, while addition of basic residues to the membrane binding domain of MA resulted in a dramatic increase of particles released (5). Moreover, for HIV, budding from the plasma membrane can be restored for MA mutants that interact with intracellular membranes by addition of a lysine residue in the basic domain (26). Thus, it is possible that the positive charges at positions 33 and 39 of M-PMV MA are critical for capsids to interact with negatively charged phospholipids at the plasma membrane in order to trigger release of myristate from MA to the hydrophobic portion of the plasma membrane. A defect in release of myristate for these basic MA mutants would be much like the hydrophobic MA mutants we have described previously which also exhibited capsid accumulation at the plasma membrane (38, 53). The model in which myristate is sequestered in the M-PMV MA hydrophobic core is supported by the partially buried myristate in the structure of myristylated HIV MA (56). A combination of electrostatic interactions between basic amino acids and acidic phospholipids and

the insertion of myristic acid in the membrane likely facilitate capsid-membrane binding and provide the driving force for initiation of capsid envelopment.

Immature capsids for the MA mutants R10A, R22A, and K27A exhibited a substantial delay in the release of capsids. Release of capsids for the K27A mutant was decreased by 75% relative to that of wild type after a 4-h chase, but only trace amounts of R10A and no detectable levels of processed Gag were observed for R22A. R10A was not entirely defective for capsid release, however, since after a 24-h chase levels of virus in the supernatant were approximately 60% that of wild type. In contrast to the basic-charge mutants that are delayed in the initial stages of membrane envelopment, these mutant capsids can be observed by electron microscopy to be accumulated in the cytoplasm under (R22A) or traversing through (R10A, K27A) the peripheral filamentous actin layer located beneath the plasma membrane (16, 40, 60). This would suggest that there is a rate-limiting delay in capsid transport through the dense cortical actin patches for these mutants. With R22A, large accumulations of capsids can be observed and few if any reach the plasma membrane. This unusual phenotype is very similar to that of an endogenous virus variant (enJS56A1) of Jaagseike Sheep Retrovirus in which a similarly located arginine (R21) is substituted by tryptophan (23). Interestingly, this arginine residue is conserved in all betaretroviruses (23).

A block in transport at the cortical actin argues that capsid transport must switch from a primarily microtubule-based system to a primarily actin-based process in order to reach the plasma membrane. While capsid transport from the peri-centriolar region to the plasma membrane can be blocked by a combination of microtubule and actin inhibitors (LaCasse and Hunter, in preparation), these mutants argue that the basic charges

within MA directly facilitate capsid transport through the cortical actin patches. It is possible, therefore, that the arginine residues in M-PMV MA mediate the association of capsids with actin in a way similar to that of the basic effector domain of the MARCKS protein which has been shown to bind actin (17).

Surprisingly, M-PMV MA K16A and K20A capsids can be observed surrounding and budding into intracellular vesicles. Moreover, K16A capsids, which show both C-type and D-type assembly morphogenesis, were released with kinetics faster than that of wild type. Mutant capsids for K20A were released just 15 min slower than those of wild type, while the double K16A/K20A mutant has a release intermediate to that of each of the single mutants. It is likely that the rapid release of K16A virions can be attributed to the mixed C-type and D-type assembly morphology since the kinetics of K16A are similar to that of R55W, the MA mutant that assembles capsids at the plasma membrane through the C-type assembly pathway (37). The K16A, K20A and K16A/K20A mutant capsids appear to associate with a variety of intracellular membranes and colocalize with sorting endosomes and multi-vesicular bodies in addition to the recycling endosomes with which wild-type capsids associate (45). This phenotype is similar to one described for an HIV mutant in which lysine residues in MA were substituted with glutamic acid, and this resulted in Gag colocalization with CD63 (25).

Not only do the K16A and K20A M-PMV MA mutants show altered trafficking through the cell; they can also be seen budding into intracellular vesicles. It appears therefore that loss of the basic amino acids in helix A results in mutants that initiate budding on the first available membrane, perhaps because they are unable to sequester the myristate moiety. MA mutants in which myristate may be constitutively exposed,

thereby allowing association of the Gag protein with the first available membrane, have been described for HIV MA. In the case of these mutants, which alter or abolish the globular head of MA, virus particles could also be seen assembling at and budding into intracellular vesicles (9, 10, 28, 36). Thermodynamic calculations show that a single myristate moiety has insufficient binding energy to facilitate the binding of a protein to a membrane (31), so there is likely to be a requirement for Gag to undergo some oligomerization prior to a high affinity association with membranes. This is consistent with observations that the I-domain of HIV Gag facilitates membrane association through oligomerization of Gag (7, 42). In contrast, assembled M-PMV capsids contain upward of 2,000 myristic acid moieties (30); thus, it is possible that a mutant which is unable to sequester myristic acid would associate with the first available membrane through hydrophobic interactions – yielding the phenotype observed with K16A and K20A. Although this hypothesis is one that we favor, it is also possible that the K16A and K20A M-PMV mutants initiate myristate exposure and intracellular membrane extrusion through an altered electrostatic interaction between MA and lipids on the endosomal compartments. Phosphatidylinositol 4,5-bisphosphate has been shown to be important in targeting HIV Gag budding to the plasma membrane (24), and it also appears to be important for plasma membrane budding of M-PMV (Stansell, unpublished data, 24). Thus, by interacting with an alternate phospholipid molecule such as $PI(3,5)P_2$, the mutants might also be able to initiate the budding process intracellularly.

The budding process has been shown to involve a complex cellular machinery, Endosomal Sorting Complexes Required for Transport (ESCRT), which is normally involved in multivesicular body formation (20, 58). Retroviral late domains have been

shown to interact with a series of ESCRT protein complexes (I-III) that function in the pinching off stages of capsid release (22), and M-PMV encodes two late domain motifs (PSAP and PPPY) in the pp24 region which is C-terminal to MA (3, 14, 50, 61). These motifs are necessary for M-PMV to complete the budding process (14, 61). Since K16A and K20A mutants colocalized with vesicles that appear to be derived from the late endosomal pathway (CD63-MVB), it is tempting to speculate that cellular ESCRT components associated with these vesicles might be recruited to facilitate intracellular budding. It has not been established when the ESCRT machinery associates with wild-type M-PMV capsids, since they do not normally associate with the MVB; however, it is tempting to speculate that at least some of the ESCRT components associate with M-PMV during capsid assembly in the pericentriolar region of the cell. These initial interactions may then recruit additional components of the ESCRT complex once the myristyl-switch mechanism has initiated membrane envelopment and release of capsids from the plasma membrane.

The results presented here demonstrate that basic amino acids in helix A of the M-PMV MA function in myristate sequestration. Additional positive charges in helix B appear to function in triggering a change in orientation of the myristate moiety, perhaps through a conformational change in the M-PMV MA protein. Substitution of these charged residues results in defects of capsid release, supporting the hypothesis that basic amino acids in MA interact with acidic phospholipids of the plasma membrane to stimulate the sequestered N-terminal myristic acid of Gag to be exposed and inserted into the hydrophobic portion of the lipid bilayer, thereby regulating a myristyl switch essential to capsid envelopment.

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CONCLUSIONS

The data presented in this thesis support the hypothesis that molecular interactions between the Mason-Pfizer monkey virus (M-PMV) matrix domain (MA) and phospholipids on the inner leaflet of the plasma membrane initiate the membrane envelopment of an assembled retroviral capsid, likely through an electrostatic myristyl-switch mechanism. M-PMV assembles capsids from approximately 2,000 Gag polyprotein precursors at the pericentriolar region of the cell. The capsids are then transported to the plasma membrane, where these assembled structures must interact with the membrane in a way that stimulates wrapping of the spherical structure with the lipid bilayer. The MA of M-PMV Gag has two characteristics that act synergistically to promote protein-membrane interactions. MA is cotranslationally modified with myristic acid, a 14-carbon saturated fatty acid, and has a positive charge density on the outer surface of the protein. Since M-PMV capsids are specifically targeted to the plasma membrane for release, it is hypothesized that myristate is sequestered in MA with the positive charge amino acids oriented toward the outer surface of the assembled capsid and that specific electrostatic interactions between the positively charged amino acid residues of MA and negatively charged phospholipid head groups on the inner leaflet of the plasma membrane induce a conformational change in MA that switches the myristate from the core of the protein into the hydrophobic portion of the membrane. These interactions are reasoned to provide the driving force necessary to initiate membrane extrusion of an assembled capsid. To provide support for this hypothesis, mutational studies were

conducted in which the hydrophobicity of the MA core was increased and in which the positive charge on the surface of MA was diminished.

The MA domain of M-PMV Gag is known to be important for transport of capsids from the site of assembly to the plasma membrane (306). The studies described in this thesis show that tyrosine at position 82 and lysine at position 25 are critical in the transport of assembled capsids to the plasma membrane since replacement of either of these amino acids (K25A and Y82F) resulted in a phenotype in which capsid release was delayed and assembled capsids were dispersed throughout the cytoplasm. This is in contrast to wild-type infected cells, where few capsids are in the cytoplasm and the majority of capsids either are released from the cell or are at various stages (early, middle and late) of membrane extrusion. For the MA mutant where tyrosine at position 82 was replaced with phenylalanine (Y82F), capsids were also accumulated at the pericentriolar region of the cell, suggesting that the initiation of transport from the site of assembly was delayed. This transport-defective phenotype was similar to that of the previously characterized A79V transport-defective MA mutant in which large numbers of immature capsids accumulated in the cytoplasm (306). Since efficient transport of immature capsids from the peri-centriolar region is dependent on a functional endocytic pathway and on the presence of the M-PMV envelope protein (333, 343), it is possible that the tyrosine residue 82 and alanine residue 79 modulate the Gag polyprotein-envelope glycoprotein interaction necessary for capsid transport. The MA mutant in which lysine at position 25 was replaced with alanine (K25A) did not accumulate capsids in the pericentriolar region of the cell, suggesting that capsids can initiate intracellular transport but are inefficiently transferred to the plasma membrane. The K25A single

mutant exhibits a greater defect in release than Y82F, which may reflect the fact that this mutant is not only defective for capsid transport but also defective in an early stage of membrane extrusion since the few K25A mutant capsids that reach the plasma membrane were blocked at an initial stage of budding.

The release of capsids was substantially delayed for the MA mutants in which charge was diminished on one side of helix A (R10A and R22A) or a lysine residue in the loop between helices A and B, at position 27, was replaced with alanine (K27A). In electron micrographs, assembled capsids for these mutants could be observed to be accumulated in the cytoplasm juxtaposed to (R22A) or traversing through (R10A, K27A) the peripheral filamentous actin (F-actin) layer located beneath the plasma membrane (157, 309, 393). For R22A, large accumulations of capsids can be observed and few if any reach the plasma membrane. This phenotype is similar to that of an endogenous virus variant (enJS56A1) of Jaagseike sheep retrovirus. In the enJS56A1 Jaagseike sheep retrovirus variant, a similarly located arginine (R21), an arginine residue conserved in all betaretroviruses, is substituted by tryptophan; interestingly, this mutant acts in a dominant negative fashion to block release of virions (247). The accumulation of capsids at the peripheral F-actin suggests that these mutants are defective for capsid release due to a defect in capsid transport through the dense cortical actin patches, which are typically found at the leading edge of a cell (310). These mutants argue that the basic charges within MA directly facilitate capsid transport through the cortical actin patches, which are typically found at the plasma membrane in "active" areas of membrane curvature and cellular exocytosis (309, 376, 393). It is possible, therefore, that the arginine residues at positions 10 and 22, along with lysine at position 27, in M-PMV MA

mediate the association of capsids with actin by interacting directly with actin in a manner similar to that of the basic effector domain of the MARCKS protein, which has been shown to bind actin (164, 260).

This finding strongly suggests that the cellular actin filamentous network is involved in the transport of M-PMV capsids to the plasma membrane and argues that capsid transport must switch from primarily a microtubule-based system to primarily an actin-based process. This concept is supported by the slight decrease in the rate of capsid release in the presence of either an actin-depolymerizing agent (cytochalasin D) or a microtubule-depolymerizing agent (nocodazole); however, when cells are treated with both microtubule and actin depolymerizing agents, capsid release is delayed significantly (LaCasse and Hunter, in preparation).

Increasing the hydrophobicity of the MA protein core or diminishing the positive charge in helix B resulted in M-PMV MA mutants capsids accumulated at the plasma membrane in early stages of budding, a finding consistent with a rate-limiting delay in the initiation of membrane extrusion. For the mutants in which tyrosine residues (Y11, Y28, and Y67) which are oriented toward the protein core in the nuclear magnetic resonance structure of the MA protein (94) were replaced with the more hydrophobic phenylalanine residue, the increased hydrophobic interior of the MA domain may sequester the myristate moiety more efficiently and interfere with the insertion of the myristate into the membrane. 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 (265, 270). 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, a finding consistent with effects on myristate exposure. The M-PMV Y28F MA mutant exhibited a phenotype similar to HIV-1 MA mutants in which a single conservative substitution increased hydrophobicity and blocked viral budding, since Y28F was essentially defective in capsid release. In contrast to the HIV-1 MA mutants, however, the Y28F mutant had no significant effect on immature capsid assembly. This may reflect the different mechanisms by which Gag proteins assemble in the two viruses, since for HIV assembly appears to be dependent on the tight membrane association that is mediated by both positive charges and myristate (146, 302, 328, 389). The other two M-PMV MA mutants, Y11F and Y67F, did accumulate capsids at the plasma membrane despite the fact that release of capsids for these mutants was not drastically impaired. The delay of release for the Y67F mutant might reflect not only a defect in initiation of membrane extrusion but also a defect in transport, since assembled capsids accumulate at the pericentriolar region of the cell in both indirect immunofluorescence and electron micrographs. The different phenotypes of the hydrophobic core mutants Y11F, Y28F, and Y67F 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 model that myristate

is sequestered in the M-PMV MA hydrophobic core is supported by the partially buried myristate in the structure of myristylated HIV MA (361).

Immature capsids accumulating in the initial stages of budding were also characteristic of MA mutants with substitutions of lysine residues at positions 33 and 39 that are located in helix B. While the release of capsids for each of these mutants was initially similar to the kinetics of release seen for wild type, after 2 h the kinetics of release for these mutants were delayed, resulting in residual uncleaved Gag which may represent the capsid population accumulated at the plasma membrane. Since few capsids were observed in the cytoplasm of transfected cells, it is likely that the efficiency of intracellular transport was similar to that of wild type. The kinetics of release for the double mutant K33A/K39A were consistent with an additive block to release, and capsids could be seen in electron micrographs in an early stage of membrane extrusion.

The accumulation of capsids at the plasma membrane in an early stage of membrane extrusion suggests that these may be defective in an initial electrostatic interaction with the negatively charged inner leaflet of the plasma membrane. Positively charged amino acids in HIV MA have been shown to facilitate Gag-plasma membrane interactions (420, 430), and the basic cluster from the HIV MA can be introduced into the Rous sarcoma virus matrix domain of Gag and restore budding of membrane-binding domain mutants (27). Similarly, loss of positive charge residues in RSV MA resulted in defects in Gag association with the plasma membrane, while addition of basic residues to the membrane-binding domain of MA resulted in a dramatic increase of particles released (75). Moreover, for HIV, budding from the plasma membrane can be restored for MA mutants that interact with intracellular membranes by addition of a lysine residue in the

basic domain (264). Thus, it is possible that the positive charges at positions 33 and 39 of M-PMV MA are critical for capsids to interact with negatively charged phospholipids at the plasma membrane in order to trigger release of myristate from MA to the hydrophobic portion of the plasma membrane. A defect in the release of myristate for these basic MA mutants would be similar to the proposed defect of the hydrophobic MA mutants that exhibited a phenotype of capsid accumulation at the plasma membrane. A combination of electrostatic interactions be-tween basic amino acids and acidic phospholipids and the insertion of myristic acid in the membrane likely facilitate capsid-membrane binding and provide the driving force for initiation of capsid envelopment.

Surprisingly, M-PMV MA K16A and K20A capsids can be observed surrounding and budding into intracellular vesicles. Moreover, K16A capsids, which show both type C and type D assembly morphogenesis, were released with kinetics faster than that of wild type. Mutant capsids for K20A were released just 15 minutes slower than those of wild type, while the double K16A/K20A mutant has a release intermediate to that of each of the single mutants. It is likely that the rapid release of K16A virions can be attributed to the mixed C-type and D-type assembly morphology since the kinetics of K16A are similar to that of R55W, the MA mutant that assembles capsids at the plasma membrane through the C-type assembly pathway (305). The K16A, K20A and K16A/K20A mutant capsids appear to associate with a variety of intracellular membranes and colocalize with sorting endosomes and multi-vesicular bodies in addition to the recycling endosomes with which wild-type capsids associate. This phenotype is similar to one described for an HIV mutant in which lysine residues in MA were substi-

tuted with glutamic acid, and this resulted in Gag colocalization with multi-vesicular bodies (263).

Not only do the K16A and K20A M-PMV MA mutants show altered trafficking through the cell; they can also be seen budding into intracellular vesicles. It appears therefore that loss of lysine amino acids at positions 16 and 20 of M-PMV results in mutants that initiate budding on the first available membrane, perhaps because they are unable to sequester the myristate moiety. MA mutants in which myristate may be constitutively exposed, thereby allowing association of the Gag protein with the first available membrane, have been described for HIV MA. In the case of these mutants, which alter or abolish the globular head of MA, virus particles could also be seen assembling at and budding into intracellular vesicles (125, 135, 270, 301, 348). Thermodynamic calculations show that a single myristate moiety has insufficient binding energy to facilitate the binding of a protein to a membrane (278), so there is likely to be a requirement for Gag to undergo some oligomerization prior to a high affinity association with membranes. This is consistent with observations that the I-domain of HIV Gag facilitates membrane association through oligomerization of Gag (107, 319). In contrast, assembled M-PMV capsids contain upward of 2,000 myristic acid moieties (273); thus, it is possible that a mutant which is unable to sequester myristic acid would associate with the first available membrane through hydrophobic interactions – yielding the phenotype observed with K16A and K20A. Although this hypothesis is one that we favor, it is also possible that the K16A and K20A M-PMV mutants initiate myristate exposure and intracellular membrane extrusion through an altered electrostatic interaction between MA and lipids on the endosomal compartments. Phosphatidylinositol 4,5-

bisphosphate has been shown to be important in targeting HIV Gag budding to the plasma membrane, and it also appears to be important for plasma membrane budding of M-PMV (Stansell, unpublished data, 262). Thus, by interacting with an alternate phospholipid molecule such as $PI(3,5)P_2$, which is thought to be localized on the late endosome (26), the mutants might also be able to initiate the budding process intracellularly.

The budding process has been shown to involve a complex cellular machinery, Endosomal Sorting Complexes Required for Transport (ESCRT), which is normally involved in multivesicular body formation (197, 388). Retroviral late domains have been shown to interact with a series of ESCRT protein complexes (I-III) that function in the pinching off stages of capsid release (245). M-PMV encodes two late domain motifs (PSAP and PPPY) in the pp24 domain, C-terminal to MA, which are necessary for capsid release (50, 147, 347, 411). Since K16A and K20A mutants colocalized with vesicles that appear to be derived from the late endosomal pathway, it is tempting to speculate that cellular ESCRT components associated with these vesicles might be recruited to facilitate intracellular budding. It has not been established when the ESCRT machinery associates with wild-type M-PMV capsids, since they do not normally associate with the MVB; however, it is tempting to speculate that at least some of the ESCRT components associate with M-PMV during capsid assembly in the pericentriolar region of the cell. These initial interactions may then recruit additional components of the ESCRT complex once the myristyl-switch mechanism has initiated membrane envelopment and release of capsids from the plasma membrane.

The model that the results presented here strongly support is one in which the myristic acid moiety is sequestered in the matrix domain with the positively charged amino acids of MA on the outer surface of the pericentriolar assembled capsid (Fig. 1). Transport of capsids specifically to the plasma membrane is regulated by basic amino acids in helix A of the M-PMV MA which function in myristate sequestration as well as in capsid transport through cortical actin. At the plasma membrane positively charged lysine residues in helix B trigger a change in orientation of the myristate moiety, perhaps through a conformational change in the M-PMV MA protein, to initiate membrane extrusion. This model supports the hypothesis that basic amino acids in MA interact with acidic phospholipids of the plasma membrane to stimulate the sequestered N-terminal myristic acid of Gag to be exposed and inserted into the hydrophobic portion of the lipid bilayer, thereby regulating a myristyl switch essential to capsid envelopment.

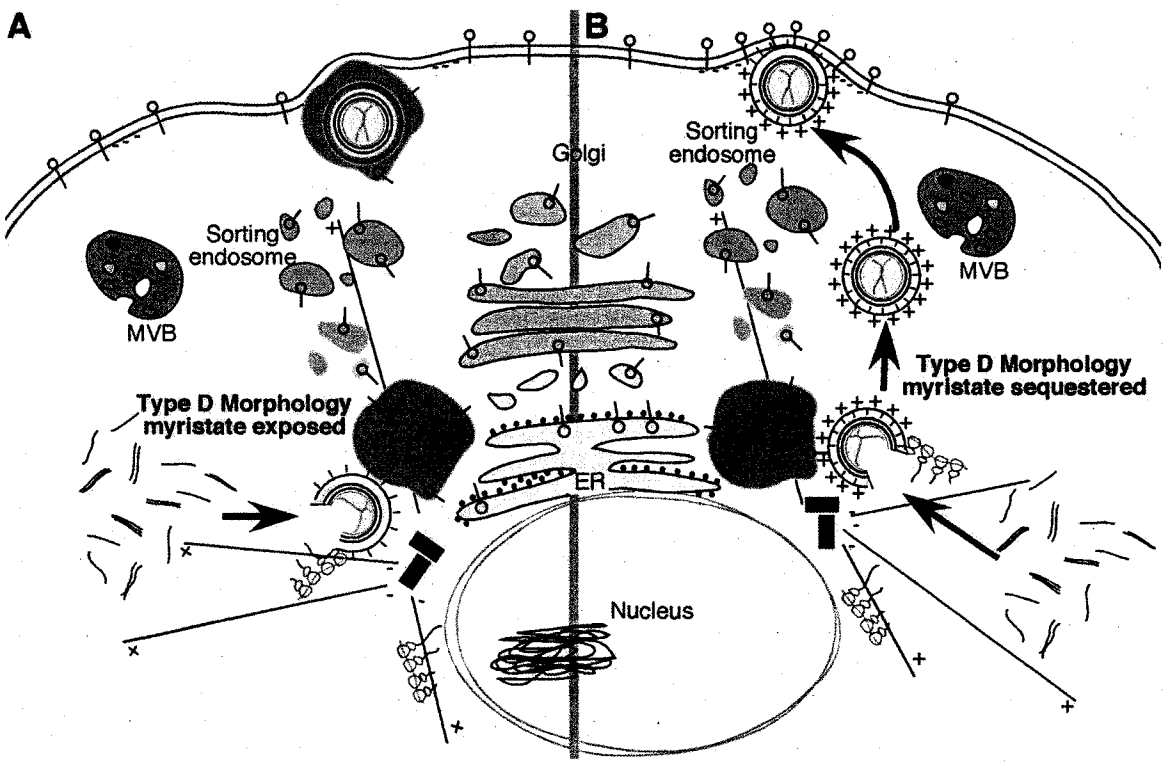

FIG. 1. Type D assembly morphology with myristate exposed or sequestered. (A) Theoretical model of capsid interactions if myristate is exposed upon capsid assembly. Myristate exposure would create a large hydrophobic density that would likely direct the interaction and release of capsids with the first membrane in the vicinity. This is not consistent with the observation that wild-type M-PMV does not bud into internal vesicles. (B) Model of capsid assembly with the myristate moiety sequestered in the matrix domain. This would allow capsids to be transported through the cytoplasm specifically to the plasma membrane, where they are released. This is consistent with the data presented in these studies.

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I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that she may be recommended for the degree of Doctor of Philosophy.

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