STUDIES OF DELTARETROVIRUS ASSEMBLY AND RELEASE

DISSERTATION

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By

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* * * *

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ABSTRACT

The assembly of retrovirus particles requires the expression of the Gag polyprotein precursor (PrGag), which is used as a principle building scaffold for retrovirus assembly and budding from infected cells. The retrovirus Gag polyprotein has all the necessary information to mediate intracellular transport to the cell membrane, to package full-length retroviral genomes, to direct assembly of virus particles, and to catalyze the final budding process.

This dissertation was focused on the virus particle assembly of Deltaretroviruses, namely human T-cell leukemia virus type-1 (HTLV-1) and bovine leukemia virus (BLV). These viruses replicate to low titers in their natural hosts and are poorly infectious in cells culture. Information regarding the molecular details of their life cycles, including virus assembly, is limited.

Virus-like particle (VLP) assay model systems were developed for BLV and HTLV-1 to test five hypotheses. The first hypothesis was that the myristylation signal in the matrix (MA) domain of BLV Gag was required for membrane targeting and binding of BLV Gag. Mutations that disrupted the amino-terminal glycine residue (which would block the addition of myristic acid) led to a drastic reduction in VLP production but did not eliminate Gag membrane
localization, suggesting that other residues in Gag were involved in membrane targeting and binding. The second hypothesis was that the PPPY domain within BLV MA was required for virus release. Mutation of the PPPY motif significantly reduced VLP production and this reduction was more severe in the presence of an active viral protease. Examination of particles by electron microscopy revealed an abundance of particles that began to pinch off from the plasma membrane but were not completely released from the cell surface, indicating that the PPPY motif functions as a late domain (L domain).

The third hypothesis tested was that the MA and nucleocapsid (NC) domains were protein determinants of BLV RNA packaging. Mutagenesis of conserved basic residues as well as residues of the zinc-finger domains in the BLV NC domain of PrGag revealed residues that led to a reduction in viral RNA packaging. Interestingly, when conserved basic residues in the BLV MA domain of PrGag were mutated to alanine or glycine, but not when mutated to another basic residue, reductions in viral RNA packaging were also observed. The ability of PrGag to be targeted to the cell membrane was not affected by these mutations in MA, indicating that these basic residues in the MA domain of PrGag influence RNA packaging, without influencing Gag membrane localization. It was further observed that i) a MA/NC double mutant had a more severe RNA packaging defect than either mutant alone, and ii) RNA packaging was not found to be associated with transient localization of Gag in the nucleus. These observations indicate that both the MA and NC domains of BLV Gag are involved in RNA packaging.
The fourth hypothesis tested was that both the PPPY and PTAP motifs in the C-terminus of HTLV-1 MA function as the L domain and influence virus release. Mutation of either motif (i.e., PPPY changed to APPY or PTAP changed to PTRP) reduced budding efficiencies. Further analysis revealed that PPPY plays an essential role in HTLV-1 particle budding from the plasma membrane and could not be replaced by other late domain motifs, i.e. PTAP or YPDL, whereas the PTAP motif plays a subtler role in the virus release.

The fifth hypothesis tested was that HTLV-1 particles could assembly at both the plasma membrane and multivesicular bodies (MVBs) and PTAP directs the Gag to the plasma membrane. I demonstrated that when HTLV-1 PTAP motif was altered, an accumulation of Gag proteins and virus particles were observed in intracellular compartments. These compartments were CD63-positive multivesicular bodies (MVBs). Further analysis excluded the possibility that these particles accumulated inside MVBs were a result of re-internalization of extracellular particles. It was further found that (i) the particle-containing MVBs traffic along microtubules using dynein-dynactin complexes recruited by RILP and these particles could therefore exit the cell by exocytosis. (ii) PI3P is likely to be a Gag receptor on MVBs as Inhibition of PI3K disrupted the MVB pathway.
Two roads diverged in a wood, and I -

I took the one less traveled by,

And that has made all the difference.

By Robert Frost
This work is dedicated to my
Mom and Dad,
Thank you for everything
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CHAPTER 1

INTRODUCTION

Significance of the Dissertation Topic

Retroviruses are associated with a variety of diseases, including immunological and neurological disorders, and various forms of cancer. Deltaretrovirus is a unique subgroup within the retrovirus family. These viruses all cause lymphoproliferative diseases. In contrast to other retroviruses such as HIV, the replication cycle of these viruses have been underexplored. Most of the assumptions about the strategy and mechanism of their replication are based on extrapolation from other retroviruses. Molecular studies of both bovine leukemia virus and human T-cell leukemia virus-1 have been hindered by the highly restricted expression of these viruses. Usually, infection and provirus formation are followed by relatively low virus production. In vitro infection with cell-free virus is extremely inefficient, and cocultivation is usually necessary for transmission of the virus to uninfected cells.

The general goal of this dissertation was to investigate the molecular aspects of the late events of the HTLV-1 and BLV replication cycle, i.e. the assembly and release of newly formed viral particles. These studies not only increase our knowledge of deltaretrovirus life cycle but also enrich the entire field
of retroviral assembly and release. A better understanding of these late events will help the identification of novel antiviral drug target and treatment.

Drugs currently available for the treatment of retrovirus infection target the important viral proteins involved in early steps of retrovirus life cycle including reverse transcriptase (RT), protease (PR), and envelope protein. However, the rapid emergence of resistant variants, inadequate therapeutic durability and increased awareness of the toxicities related to chronic administration of many of the current agents emphasizes the need for new treatment options. One potential target that has yet to be exploited is the late steps of the virus life cycle, i.e. viral assembly and/or maturation.

Data presented in Chapter 2 describe a novel virus-like particle production system. The first application of this new system was to study the role of myristylation of Gag polyprotein in membrane targeting of Gag. It was shown that myristylation of Gag in the N-terminus is required for efficient targeting of Gag to plasma membrane. The second part of the study identified the late domain motif of BLV. The PPPY motif within matrix domain was shown to function in the late stage of virus assembly. When PPPY was replaced by other residues, the particle release was arrested at the very last step of virus budding.

Data presented in Chapter 3 are the second application of the novel BLV VLP system. We tested the hypothesis that BLV matrix protein is likely to be involved in BLV RNA encapsidation. Alanine scanning mutagenesis was performed to identify the basic amino acid residues of matrix domain of BLV that
are possibly involved in BLV RNA encapsidation. Three basic residues were thought to be important for BLV RNA encapsidation.

Data presented in Chapters 4 and 5 are focused on HTLV-1. In Chapter 4, the HTLV-1 late domain was identified. Both PPPY and PTAP were shown to function in HTLV-1 particle release. However, PPPY was found to play a more important role than PTAP. In Chapter 5, HTLV-1 particle assembly and budding were further explored. Confocal microscopy and transmission electron microscopy were used to show that HTLV-1 Gag can not only be targeted to the plasma membrane but also to the multi-vesiculuar bodies (MVB). In both cases, cellular machinery normally used for MVB biogenesis was exploited by HTLV-1 for its own assembly and release. We further showed an unexpected role of PTAP motif in targeting Gag to the plasma membrane.

The first part of Chapter 1 will provide the general information on Deltaretrovirus with a focus on HTLV-1 and BLV. The second part will detail the function of various domains of Gag protein since this protein is such a critical player in late steps of the retroviral life cycle. The last portion will focus on the molecular aspects of three major late events that have been studied including the membrane targeting and binding of Gag polyprotein to plasma membrane, the viral genomic RNA encapsidation, and virus release from the cell.

**Deltaretroviruses**

The family *Retroviridae* comprise a variety of enveloped RNA viruses, such as endogenous retroviruses, leukemia viruses or notorious HIV-1. The
hallmark of this family is its replicative strategy which includes as essential steps reverse transcription of the virion RNA into linear double-stranded DNA and the subsequent integration of this DNA into the genome of the cell.

Retroviruses are broadly divided into two categories—simple and complex—distinguishable by the organization of their genomes. They are further divided into seven genuses defined by evolutionary relatedness. Deltaretroviruses are a unique genus within the retrovirus family, characterized by a distinct genetic content, organization and strategy for gene expression. This genus of retroviruses include distantly related viruses such as human T-cell leukemia/lymphoma viruses types I and II (HTLV-1 and HTLV-2 respectively), bovine leukemia virus (BLV) and simian T-cell leukemia/lymphoma viruses type I and II (STLV-1 and STLV-2).

**HTLV-1**

In humans, HTLV-1 is the etiological agent of two diverse diseases: adult T-cell leukemia/lymphoma (ATLL) (48, 88), as well as the neurological disorder tropical spastic paraparesis/HTLV-1-associated myelopathy (TSP/HAM) (31, 83).

ATLL is an aggressive lymphoproliferative disease whose clinical course can be classified into five different stages: asymptomatic, pre-Leukaemic, chronic/smouldering, lymphoma, and acute (114). The majority of HTLV-1 infected individuals are asymptomatic, but capable of transmitting the virus.
Morphologically abnormal T-cells with highly lobulated or flower-shaped nuclei are pathognomonic of HTLV-1 infection.

TSP/HAM is a chronic demyelinating disease affecting women more often than men, usually beginning in adulthood (83). In contrast to ATLL, TSP/HAM can develop in some patients within years of HTLV-1 infection, which is often a result of a blood transfusion. TSP/HAM is characterized by weakness and spasticity of the extremities, mild peripheral sensory loss, and hyperreflexia.

It is estimated that 10-20 million people worldwide are infected with HTLV-1, which is endemic to southern Japan, Africa, the Caribbean, and eastern parts of South America. HTLV-1 is transmitted sexually or by blood, blood products, or breast milk and induces a lifelong chronic infection. In the infected individual, HTLV-1 is present mainly in CD4+ T-lymphocytes, although rarely it has also been found in CD8+ cells. Unlike HIV-1, HTLV-1 virions are poorly infectious in vitro and transmission of HTLV-1 occurs mainly through cell-to-cell contact. ATLL occurs in 1-2% of infected carriers generally 20-30 years after infection (114). Epidemiological studies have shown that ATLL develops mainly in individuals that were infected in infancy. This long latency period of ATLL suggests that the accumulation of genetic mutations, in addition to HTLV-1 infection, is required for the induction of ATLL.

In 1982, another human retrovirus (HTLV-2), sharing 60% sequence identity to HTLV-1, was isolated from a patient with T-cell hairy cell leukemia. Although several isolates of HTLV-2 have been obtained from patients with lymphoid malignancies and with myelopathy, epidemiologic evidence linking it to
any particular disease is still lacking. Retroviruses related to HTLV-1, designated simian T-lymphotropic viruses, were also identified in some Old World monkeys, both African and Asian, in some of which they are associated with lymphoid malignancies.

**BLV**

Another important member of the Deltaretrovirus genus is BLV. BLV primarily affects the lymphoid tissue of dairy and beef cattle and causes malignant lymphoma. It is characterized by a long latency period which may be followed by the development of tumors (32). Most BLV infections are asymptomatic and can be diagnosed by serologic assay to detect viral-specific antibodies. Clinical signs of malignant lymphoma become evident as tumors invade different tissues. These signs can include enlarged lymph nodes, weight loss, decreased milk production, fever and loss of appetite. There is no curative treatment for this disease. The predominant target of BLV is the B cell, but other cellular types, such as T cells or even macrophages, might be infected. BLV can infect in vitro a variety of cells and can propagate in different animal species. It has a financial impact on the cattle industry. It is estimated that more than 10 and 30% of the dairy and beef cattle in the United States and Argentina, respectively, are infected with BLV. Economic losses due to BLV infection can come from reduced milk production, reduced reproductive efficiency, increased replacement costs, and increased veterinary costs. (87).
Genome Organization of Retrovirus

The retroviral genome is flanked by a long terminal repeat sequence (LTR). This region contains all signals necessary for gene expression, including the enhancer, promoter, transcription initiation, transcription terminator and polyadenylation signal. The integrated provirus has two LTRs; the 5’ LTR normally acts as an RNA polymerase II promoter whereas the 3’ LTR functions as the terminator sequence.

The viral RNA encodes four genes: *gag*, *pro*, *pol* and *env*. The *gag* gene encodes the internal structural protein of the virus (Gag protein, from the original name “Group specific antigens”). Gag is proteolytically processed into the mature proteins MA (matrix), CA (capsid), NC (nucleocapsid), and sometimes others, of uncertain function. The *pol* gene gives rise to the reverse transcriptase (with RNase H activity) and integrase. The *pro* gene encodes the viral protease (PR), which acts late in assembly of the viral particle to proteolytically process the proteins encoded by *gag*, *pro*, and *pol*, and in some cases also *env*. The *env* gene gives rise to two polypeptides: the transmembrane ‘spike’ protein (gp41 in HIV) and the knob-like surface protein gp120. Together these form the surface antigen gp160 characteristic to retroviruses. These proteins are initially inserted into the host cell membrane and are acquired by the virus particle at a later stage during budding.
In addition to the *gag*, *pro*, *pol*, and *env* common to all retroviruses, members of the Deltaretrovirus and the Lentivirus genus have accessory genes that regulate virus transcription and allow those viruses to replicate in differentiated cells.

HTLV-1 uses alternative splicing and internal initiation codons to produce several regulatory and accessory proteins encoded by four open reading frames (ORFs) predominantly located in the pX region (pX ORF I to IV) of the viral genome between *env* and the 3' long terminal repeat (2, 53). A bicistronic mRNA encodes the regulatory proteins Tax (pX ORF IV) and Rex (pX ORF III). Tax potently activates transcription not only from the viral promoter (Tax-responsive element) but also from the enhancer elements of many cellular genes involved in host cell proliferation. Rex is responsible for nuclear export of unspliced or singly spliced viral RNA.

In addition to Tax and Rex, pX ORFs I and II produce alternatively spliced forms of mRNA, which encode four accessory proteins, p12\textsuperscript{I}, p27\textsuperscript{I}, p13\textsuperscript{II}, and p30\textsuperscript{II}. These accessory proteins are critical for viral infectivity and maintenance of high viral loads, host cell activation, and regulation of gene transcription (2).

**Gag Protein**

Not only is Gag protein the precursor to the internal structural protein of all retroviruses, but it plays important role in various aspect of retrovirus life cycle.

Expression of *gag* alone leads to assembly of immature virus-like particles that bud from the plasma membrane. These particles lack the surface projections
comprising the Env proteins, but they are otherwise indistinguishable in thin-section electron micrographs from virions formed by complete genomes with an inactivating mutation in protease. In virion assembly, Gag proteins must interact with each other, with components in the plasma membrane, with the genomic RNA, and with Env proteins and cellular proteins.

Gag protein is organized into regions, which are proteolytically liberated as the separate mature Gag proteins during viral maturation. Structural proteins that comprise mature infectious virus particles are matrix (MA), capsid (CA), and nucleocapsid (NC). The outermost shell underlying a lipid bilayer, derived from the plasma membrane during budding, is composed of MA. There is an inner core comprised of a shell assembled from the CA protein and inside this core is a ribonucleoprotein containing NC complexed to the diploid genomic RNA and the replicative enzymes. All Gag proteins are organized in the same order from the amino terminus to the carboxyl terminus, with domains that are cleaved into the following proteins: (NH2)-MA-X-CA-NC-Y-(COOH) (for review, see ref. (112). X and Y represent segments that each may be cleaved into one or more small proteins or peptides or may be absent altogether. Thus, the "minimal" Gag protein is the unit MA-CA-NC. Examples of the structural organization of Gag proteins of prototypic retroviruses are given in Figure 1.1. The structure and role of each domain will be discussed below.
MA Protein

In all retroviruses, the amino-terminal domain of Gag gives rise to MA protein (membrane-associated, or matrix). The MA domain of Gag is the region that forms the final contacts with the plasma membrane of the host cell and interacts with gp120. In virions, the MA domain is visualized as a thin membrane-bound layer by cryoelectron microscopy (110).
**Interaction of MA with Lipid Membrane**

Gag proteins of most retroviruses are modified by myristylation at their amino termini (46). Myristate is a 14-carbon fatty acid that is added cotranslationally to many cellular proteins associated with membranes and also to some proteins that remain cytosolic (39). The consensus sequence for myristylation is Met-Gly-X-X…Ser/Thr. The fatty acid is linked via an amide bond to the free amino group of the glycine residue after the initiating methionyl residue is removed. Mutagenesis was used to show that for MLV and HIV-1 myristylation is essential for retroviral assembly. Mutation of the glycine residue results in a block in the budding of particles and an accumulation of Gag inside the cell (15, 38). Prevention of myristylation of the M-PMV Gag protein does not prevent formation of the immature particles in the cytoplasm, but rather prevents their transport to, or stable association with, the plasma membrane (96).

Myristylation is not the only requirement for MA association with the membrane. A patch of basic residues with multiple positive charges of HIV-1 MA, downstream from the amino terminus is believed to strengthen the Gag-membrane interaction through electrostatic interaction with the head of acidic phospholipids (120). Efficient binding is dependent both on the fatty acid modification at the amino-terminal glycine residue and on the basic sequences further downstream. Many, but not all, MA proteins also have clusters of basic residues in this region.

The three-dimensional structure of HIV-1 and SIV-1 MA proteins has been deduced by nuclear magnetic resonance (NMR) (73, 74) and X-ray
crystallography (47, 94). In solution, MA is a monomer consisting of five helices joined by short loops or β-stands, four of the helices surrounding a fifth to form a hydrophobic core. In this structure, most of the basic residues, including those near the amino terminus, are positioned at one end of the molecule. Both of these lentiviral MA proteins are trimeric in the crystal, with a globular amino-terminal domain and a smaller carboxy-terminal domain projecting away. The strongly positively charged regions on one side of each of the globular domains come together to form a kind of platform. The myristylated amino termini of the three subunits are positioned in the same region. The shape of the trimer is thus consistent with the notion that the myristates insert into the hydrophobic portion of the membrane and the basic charges interact with the phosphate of the lipid head groups.

**Other Roles of MA**

In HIV-1, MA has been found to accompany the newly synthesized viral DNA into the nucleus (17). It may be a factor that directs migration of the preintegration complex. During the initial stages of virus infection, a small population of MA dissociates from the membrane, exposing a nuclear localization signal, and becomes incorporated into the preintegration complex (16, 28). Interestingly, one region of this nuclear localization signal lies in the highly basic region of MA that is involved in membrane binding and thus membrane binding must be somehow weakened to allow for MA dissociation. MA may also interact with the Env proteins during budding. Portions of MA can be deleted from ASLV and HIV-1 Gag proteins without impairing its ability to assemble and bud from the
membrane. Some of these deletions prevent incorporation of HIV envelope
glycoproteins into virions (26, 117), suggesting that at least a part of the MA
domain forms a contact with the cytoplasmic portion of Env.

MA proteins of some viruses were shown to bind RNA in vitro. ASLV MA
binds to RNA in a sequence-independent fashion (103). In contrast, the MA
protein from BLV apparently can recognize RNAs carrying sequences near the 5′
end of the genomic RNA (54), raising the possibility that this protein domain has
a role in packaging of RNA into virions.

**CA Protein**

Sequentially located in the center of the Gag is the CA domain. The exact
structural function of CA in the mature viral particle has not been elucidated, but
the protein is believed to form a shell surrounding the ribonucleoprotein complex
that contains the genomic RNA. This shell is most appropriately referred to as the
"capsid." The capsid together with the components it encloses are then referred
to as the "core". CA provides structural stability to the virion and also plays a key
role in forming the protein–protein contacts required for productive assembly.

CA can be divided into two domains, an N-terminal domain (NTD) and a
C-terminal domain (CTD) (Fig. 1.3.). The structure of these separate domains as
well as the whole protein has been solved (29, 33, 79). The N-terminal domain
consists of five long helices forming a stable coiled-coil structure, two short ones,
two E-hairpins, and a Pro-rich loop. The C-terminal half of CA contains four
conserved helices and a highly conserved sequence among retroviruses known
as the major homology region (MHR; 285–304 in HIV-1; see Fig. 1.2.). This region is conserved throughout the retrovirus group (112) and thus offers a novel and stable target for viral vaccines. The role of the MHR sequence has been investigated using mutagenesis and biophysical strategies. Various mutations within the MHR block viral replication at different and distinct stages, such as assembly, maturation, or target cell infection in vivo, indicating that the MHR may play a role in Gag interactions with viral protein, the host membrane, or viral RNA (67).

![Diagram of NTD and CTD domains of CA protein with CypA binding loop](image)

Fig. 1.2. Linear representation of the HIV Gag proteins CA.

Several lines of evidence suggest that CA is important early in infection. One of them involves the recruitment of Cyclophilin by CA into virions. Cyclophilin A is recruited into the virions through interactions with a proline-rich stretch in the N-terminal region of the capsid domain (13). The biological function of cyclophilin A binding to CA might be to destabilize the shell of CA proteins, thereby facilitating disassembly upon infection.
Numerous molecular genetic analyses have been carried out to study the function of CA in assembly. Evidence exists for its essential role in particle assembly (1, 109) and in vitro mature capsid alone has the capacity to assemble into particles (25).

**NC Protein**

The nucleocapsid (NC) protein is a small basic protein, typically about 60–90 amino acid residues long. Its primary role is to tightly bind and protect genomic RNA. In all retroviruses except those of the spumavirus group, NC has one or two characteristic motifs made of regularly spaced cysteine and histidine residues (Fig. 1.3.). The retroviral Cys-His motif has the structure CX$_2$CX$_4$ HX$_4$C (here abbreviated CCHC). The CCHC motif is similar to other short cysteine and histidine-containing structures, called "zinc fingers," that coordinate a Zn$^{++}$ ion and that have a role in binding of certain proteins to nucleic acids. Typically, clusters of lysine or arginine residues follow the CCHC motifs. Deletions or major alterations of the CCHC result in the absence of viral RNA in virions or alterations of the specificity of RNA packaging. Thus, this NC motif probably interacts with the "packaging signals" near the 5$'$ end of retroviral genomic RNAs (11, 66). Both the runs of basic residues and the CCHC motifs are important for the specific interaction with genomic RNA (24, 35, 78).

Other roles of NC include catalyzing the annealing of primer tRNA to primer binding site before or during assembly; catalyzing the dimerization of genomic RNA.
**Other Gag Proteins and Peptides**

In addition to the proteins discussed above, many retroviral *gag* genes encode polypeptide segments that lie between MA and CA, between CA and NC, and/or downstream from NC. In most cases, the functions of these segments are poorly understood. Two examples are given below.

The segment of Gag between the ASLV MA and CA regions is processed into three polypeptide fragments, p10, p2a and p2b. p2b contains the sequence Pro-Pro-Pro-Tyr (PPPY), which is found in a large number of retroviral Gag proteins. Mutation or ablation of this motif results in a defect in particle release.

In HIV-1 and other lentiviruses, a polypeptide of approximately 60 amino acids is cleaved from the Gag protein downstream from NC in a region partially overlapping the *pro* reading frame. This "p6" domain has a role in release of virus
in the final steps of budding. Viral particles from mutants with p6 deleted or altered remain tethered to the plasma membrane (37). The amino acid sequence that are important for release of the virus has been mapped to a conserved segment, Pro-Tyr-Ala-Pro (PTAP), near the amino-terminal domain of p6 (50).

**Retroviral Replication Cycle**

A summary of the retroviral replication cycle is shown in Figure 1.4. In brief, the virion recognizes and attaches to a specific receptor located on the surface of a target cell membrane. The viral envelope fuses with the cell membrane, and the viral core is injected into the cytoplasm. Reverse transcription takes place in the cytoplasm, viral genomic RNA is reverse transcribed into double stranded DNA. The viral DNA enters the nucleus and integrates into the cellular DNA. After integration, the viral RNA is transcribed by the cellular RNA polymerase II. The RNA is capped and processed like normal cellular mRNA, and utilizes cellular export and translation machinery. Both spliced and unspliced viral RNA are then exported to the cytoplasm and undergo translation. The viral proteins and genomic RNA assemble into virions at the cell periphery and the plasma membranes. After budding from the cell, progeny virions undergo proteolytic maturation into infectious virus particles.
Figure 1.4. A brief summary of the retroviral replication cycle. The retrovirus attaches to a target cell via specific receptors. The viral envelope protein then fuses with the cellular membrane. Reverse transcription in the cytoplasm generates a DNA copy of the RNA genome. The viral DNA is transported into the nucleus and integrates into the host chromosomal DNA. The cellular RNA polymerase II transcribes the viral RNA. Both full length and spliced versions of the viral RNA are created, and exported to the cytoplasm, where translation occurs. Viral proteins are assembled into progeny virions at the cell membrane, and package full length viral genomic RNA. The progeny virions then bud from the cell membrane.

Retroviral Assembly and Release

Gag and Gag-Pro-Pol proteins are synthesized on free polyribosomes in the cytosolic spaces of the cell. Env polyproteins are synthesized on the membrane of the rough ER. Once these proteins are synthesized, they come
together (along with two copies of viral RNA and tRNA primers) at a common site on the cell membrane to assemble viral particles. Very late or immediately after budding, the Gag and Gag-Pro-Pol proteins within the immature virion are cleaved by the viral protease to produce the mature, infectious virion.

It is Gag protein that alone provides the driving force for the assembly and release of viral particles. Over the past years, these late events and the role of Gag in these events have been explored in depth. Three of these major events will be discussed in this section.

**Membrane Binding and Targeting of Gag Proteins**

Retroviral morphogenesis follows one of several distinct pathways, characterized by where viral structures are initially assembled and visualized by electron microscop. For viruses that follow the type C pathway, such as RSV, HIV, and MLV, Gag proteins are targeted to the plasma membrane where they form dense aggregates just under the inner leaflet of the plasma membrane. Type B and type D retroviruses assemble viral cores within the cytoplasm, which are subsequently targeted to the plasma membrane for budding. A third pathway is followed by defective endogenous retroviruses, which form intracisternal A-type particles that are released into the endoplasmic reticulum (ER). Like many cellular proteins, the Gag polyprotein is initially synthesized on free ribosomes in the cytoplasm. Subsequently, Gag proteins are directed to the site of assembly. Specific targeting of Gag protein to the site of assembly is directed by the
retroviral Gag polyprotein, which is the only viral protein needed to drive the assembly process.

**Bipartite Membrane-Binding Signal**

For type C retrovirus, while the mechanisms underlying how Gag proteins are selectively directed to the plasma membrane rather than to intracytoplasmic membrane remains very poorly understood, the sequences involved in membrane binding have been identified for several viruses. For HIV-1, stable membrane association is accomplished using a bipartite membrane-binding domain consisting of the fatty acid myristate, added cotranslationally to the N terminus of Gag (15), (38, 100), in concert with a patch of basic residues between amino acids 15 and 31. Myristate provides a hydrophobic interaction with the lipid membrane, while the basic residues are believed to strengthen the interaction by forming the electrostatic interactions with acidic phospholipids that are enriched at the cytoplasmic face of the plasma membrane (85, 120).

Although the primary sequence of matrix proteins from different retroviruses can be very different, their three-dimensional structures appear to share similar characteristics. The structures of the matrix proteins from BLV and HTLV-II have been reported (18, 75). Both are similar to the structure of the HIV-1 matrix protein. They are highly helical and contain an exposed cluster of basic residues that could bind acidic membranes. HTLV-I shares 58% overall sequence identity to HTLV-II and are therefore likely to adopt very similar structures. A primary sequence alignment supports this conclusion since
residues that form the hydrophobic core and the exposed basic patch are well conserved between the two proteins. The mechanism underlying BLV and HTLV-1 Gag targeting and binding to cellular membrane was investigated in Chapter 2 and Chapter 5.

**Genomic Viral RNA packaging**

The retrovirus particle contains a genomic complement of two molecules of viral genomic RNA, but a provirus also may give rise to a variety of smaller, singly or multiply spliced subgenomic mRNAs. Although viral spliced RNAs and cellular RNAs may be packaged into virus particles, the packaging of viral genomic RNA is essential for the newly formed virus to be infectious. Thus, the encapsidation of the retroviral genomes must be selective enough to overcome the high background of cellular RNAs and subgenomic viral RNAs. Two components are involved in specific encapsidation of viral genomic RNA: the secondary and tertiary structures of genomic viral RNA and the Gag or Gag-Pol proteins which form assembling virus particles.

**RNA Packaging Signal**

The specificity with which retroviral full-length genomic RNAs are packaged suggests that specific sequences are required for RNA selection. These cis-acting sequences are referred to as the encapsidation signal (E) or packaging signal ($\psi$), which have been identified in avian, murine, and primate retroviruses (56, 61, 65, 98). In most retroviruses, the location of the packaging
signal is in the untranslated leader region between the major subgenomic splice donor and the start of the *gag* codon; therefore, spliced messages would lack the entire packaging signal.

Since the specificity of packaging can be exchanged by the substitution of sequences that have no sequence similarity, the process of packaging is likely to involve recognition at the structural level (14, 69, 115). Predicted secondary RNA structure suggested stem/loop structures as packaging signals for many simple and complex retroviruses (42, 43). Although the significance of these structures to packaging in most retroviruses has not been established yet, in some retroviruses, simple as well as complex stem loop structures have been empirically shown to be important for packaging (8, 9, 60, 69, 115). In HIV-1 the primary encapsidation signal is located downstream of the primer-binding site (pbs), overlaps with the major splice donor, and extends into the *gag* gene (76). The stable RNA secondary structures in this region consist of four hairpin loops, referred to as stem-loop 1-4 (SL1, SL2, SL3, SL4) (Fig. 1.5 A). The RNA packaging signal region of BLV was initially mapped to two important regions (68). The first region includes sequences downstream of the pbs and near the *gag* gene start codon. The second region was a 132 nucleotide base sequence within the *gag* gene. Structure-function analysis (69) has provided genetic evidence that the primary packaging signal region of BLV consists of two stable RNA stem-loop structures (Fig. 1.5 B). HTLV-1 and HTLV-2 have similar SL1 and SL2 structures downstream of the *gag* start codon (Fig. 1.5 C).
Fig. 1.5. Characterized complex retrovirus RNA encapsidation signals. (A) The HIV-1 RNA packaging signal is located downstream of the pbs and overlaps with the AUG start codon of gag (boxed sequence). The RNA secondary structure of this region includes four stable stem–loops, designated SL1, SL2, SL3 and SL4. (B) BLV primary RNA packaging signal. The SL1 and SL2 of BLV, as well as the gag start codon, are indicated. (C) HTLV-1 primary RNA packaging signal. The SL1, SL2 and gag AUG are indicated.

**Protein Domains Involved in RNA Binding**

The viral protein components needed for specific viral RNA encapsidation are in Gag since the Gag-only particles can packaging viral RNA. Of the three major domains of Gag, only mutations in NC have been shown to block packaging of viral RNA into virus particles (3, 36, 63), suggesting NC plays an important role in viral RNA packaging (11, 52).
The NC proteins of all retroviruses share two characteristics: a high percentage of basic residues and, with the exception of spumaviruses, a zinc-binding motif composed of regularly spaced cysteine and histidine residues (59) of the form –Cys-X2-Cys-X4-His-X4-Cys– (CCHC). These sequences are found either once or twice depending on the viral species (10). Over the years, the function of this conserved motif has been investigated by a variety of approaches, including mutational analysis. Mutations that abolished zinc binding produced non-infectious virions that lacked their genomes and increased the incorporation of spliced viral RNA and cellular mRNAs (40). Other experiments that altered the basic residues flanking the zinc finger motifs resulted in a general decrease in the ability of NC to bind RNA (19, 21, 49, 62, 89). These results suggest that both zinc finger motifs and flanking basic residues control the packaging of retroviral genomic RNA.

While the NC domain is likely to have a major role in genome recognition and RNA encapsidation, evidence in the literature implicates the MA protein of BLV in these events. In particular, it has been previously determined, using an RNA gel mobility shift assay, that the BLV MA protein specifically binds to RNAs representing that 5’ end of the BLV RNA genome (54, 55). Thus, the MA domain of BLV is very likely to be involved in RNA packaging process. Data presented in Chapter 3 of this dissertation provide further evidence to confirm the hypothesis.

*The Traffic of Viral Genomic RNA*
For most retroviruses, the interaction of full-length viral genomic RNA and Gag has long been believed to occur in the cytoplasm after the unspliced viral genomic RNA is transported out of the nucleus and Gag proteins are synthesized on free ribosomes in the cytoplasm. However, a recent study has discovered an unexpected step in the RSV assembly pathway (99). RSV Gag was found to relocate into the nucleus after synthesis in the cytoplasm. It has been proposed that Gag proteins enter the nucleus where they might interact with unspliced viral RNA transcripts. The Gag-RNA nucleoprotein complex is then transported through the nuclear pore complex via the CRM1 export pathway. Elimination of this nuclear step correlates with a defect in RNA packaging. Data presented in Chapter 3 suggest that BLV Gag-RNA interaction may not take place inside the nucleus.

Retrovirus Release

Viral L Domain

The final step in the life cycle of enveloped viruses is a membrane fusion event that releases the nascent virion from the host cell. Studies on the HIV-1 suggested that a virus-encoded function within the Gag protein was necessary for this late budding (37). Early mutational analyses demonstrated that deletion of the p6 protein, which is located at the carboxyl terminus of Pr55Gag, produced a striking defect in the production of virus particles (37, 50). Rather than budding off from the plasma membrane, mutant virions instead remained attached to the cell surface via a thin tether. Mutations in a highly conserved PTAP motif
between p6 residues 7 and 10 produced the same phenotype; each of these four residues appeared to be crucial for virus release (23, 50). These results identified p6, and in particular the PTAP motif within p6, as playing a crucial role in HIV-1 budding.

Following the identification of the p6 PTAP motif as a major contributor to the HIV-1 budding process, domains with analogous functions were defined in the Gag proteins of several other retroviruses and in the matrix proteins of the rhabdoviruses and filoviruses (for reviews, see (27, 90). These domains are referred to as `late´ or `L´ domains to reflect their role late in the virus life cycle.

Three different classes of viral L domains have been identified: PTAP, PPXY, and YPDL (Fig. 1.6). A PTAP motif in p6 confers HIV-1 L domain activity (37); the same motif in Ebola VP40 has also been reported to contribute to particle release (71). PPXY motifs, which appear to be the most common sequence associated with L domain function, stimulate budding of Rous sarcoma virus (RSV) (111, 113), Mason-Pfizer monkey virus (M-PMV) (116), murine leukemia virus (MLV) (118, 119), the rhabdoviruses (20, 45, 51), and the filoviruses (44). Equine infectious anemia virus (EIAV) L domain activity is provided by a YPDL motif (91). A number of retroviruses, and the Ebola filovirus, contain adjacent or overlapping PTAP and PPXY sequences. In several cases, viral L domains are functionally interchangeable and can exert their activity when positioned at different locations within a retroviral Gag protein. (71, 84, 118). Data presented in Chapter 2 and Chapter 5 are among the first studies to identify the late domains of BLV and HTLV-1.
FIG. 1.6. Location of L domains in retroviral Gag proteins. The retroviral Gag proteins that harbor L domains are shown in pink; matrix, capsid, and nucleocapsid proteins are depicted in green, blue, and purple, respectively. L domain motifs (PTAP, PPXY, and YXXL) are indicated.

All three late domains, PXXP, PPXY and/or YXXL, are known to be involved in protein–protein interactions among cellular proteins. The presence of these highly conserved protein–protein interaction motifs within L domains raised the possibility that viral L domains function by associating with host factors. Indeed, recent studies strongly support the concept that L domains interact with the components of the cellular machinery responsible for sorting cargo into multivesicular bodies (MVBs) (30, 71, 107).
Endosomal Sorting Pathway and MVB

Regulated degradation of cell surface molecules, particularly receptors involved in myriad signaling pathways, is essential for the control of many biological processes, including cell growth, tissue morphogenesis, and host defense (101). Endocytic membrane cargo can be partitioned into recycling vesicles, for delivery back to the cell surface, or into late endosomes, for delivery to degradative compartments (41). Recognition of cargo at the limiting membrane of the late endosome results in invagination of the bilayer, budding of cargo into intraluminal vesicles, and formation of multi-vesicular bodies (MVBs). The vesicles and their contents are then degraded by proteases and hydrolases after fusion of the MVBs to lysosomes. (93, 107). Monoubiquitination acts as a signal for directing proteins into MVBs, although it might not be the only signal, given that membrane proteins that are not ubiquitinated can also be transported to the MVBs. Genetic and biochemical studies during the last several years have led to the identification of so-called ‘class E´ family of vacuolar protein sorting (Vps) proteins required at different stages of MVB morphogenesis. These include components of the three high molecular weight protein complexes, which were first characterized in yeast and are collectively known as the endosomal sorting complexes required for transport (ESCRTs) (4, 5, 57). ESCRT-I and ESCRT-II each contain one subunit that binds ubiquitin. ESCRT-II is believed to function downstream of ESCRT-I and functions to recruit ESCRT-III to the membrane. Recent studies have confirmed the interaction between proteins of ESCRTs- I
and II and between those of ESCRTs -II and -III (70, 108). The full ESCRT complex is released at late stages of budding by the AAA (ATPase associated with diverse cellular activities) protein, Vps4 (7, 81).

**L domain and endosomal sorting machinery**

The notion that endosomal sorting pathways are intimately involved in retrovirus budding is first derived from the identification of a HIV p6-binding protein, TSG101. A variety of physiological activities have been attributed to TSG101, but it appears likely that its primary function is in endosomal protein sorting. TSG101 and its yeast ortholog Vps23, are members of Vps proteins (6). Both the mammalian and the yeast proteins have been shown to recognize Ub and act in the removal of endosomal protein-Ub conjugates through a multivesicular body (MVB) (64). In both mammalian and yeast cells, TSG101/Vps23 associates with ESCRT-I (12). Binding between p6 and TSG101 mapped to the PTAP motif of p6 and the amino-terminal, E2-like domain of TSG101(22, 30, 107). Emerging evidence suggests that the interaction between PTAP and TSG101 leads to the recruiting of ESCRT-I to the sites of virus budding, where it recruits two other complexes, ESCRT-II and ESCRT-III, to initiate virus budding (72, 108).

Other types of L domains, PPXY and YXXL, do not directly interact with TSG101. Nevertheless, they have been reported to recruit other host cell factors to facilitate viral budding. The PPXY L-domain sequences of Rous sarcoma virus, Ebola virus and vesicular stomatitis virus have each been reported to bind
to a particular class of WW domain-containing proteins, namely, the Nedd4-like E2 ubiquitin ligases (44, 45, 58). For EIAV, the YPDL motif conforms to the YXXL consensus sequence that mediates endocytosis of certain transmembrane proteins via interaction with the AP2 clathrin adaptor. Accordingly, EIAV p9 was shown to bind to the AP50 subunit of the AP-2 complex in vitro and induce AP-2 relocalization to sites of viral assembly in infected cells (92). Although PPXY- and YPDL- type L domains do not require TSG 101 and an intact ESCRT-I, it appears at least a subset of the class E VPS factors are required for viral budding since the activities of all three viral L domains are inhibited by a dominant-negative VPS4A protein (30, 72) and both PTAP- and YPDL-type L domain binds to a mammalian orthologue of the yeast VPS factor, Bro1 (AIP-1/ALIX) (70). It is likely that PPXY- and YPDL-type L domains simply bypass ESCRT-I and interact directly or indirectly with downstream factors or that PPXY and YPDL motifs recruit an alternative activity that can functionally replace that of ESCRT-I.

It appears clear that the processes of vesicle budding at late endosomes and retroviral budding at the plasma membrane are topologically equivalent and the same set of factors mediates both processes in a mechanistically analogous manner.

**Virus Budding into MVBs**

Retroviruses, have until recently been thought to bud exclusively from the plasma membrane of infected cells (34, 97, 105). However, surface budding cannot be the only mechanism of retroviral egress. Observations made a decade
ago suggested that HIV-1 infected macrophages tend to accumulate infectious virus particles in intracellular large compartments or vacuoles (77). Recent studies have identified these intracellular vacuoles as CD63-positive MVBs. (86, 95, 104, 106). These studies further showed that the ability of retroviruses to bud into MVBs can be observed in standard tissue culture cell lines (293 and HeLa cells) previously believed to only support budding at the plasma membrane (80, 82, 102, 108). MLV and HIV Gag were specifically targeted to the cytoplasmic face of late endosomal membranes where virus-like particles or matures virions were detected budding into the lumen of the vesicle. These results indicate that Gag association with endosomal membranes and subsequent budding into MVBs is not restricted to macrophages but exists as a constitutive pathway in multiple cell lines. Thus, the retrovirus does not only hijack MVB biogenesis machinery to the plasma membrane for its budding, but also that viral assembly and budding can take place at MVBs where the machinery is usually active. The intracellularly accumulated particles may be transported to the cell surface along microtubues (80) where the MVB fuses with plasma membrane and releases the viruses as exosomes (80, 95, 104, 106).

To this date the MVB budding pathway has only been exensively studied in HIV. Data presented in Chapter 5 demonstrates that existence of MVB pathway for HTLV-1.

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CHAPTER 2

ANALYSIS OF BOVINE LEUKEMIA VIRUS GAG MEMBRANE TARGETING AND LATE FUNCTION

Abstract

Assembly of retrovirus-like particles only requires the expression of the Gag polyprotein precursor. We have exploited this in the development of a model system for studying the virus particle assembly pathway for bovine leukemia virus (BLV). BLV is closely related to the human T-cell leukemia viruses (HTLVs) and all are members of the deltaretrovirus genus of the Retroviridae family. Overexpression of a BLV Gag polyprotein containing a carboxy-terminal influenza virus hemagglutinin (HA) epitope tag in mammalian cells led to the robust production of virus-like particles (VLPs). Site-directed mutations were introduced into the HA-tagged Gag to test the usefulness of this model system for studying certain aspects of the virus assembly pathway. First, mutations that disrupted the amino-terminal glycine residue that is important for Gag myristylation led to a drastic reduction in VLP production. Predictably, the nature of the VLP production defect was correlated to Gag membrane localization. Second, mutation of the PPPY motif (located in the MA domain) greatly reduced VLP production in the absence of the viral protease. This
reduction in VLP production was more severe in the presence of an active viral protease. Examination of particles by electron microscopy revealed an abundance of particles that began to pinch off from the plasma membrane but were not completely released from the cell surface, indicating that the PPPY motif functions as a late domain (L domain).

Introduction

The assembly of retrovirus particles requires the expression of the Gag polyprotein precursor (PrGag), which is used as a principal building scaffold for retrovirus assembly and budding from infected cells (42, 49). During or after the process of particle release, the action of the retroviral protease cleaves, except for the spumaviruses, PrGag into mature matrix (MA), capsid (CA), and nucleocapsid (NC) proteins (16, 42, 49).

The retrovirus Gag protein has all the necessary information to mediate intracellular transport to the cell membrane, to direct assembly of virus particles, and to catalyze the budding process (9). The events associated with Gag-mediated budding appear to be similar among different retroviruses. After synthesis of Gag in the cytoplasm, Gag-Gag interactions, Gag-RNA interactions, Gag-membrane interactions, and Gag-host protein interactions occur. The target of Gag to the cell membrane leads to a bulging out of the nascent virus particles. It is clear that each step of the virus assembly process requires a great degree of precision and involves highly specific macromolecular interactions including protein-protein and protein-RNA interactions (9, 16).
The expression of retroviral PrGag alone can lead to the formation of virus-like particles (VLPs). The formation and release of VLPs occurs in the absence of expression of the viral envelope glycoproteins, reverse transcriptase, or of full-length viral RNA (46). Retroviral RNA can act as a scaffold to facilitate virus particle assembly (27, 38). In the absence of viral RNA, cellular RNAs are used and can be found in VLPs.

There are three functional assembly domains in PrGag (31). Each domain is responsible for a separate function in the assembly process. The three functional domains are the membrane-binding domain (M domain), the late domain (L domain), and the interaction domain (I domain). The M domain for most retroviruses resides primarily in the MA domain and contains a myristylation signal (2, 39) while the I domain resides in the CA and NC domains of Gag.

L domains have been observed to be critical for efficient pinching off of the virus particle. L-domains have been identified by extensive mutational analysis in many retroviruses (3, 10, 11, 22, 34, 48, 50-52, 54). Different retroviruses may utilize different viral proteins and structural motifs to accomplish the same late budding function. Rous sarcoma virus (RSV), murine leukemia virus (MLV), and Mason-Pfizer monkey virus (MPMV) all have L-domains that consist of a highly conserved PPPY motif as the core sequence and are located near the junction of the MA and CA domains in Gag (5, 31, 47, 50-52). A PPPY motif has been also found in the matrix protein of rhabdoviruses and can function as an L domain (5). In contrast, the L-domains of lentiviruses are located at the C terminus of the PrGag and have distinct core motifs, PTAP in HIV-1 and YXXL in equine
infectious anemia virus (3, 11, 34). The retroviral L-domains are protein-interaction domains, and most likely function by binding to specific cellular proteins that facilitate the late stages of retroviral particle release (45).

Ubiquitination has been found to be involved in virus particle release from infected cells, extending earlier studies that had identified ubiquitin in retrovirus particles (36, 45). Recently, proteins associated with the ubiquitin pathway have been found that associate ubiquitin with virus particle release. Specifically, cellular factors that are associated with the ubiquitin pathway have been shown to interact with L domains (12, 20, 33, 35, 44). The Nedd4-like family of E3 ubiquitin protein ligases (specifically LDI-1) has been identified as the cellular protein that interacts the PPPY motif (20, 33). TSG101, a putative Ub regulator that is involved in trafficking of endosomal proteins, has been reported to interact with the PTAP motif and therefore be involved in L domain function (6, 12, 44).

The Deltaretrovirus genus of the Retroviridae family includes bovine leukemia virus (BLV), human T-cell leukemia virus type 1 (HTLV-1), and human T-cell leukemia type 2 (HTLV-2). These viruses replicate to low titers in their natural hosts and are poorly infectious in cell culture. Cocultivation is typically used to infect permissive host cells. Because of these difficulties, information regarding the molecular details of their life cycles, including virus assembly, is limited. Aspects of the deltaretrovirus assembly process that have been studied in some detail include BLV RNA encapsidation, BLV and HTLV-1 Gag myristylation, and the role of basic residues in MA of HTLV-1 Gag membrane localization and virus production (1, 17, 21, 23, 24). The primary encapsidation
signal of BLV consists of two stable RNA stem-loop structures (i.e., SL1 and SL2) located just downstream of the gag start codon (24).

To more easily study the steps involved in the virus assembly pathway of BLV, we have developed a VLP model system. In this system, the BLV Gag protein containing a C-terminal influenza virus hemagglutinin (HA) epitope tag was overexpressed in mammalian cells and led to the robust production of VLPs. A series of site-directed mutations were introduced to assess the validity of this model system. First, mutations that disrupted the myristylation signal led to a dramatic reduction in VLP production. Second, mutations in a PPPY motif in the MA domain reduced VLP production and resulted in particles that budded but were not released from cells, as determined by electron microscopy, indicating that this motif functions as a L domain.

Materials and Methods

Plasmid construction

PCR amplification of the BLV gag gene from BLV-SVNEO (8) was performed to create a HindIII site immediately upstream of the start codon and an EcoRI site immediately downstream of the stop codon by using

5'-AAAAAAAAGCTTGATGGGAAATTCCCCCTAT-3' as the upstream primer and 5'-AAAAAAGAATTCTCGTTTTTTGATTTGAGGGTTGG-3' as the downstream primer. The amplified fragment was digested with HindIII and EcoRI and cloned into a mammalian HA tagged expression vector, pMH (Boehringer Mannheim, Indianapolis, IN). The resulting plasmid, pGag-HA, has a HA epitope.
tag fused to the C-terminus of the BLV PrGag. In these studies, pGag-HA is the parental construct for many of the mutants studied and is considered the wild-type (wt) construct. The plasmid pHA-Gag has a HA epitope tag fused to the N-terminus of the BLV PrGag was generated similarly by cloning the BLV gag gene into pHM6 (Boehringer Mannheim). The BLV vector pPR+ contains intact gag and pro genes and was created from BLV-SVNEO by digestion with XbaI and EcoRI to remove the majority of the vector sequences, then filling-in the sticky ends with the Klenow fragment of DNA polymerase and ligating the ends with T4 DNA ligase. The plasmid pU5-Gag-HA was created by PCR amplifying the BLV proviral DNA that contains the U5 region of the 5’ long terminal repeat, the untranslated region between U5 and the gag gene start codon, and the entire gag gene and cloning it into the HindIII and EcoRI sites of pMH.

**Site-directed mutagenesis**

Alanine-scanning mutagenesis was done using the QuickChange XL kit (Stratagene) according to the manufacturer’s instructions. The plasmid pHA-Gag was created to test the influence of an N-terminal HA tag on VLP production. To restore a glycine residue as the first residue after the methionine start (but before the HA tag), mutagenic primers were designed to create this mutant. The upstream primer used was 5’-CAAGCTGGAGACCATGGGTACCCCATACGACGTCC-3’ and downstream primer used was 5’-GGACGTCGTATGGGTACCCCATGGTCTCCAGCTTG-3’. The plasmid pGag-HA was used as the parental construct to generate the
following mutants: G2A, APPY, PAPY, PPAY, PPPA and AAAA. The following oligonucleotides were used in the mutagenesis reactions:

for G2A, 5'-TTTTTGAGCTTGATGGCAAATTCCCCCTCTATAA-3' and 5'-TTATAGGAAGGGGAATTTGCCATCAAGCTCAAAAA;

for APPY, 5'-GCCCCAGAAGAACAAGCCCCGCCTTATGACCCCGC-3' and 5'-GCCGGTGTCATAAGGCGGGGCTTGTCTTCTTGGG;

for PAPY, 5'-CCAGAAGAACAACCCGCCCCTTATGAACCCCGC-3' and 5'-GGGGGTCTATAAGGGGC-3';

for PPAY, 5'-GAAGAAACAACCCCCGGCTTATGACCCCGCCAT-3' and 5'-ATGGCGGGGTCATAAGCGGCGGTTGTTCTTC-3';

for PPPA, 5'-GAACAACCCCCGCTGCTGACCCCCGCT-3' and 5'-AAAATGGCGGGGGGTCTAGCGAGCGGTTGTTCT-3';

for AAAA, 5'-GAAGAAACAAGCCGCGGTCTGCTGACCGCCGT-3' and 5'-ACGGCGGGGTCAGCGAGGCGGTTTCTTCC-3'. In order to create PR+/AAAA, the primers 5'-GAAGAAACAAGCCGCGGTCTGCTGACCGCCGT-3' and 5'-ACGGCGGGGTCAGCGAGGCGGTTTCTTCC-3' were used.

**Cell culture and transfections**

All cells were grown in 100-mm or 60-mm-diameter dishes in Dulbecco's modified Eagle medium (DMEM)(GIBCO BRL, Gaithersburg, MD) supplemented with 10% Fetal Clone III serum (Hyclone, Logan, UT). Superfect (Qiagen, Valencia, CA) was used to transfect COS-1 or 293 cells. Two days post-transfection, cells were placed under G418 selection until resistant colonies
formed (~ 3 weeks). Approximately 100 G418-resistant colonies were pooled and used for VLP analysis.

**Analysis of VLPs**

Cell culture supernatant containing VLPs were harvested from pooled stable cell clones. To prepare VLP samples, the cell culture supernatant was clarified (5 min at 700 x g) and the VLPs were pelleted by centrifugation for 1 h at 20,000 x g. The pelleted VLPs were resuspended in RIPA buffer (1% IGEPAL CA-630, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% DOC, 5 mM EDTA, 0.1% SDS). Cell lysates were prepared by trypsinizing and pelleting VLP-producing cells, then resuspending them in RIPA buffer.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis were used to analyze Gag protein expression (45). Western blot analysis was done using a primary antibody directed against the HA epitope tag (Covance, Berkeley, CA) and anti-mouse Ig and a sheep horseradish peroxidase linked whole antibody as the secondary antibody with the ECL western analysis kit (Amersham, Arlington Heights, IL). In the analysis of the PPPY domain with the PR+ and the PR+/AAAA constructs, the primary antibody used was a monoclonal antibody directed against the BLV MA (BLV3) (VMRD, Pullman, WA). The efficiency of VLP production was estimated by determining the percentage of VLP-associated Gag protein normalized to the amount of Gag in cells. Quantitation of band intensities was done using the Quantity One software package with the Chemi Doc 2000 Documentation System (BioRad, Richmond, CA).
**Electron microscopy**

For thin sectioning, stable COS-1 cell pellets were fixed with 2.5% glutaraldehyde. After dehydration in a grade series of cold ethanol, the samples were embedded in Epon 812 resin. Ultrathin sections (90nm) were then stained with uranyl acetate. The stained sections were observed with a Philips CM 12 electron microscope.

**Analysis of VLPs by sucrose gradient ultracentrifugation**

VLPs pelleted from the culture supernatant of transfected COS-1 cells were resuspended in 0.1ml of PBS. Resuspended VLPs were placed onto a sucrose gradient composed of 10, 20, 30, 40, 50, and 60% sucrose layers in PBS (0.6 ml each). The gradients were centrifuged at 33, 200 rpm at 4C for 16 h in a Beckman SW60Ti rotor. Eleven fractions were collected starting from the top of the centrifuge tubes. Fractionated samples were analyzed for BLV Gag expression by Western blot analysis.

**Confocal microscopy**

Transfected cells were grown on coverslips and fixed with 4% paraformaldehyde and permeabilized with Triton X-100, both diluted in phosphate-buffered saline (PBS). The cells were then incubated with anti-HA antibody followed by incubation with Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR). Images were collected with a Biorad MRC 600 confocal microscope using an attached Argon laser.
Results

Expression of BLV Gag leads to the production of virus-like particles in mammalian cells

To express BLV PrGag in mammalian cells, we constructed pGag-HA, which contains the entire BLV Gag protein coding sequence under the control of CMV promoter (Fig. 2.1A). COS-1 cells were transfected and placed under G418 selection to obtain stable cells expressing PrGag. The expression of Gag in COS-1 cells was monitored by Western blot using a monoclonal antibody directed against the HA epitope tag (Fig.2.1B). The expressed protein was approximately 44 kDa and it corresponded to the predicted size of the BLV PrGag with the HA tag. To initially test the prediction that the expressed BLV PrGag could assemble and subsequently be released into the medium as VLPs, the supernatants were collected from pooled stable cell lines, cleared of cell debris, and subjected to ultracentrifugation. The pelleted material was examined by Western blot to determine the amount of Gag released from cells. Figure 1B shows that Gag was readily detectable from the pelleted material.
Figure 2.1. Overexpression of the bovine leukemia virus (BLV) Gag polyprotein precursor (PrGag) leads to the production of virus-like particles (VLPs). A. Expression construct used for overexpression of PrGag. The gray box represents the cytomegalovirus promoter (CMV). The long white rectangular box represents the gag gene with the location of the corresponding matrix (MA), capsid (CA) and nucleocapsid (NC) protein domains indicated. The influenza virus hemagglutinin (HA) epitope tag is indicated by the thin black box. The bovine growth hormone polyadenylation signal (pA) is indicated by the striped box. The locations of the HindIII and EcoRI restriction sites are indicated. B. BLV PrGag overexpression. COS-1 cells were stably transfected with pGag-HA. Cell and virion lysates were used in Western blot analysis with an anti-HA antibody. The plasmid pMH6 (empty vector) was the HA epitope expression vector used to create pGag-HA and was a control for Gag expression in cells and VLP release into the cell culture supernatant. The positions of the molecular weight markers are indicated.
To confirm that the Gag detected in the pelleted material was an indication of VLP formation and release, the stably transfected COS-1 cells were examined by electron microscopy. Figure 2.2 (panels A-C) shows representative VLPs that were identified from the examination of thin sections. The VLPs do not have an electron dense core due to the absence of a viral protease. Immature retroviral particles can have an electron dense ring underneath the viral membrane. This is observed in Fig. 2.2B, but not easily observed in the other particles shown. These VLPs are similar in diameter to that of wt BLV particles produced from fetal lamb kidney cells chronically infected with BLV (FLK-BLV) (Fig. 2.2, panels D-F). The electron dense cores in wt BLV particles are readily seen. Comparison of the diameter for the VLPs with that of wt BLV particles indicates that the VLPs are very similar in size to wt particles.
Figure 2.2. Visualization of virus-like particles (VLPs) by electron microscopy. Panels A-C show representative VLPs produced from COS-1 cells. The VLPs are immature and do not have core particles because the viral protease is absent. Panels D-F show representative wild-type virus particles produced from fetal lamb kidney cells chronically infected with bovine leukemia virus (FLK-BLV). The mature cores are readily visible. Bar, 100 nm.

To further confirm that BLV Gag was being released from cells as VLPs, we used sucrose density gradient fractionation. VLP and wt BLV preparations recovered from the cell culture supernatants were loaded onto a sucrose gradient and fractionated by ultracentrifugation. Eleven gradient fractions were collected from the top to bottom, and the amount of Gag in each fraction was determined by Western blot (Fig.2.3). The majority of the Gag from wt BLV particles was
identified in fractions 5 to 7, with the peak in fraction 6 (Fig.2.3, bottom panel). The Gag derived from the VLPs was only detected in fractions 5 and 6. This indicates that the VLPs had a density similar to that of wt BLV. In total, these data indicate that overexpression of PrGag in mammalian cells leads to the production of VLPs.

Figure 2.3. Sucrose density gradient fractionation of VLPs. VLPs produced from COS-1 cells stably transfected with pGag-HA or wt BLV produced from FLK-BLV cells were layered onto a sucrose gradient composed of 10, 20, 30, 40, 50, and 60% sucrose layers. The gradients were centrifuged and eleven fractions were collected starting from the top of the centrifuge tubes. Fractionated samples were analyzed for BLV PrGag expression by Western blot analysis using either an anti-HA Ig (VLPs) or an anti-CA Ig (wt BLV). The locations of the 44 kD Gag polyprotein (PrGag) and the 24 kD capsid (p24) protein are indicated.
An N-terminal myristylation signal is required for virus-like particle formation.

The Gag protein of most retroviruses, including BLV, is myristylated at the N-terminal glycine residue. The cotranslational addition of the 14-carbon fatty acid myristic acid to the N-terminal glycine residue plays an essential role in targeting the Gag precursor to the cell membrane and in the subsequent assembly, budding and release of extracellular particles. The elimination or substitution of the glycine residue in some retroviruses abrogates particle production. To test this for BLV, three mutant constructs were made (Fig. 2.4A). First, the mutant G2A has the glycine residue immediately after the methionine start changed to an alanine residue and would prevent myristylation of the BLV PrGag. Second, HA-Gag has an HA-epitope tag fused to the N-terminus of Gag, and thereby blocking the exposure of the glycine residue to myristic acid, which would prevent the myristylation of the BLV Gag precursor polyprotein. Third, the mutant G-HA-Gag was constructed from HA-Gag by adding an extra glycine before the HA tag. The additional glycine was added to test if this residue could restore the myristylation signal.

Each vector was transfected into COS-1 cells in parallel with pGag-HA and the constructs were observed to express the Gag protein in cells at comparable levels (Fig. 2.4B). Analysis of VLP production indicated that the substitution of glycine for alanine in the G2A mutant drastically reduced VLP production to that of the parental vector (Fig. 2.4B). No BLV PrGag was detected.
for HA-Gag, indicating the complete loss of VLP production. Interestingly, by adding the additional glycine in G-HA-Gag, VLP production could be detected (Fig. 2.4B).

To further examine the nature of the mutant phenotypes, we used confocal microscopy to analyze the subcellular localization of the Gag proteins. Expression of Gag from Gag-HA yielded a stippled appearance along plasma membrane (Fig. 2.4C). G2A was observed to have both a perinuclear and cytoplasmic distribution (Fig. 2.4C). Very little plasma membrane staining was observed for G2A, indicating a severe defect in plasma membrane targeting. HA-Gag demonstrated staining that was primarily intracellular, with a predominant bright perinuclear halo (Fig. 2.4C). No peripheral staining was observed with HA-Gag, indicating that translocation of Gag to the plasma membrane was severely impaired. In contrast, the localization of G-HA-Gag led to a cytoplasmic distribution, though a stippled staining pattern along the plasma membrane was also observed (Fig. 2.4C).
Figure 2.4. The amino-terminal myristylation signal is required for efficient VLP production and PrGag membrane targeting. A. Mutants made to test the role of the myristylation signal on VLP production and Gag membrane targeting. The name of each mutant is indicated at the left. The open rectangular box shows the Gag protein with the MA, CA and NC domains indicated as in Fig. 1. The HA epitope tag is indicated by the thin black box. The N-terminal amino acid sequence of MA is enlarged to identify the nature of the mutations made to create G2A and G-HA-Gag. The bold letters indicate residues that were mutated; dashes denote same as wt sequence. The wavy line at the N termini indicates the myristylation signal. B. VLP production. pGag-HA and mutants were stably transfected into COS-1 cells. Cell and virion lysates were analyzed by Western blot using an anti-HA antibody. C. Cellular distribution of PrGag. Cells stably transfected with VLP constructs were grown on coverslips, fixed and incubated with an anti-HA Ig followed by incubation with Alexa Fluor 488-conjugated anti-mouse Ig. Images were collected using a confocal microscope.
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G-HA-Gag

G2A

HA-Gag
**Mutagenesis of the BLV PPPY motif decreases virus-like particle production**

A functional L domain is required for the budding and release of many retroviruses. The RSV, MLV and MPMV Gag the L domain maps specifically to a proline-rich sequence, PPPY, located near the MA and CA protein domain junction in Gag. Examination of the protein coding sequence of BLV indicates a similar PPPY motif is located in the MA domain near the boundary with CA. To examine the significance of the PPPY motif in the process of particle budding and release, a series of mutations were introduced into pGag-HA (Fig. 2.5A). In the APPY, PAPY, PPAY, PPPA mutants, each residue within PPPY motif was individually replaced with alanine. In the mutant AAAA, all four residues in the PPPY motif were substituted for alanine residues.

To test the influence of the PPPY mutants on VLP production, COS-1 cells were transfected in parallel with the parental construct and each mutant. Analysis of Gag expression in cells and subsequent VLP production indicated that for each of the alanine substitution mutants, there was a decrease in the release of VLPs (Fig. 2.5B). The reduction in VLP production was most pronounced for APPY, PAPY and AAAA, where there was a reduction in VLP production that was approximately half (42, 45 and 42%, respectively) that of the parental construct. These results indicate that mutations in the PPPY motif can significantly reduce VLP production and suggest that this motif may function as a L domain.
Recent observations have indicated that late domain function can be cell-type dependent (7). To test this for the BLV, the panel of PPPY mutants was tested in parallel in 293 cells for expression in cells and for production of VLPs. As was observed with COS-1 cells, there was a general decrease in VLP release for each of the mutants tested (Fig. 2.5B). In particular, the APPY and AAAA mutants were most pronounced, reducing VLP production to approximately one-fifth (26 and 28%, respectively) that of the parental construct. These results provide further evidence in support of the PPPY motif functioning as a late domain.
Figure 2.5. Mutation of the PPPY motif reduces VLP production. A. Amino acid substitutions to the conserved PPPY sequence. The PrGag is shown as described in Fig. 4. The amino acid sequence of PPPY domain and its flanking sequence are expanded. The full-length black line represents wt Gag sequence. The alanine substitutions are indicated below and are aligned with the expanded sequence. The name of each mutant is indicated on the left. B. Analysis of VLP production. COS-1 cells or 293 cells were stably transfected with pGag-HA or the indicated mutants. Cell and VLP lysates were analyzed by Western blot using an anti-HA Ig.
**Effect of PPPY motif mutants on virus-like particle production in the context of an active viral protease**

Previous studies have indicated that perturbation of Gag precursor processing can severely impair the assembly of infectious virions (19, 20). In previous studies with HIV-1, a less severe L domain defect was observed when protease function was absent (15). To determine if the presence of protease activity can enhance the VLP production defect observed by mutation of the PPPY motif, we examined the phenotype of the PPPY domain mutant in the context of an active viral protease (Fig. 2.6). The construct expressing Gag and the viral protease (PR+) was transfected into 293 cells in parallel with PR+/AAAA, a derivative with the PPPY motif mutated to AAAA. The production of VLPs was monitored by Western blot using an anti-BLV CA monoclonal antibody. As anticipated for a PR+ clone, the completely processed BLV CA was detected in both PR+ and PR+/AAAA, demonstrating the expression of the viral protease (Fig. 2.6A). Although not readily observed from cell lysates, PrGag was observed with longer exposures (data not shown). VLP production for PR+/AAAA was reduced to about one-twentieth (5%) that of PR+. An incompletely processed Gag intermediate and a small amount of unprocessed Gag precursor could also be readily observed with PR+ but not in PR+/AAAA. This was presumably due to the relative abundance of VLPs produced by PR+ to that of PR+/AAAA. These observations in total indicate that the effect of the PPPY motif on VLP production was more pronounced in the context of an active protease.
Finally, we used electron microscopy to determine if the reductions in VLP production that we had observed correlated with defects in virus release. By comparing VLP production of wt BLV with that of PR+/AAAA, we observed a very high proportion of budding virus particles that did not release from cells compared to that of wt BLV (Fig. 2.6B). Interestingly, VLPs with donut-shaped (immature) cores were observed for PR+/AAAA. The high proportion of VLPs observed for PR+/AAAA that have not released from cells provide further support that the PPPY motif functions as an L domain. It is interesting to note that no long "strings" of particles were observed like those seen with HIV-1 and MLV L domain mutants.
Figure 2.6. The PPPY motif functions as a late (L) domain. A. An active protease enhances the L domain defect. 293 cells were transfected with PR+ or PR+/AAAA. Two days posttransfection cell and VLP lysates were made and analyzed by Western blot using an anti-capsid (CA) Ig. B. Visualization of the L domain defect. Representative VLPs budding but not released from 293 cells are shown (PR+/AAAA). The VLPs have mature cores because of the presence of the viral protease. Representative wild-type virus particles released from fetal lamb kidney cells chronically infected with bovine leukemia virus (FLK-BLV) are also shown (PR+). Arrows point to the location of budding particles. Bar, 100 nm.
Discussion

Development of a VLP model system for studying various aspects of the BLV assembly pathway

We have described a model system that overexpresses the BLV PrGag in mammalian cells and have demonstrated that this leads to the assembly, budding and release of VLPs. Our observations that overexpression of Gag leads to the production of virus-like particles is similar to observations made with other retroviruses, including BLV, that relied on the use of recombinant vaccinia viruses (14) or recombinant baculovirus systems (1, 17, 37, 43). 

Myristylation of the N-terminal glycine is necessary for efficient membrane targeting of Gag

Gag proteins of many retroviruses are myristylated at the N terminal glycine residue, and the covalent attachment of myristic acid is required for efficient membrane association and virion formation (2, 30, 32, 39-41). We found that mutation of the N-terminal glycine led to a dramatic reduction in VLP production, similar to previous observations made with BLV Gag expressed from a baculovirus vector in insect cells (17). The mutation of the N-terminal glycine also led to a dramatic reduction in the ability of PrGag to localize at the plasma membrane. When a HA epitope tag was fused to the N-terminus of PrGag, N-terminal myristylation of Gag was blocked, and VLP production along with
localization of Gag to the plasma membrane was severely impaired. However, addition of a glycine in front of the HA-Gag restored VLP production.

Two observations suggest that the N-terminal glycine residue alone was not sufficient for efficient PrGag membrane binding and VLP production. First, the mutant G2A severely impaired but did not completely eliminate VLP production. It is worth noting that in previous studies with BLV and HTLV-1, mutation of the N-terminal glycine eliminated detectable particle production (1, 17, 21). It is possible that the level of Gag expression in our system was high enough that low level VLP production was detected whereas it was not in previous reports. Second, the G-HA-Gag mutant restored VLP production, presumably by restoration of PrGag myristylation, though this has not been formally tested. Studies with HIV-1 have demonstrated that hydrophobic residues within the first 14 amino acids of HIV-1 Gag can enhance the interaction with the plasma membrane (28, 29, 54). Extensive mutagenesis of the N-terminal residues of the HIV-1 Gag have indicated that the first five residues blocked or impaired Gag myristylation (29), while mutation of residues 6, 7 and 8 reduced membrane binding without affecting N-terminal myristylation (28). In light of these observations with HIV-1, the restored VLP production phenotype observed with the G-HA-Gag may be due to the introduction of an N-terminal glycine residue, but that the HA sequence disrupted the myristylation signal and prevented restoration to wt levels. The nature of the restored VLP production phenotype for G-HA-Gag is currently not completely understood and needs to be further investigated. The results with HIV-1 support the myristyl switch model for
the regulation of Gag membrane binding, which proposes that membrane binding is determined by the degree of exposure or sequestration of the N-terminal myristate moiety. The electrostatic interaction between a cluster of basic amino acids positioned between residues 15 and 31 in HIV-1 MA and acidic membrane phospholipids can stabilize the protein-lipid association and can potentially contribute more binding energy than the hydrophobic myristate moiety (13, 28, 32, 54). This is further supported by the fact that the PrGag of several retroviruses, including RSV, visna virus, EIAV, and caprine arthritis-encephalitis virus, are fully capable of membrane targeting and budding without the need of myristylation (26, 49). Therefore, the proximity of myristate with downstream residues can be important for membrane targeting and VLP production. In total, analysis of the affects of site-directed mutations for all the residues near the N-terminus of the BLV PrGag will help establish the roles of these residues in Gag membrane targeting.

Basic residues in the BLV MA domain likely play a role in PrGag membrane binding. The majority of the basic residues in BLV MA are not concentrated at the N-terminus, but appear on one side of the molecule within the 3D structure (4, 25). This could create a basic surface exposed to the solvent which is ideally situated for the interaction with acidic phospholipid headgroups of the inner face of the membrane. We are currently investigating the role of the basic residues in BLV MA in PrGag membrane binding.
PPPY motif functions as a BLV L domain

We have shown that mutagenesis of the PPPY motif in the MA domain of PrGag plays an important role in VLP release. PPPY mutants displayed a reduced level of VLP production from COS-1 cells that appear more pronounced when using 293 cells. The PPPY mutant phenotype also appeared more pronounced in the presence of the viral protease. An abundance of budding particles that had not been released by cells were commonly seen with the PPPY mutants, which is in stark contrast to that observed with wt. Interestingly, most of the budding particles for the PR+/AAAA mutant that had not released had developing core particles. This indicates the presence of viral protease activity prior to release of the VLP. This is in contrast to what has been observed for HIV-1 (6). However, our observations could be influenced by the overexpression of Gag in the VLP-producing cells.

In this study, the individual exchange of an alanine residue with a residue in the PPPY motif led to a significant reduction in VLP production, as did the simultaneous exchange of the PPPY motif with four alanine residues. With MPMV, each residue in the PPPY motif was found to be critical for late domain function, but the tyrosine residue appeared to be more important (51). The individual proline residues in the PPPY motif of RSV were found to equally influence virus budding when mutated to glycine (50). The simultaneous exchange of the PPPY motif in MLV with alanine residues resulted in a significant defect in virus budding (53).
Our finding that the viral protease enhanced the phenotype of the PPPY mutants is similar to an observation made with HIV-1 where the inactivation of the viral protease alleviated the requirement of the PTAP motif in virus release (15). Interestingly, in both RSV and MPMV, protease inactivation did not alter the requirement of the late domains for budding (48, 51).

In summary, we have described a BLV-like particle model system to study virus assembly and release. The data presented indicate that aspects of Gag membrane targeting and virus release can be analyzed. Given the difficulties in studying the molecular biology of the deltaretroviruses, this model system will greatly facilitate future molecular analyses.

References


interact with the L domain of Rous sarcoma virus and are required for gag budding from cells Proc Natl Acad Sci U S A. 98:11199-11204.


CHAPTER 3

INVolvement of the maTRix AND nUceoCAPsid DOmAINs of the bovine leuKemia virus gag POLYproteIN precUrSOR IN vIRal RNA PACKAgING

Abstract

The RNA packaging process for retroviruses involves a recognition event of the genome-length viral RNA by the viral Gag polyprotein precursor (PrGag), an important step in particle morphogenesis. The mechanism underlying this genome recognition event for most retroviruses is thought to involve an interaction between the nucleocapsid (NC) domain of PrGag and stable RNA secondary structures that form the RNA packaging signal. Presently, there is limited information regarding PrGag-RNA interactions involved in RNA packaging for the deltaretroviruses, which include bovine leukemia virus (BLV) and human T-cell leukemia virus types 1 and 2 (HTLV-1 and HTLV-2). To address this, alanine-scanning mutagenesis of BLV PrGag was done using a virus-like particle (VLP) system. As predicted, mutagenesis of conserved basic residues as well as residues of the zinc-finger domains in the BLV NC domain of PrGag revealed residues that led to a reduction in viral RNA packaging. Interestingly, when conserved basic residues in the BLV MA domain of PrGag were mutated to
alanine or glycine, but not when mutated to another basic residue, reductions in viral RNA packaging were also observed. The ability of PrGag to be targeted to the cell membrane was not affected by these mutations in MA, indicating that PrGag membrane targeting was not associated with the reduction in RNA packaging. These observations indicate that these basic residues in the MA domain of PrGag influence RNA packaging, without influencing Gag membrane localization. It was further observed that i) a MA/NC double mutant had a more severe RNA packaging defect than either mutant alone, and ii) RNA packaging was not found to be associated with transient localization of Gag in the nucleus. In summary, this report provides the first direct evidence for the involvement of both the BLV MA and NC domains of PrGag in viral RNA packaging.

Introduction

The RNA packaging process for retroviruses involves a recognition event of the genome-length viral RNA by the viral Gag polyprotein precursor (PrGag), which acts to initiate the morphogenesis of virus particles (see (17) for review). The mechanism underlying this genome recognition event is poorly understood, but many biochemical and genetic analyses have revealed that this event involves the interaction between stable RNA secondary structures at the 5’ end of the viral genome and, in many cases, amino acids in the nucleocapsid (NC) domain of PrGag (1, 2, 4, 6, 7, 9, 10, 13, 15, 29, 30, 32, 36, 37).

The genome recognition event is an important step in the morphogenesis of infectious retrovirus particles. This recognition event leads to the predominant
packaging (encapsidation) of the genome-length viral RNA into assembling particles (38). This discrimination process, which is primarily a viral RNA-protein interaction, is known to strongly favor the full-length viral RNA to that of spliced viral RNAs and cellular mRNAs (38). In general, the RNA sequences necessary and sufficient for the RNA packaging process are located in a region that includes the 5'-noncoding region along with the 5'-half of the \textit{gag} gene (38). These sequences are referred to as the packaging or encapsidation signal. The packaging signal can enhance the packaging of RNAs containing the signal over those not containing the packaging signal. However, in the absence of RNAs containing a packaging signal, cellular RNAs can be packaged into particles and supports the conclusion that RNA is a structural element in retrovirus particles (31).

The Gag polyprotein precursor alone is sufficient for the formation of particles, and the presence of RNA aids in efficient particle production (5). Since activation of the viral protease does not typically occur until after virus particle release, the genome recognition event involves full-length viral RNA and unprocessed Gag polyprotein (38). Gag includes three main domains (matrix, MA; capsid, CA; nucleocapsid, NC). Attempts to address the RNA packaging process have focused on identifying RNA-binding domains within unprocessed Gag (and the processed Gag products) and demonstrating specificity in binding of viral RNA to Gag either in cell-free reactions or in the uptake of viral RNA into virus particles.
The NC proteins of all retroviruses share the characteristics of a high percentage of basic residues and zinc binding domains involved in RNA packaging and alter in many instances the specificity of RNA binding (8, 12, 14, 17, 21, 23, 33, 36, 37). It is not clear if NC actually confers all the selective recognition of the viral genomic RNA (4, 32). The p2 spacer peptide between CA and NC of HIV-1 PrGag has been suggested to play a role in selective RNA packaging (20). However, previous studies with HIV-1 indicated that MA does not play a role in RNA packaging (32).

BLV, as well as other deltaretroviruses, replicate to low titers in their natural hosts and are poorly infectious in cell culture. Cocultivation is typically used to infect permissive host cells. Because of these difficulties, information regarding the molecular details of their life cycles, including virus assembly, is limited. To more easily study the RNA packaging step in the virus assembly pathway of BLV, we have used a VLP model system to identify the protein determinants of BLV RNA packaging (39). As expected, many of the basic amino acid residues and all zinc-finger residues mutated in the NC domain of PrGag led to significant reductions in RNA packaging. Interestingly, many of the basic residues in MA were also found to significantly influence RNA packaging. Together, these observations provide the first evidence that both the MA and NC domains of BLV PrGag are involved in viral RNA packaging.
Material and Methods

VLP vector and mutagenesis

The vector used to produce VLPs, pGag-HA, has a HA epitope tag fused to the C-terminus of the BLV PrGag. The construction of this vector and the ability of the vector to produce VLPs have been previously described (39). This vector transcribes a single RNA transcript and no spliced RNA. The RNA is translated to express PrGag in the absence of the viral protease. Therefore, the VLPs do not have mature cores. The primary BLV RNA packaging signal, consisting of two stable RNA stem-loop structures (i.e., SL1 and SL2), are located just downstream of the \textit{gag} start codon (24-26). Due to the presence of the packaging signal, this RNA transcript can be packaged into VLPs through the interaction with PrGag. In this system, the VLP expression vector transcribes only one RNA species due to the absence of the BLV splice donor and splice acceptor sites.

Site directed mutations were introduced into pGag-HA using the QuickChange XL kit (Stratagene, La Jolla, CA) according to manufacturer's instructions. All derivatives made of pGag-HA were sequenced to verify the correct introduction of the desired mutation and the absence of undesired mutations.

Cells and transfections

To express BLV PrGag in mammalian cells, COS-1 cells were transfected with pGag-HA or derivative and placed under G418 selection to obtain stable cells expressing PrGag. Cells were grown in 100-mm, 60-mm, or 35-mm-
diameter dishes in Dulbecco’s modified Eagle medium (DMEM)(GIBCO BRL, Gaithersburg, MD) supplemented with 10% Fetal Clone III serum (Hyclone, Logan, UT). Superfect (Qiagen, Valencia, CA) was used for transfection of cells. Two days post-transfection, cells were placed under G418 selection until resistant colonies formed (~ 3 weeks). Approximately 100 G418-resistant colonies were pooled and used for VLP analysis.

**Quantitation of VLP production**

Supernatant from pooled stable cell clones producing VLPs was harvested and clarified by low speed centrifugation (5 min at 700 x g), then subjected to ultracentrifugation for 1h at 40,000 x g at 4°C. Pelleted VLPs were then resuspended in RIPA buffer (1% IGEPAL CA-630, 50mM Tris-HCl, pH7.5, 150 mM NaCl, 0.5% DOC, 5mM EDTA, 0.1% SDS).

Lysates prepared from VLP-producing cells and VLP samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and BLV Gag detected with a primary antibody directed against the HA epitope tag (Covance, Princeton, NJ) and an anti-mouse Ig, horseradish peroxidase linked whole antibody (from sheep) as a secondary antibody (Amersham, Arlington Heights, IL). Quantitation of band intensities was done using the Quantity One software package with the Chemi Doc 2000 Documentation System (BioRad, Richmond, CA).
Quantitation of viral RNA in cells and VLPs and determination of RNA packaging efficiencies

Total RNA was extracted from transfected cells by using a cellular RNA extraction kit (Qiagen), according to the manufacturer's instructions. Viral RNA was extracted by using a QIAamp Viral RNA kit (Qiagen), according to the manufacturers’ instructions. To control for DNA contamination, the isolated RNAs were treated with Dnase I (Invitrogen) and used in RT-PCR without the addition of reverse transcriptase to confirm the absence of DNA.

Detection of the RNA transcript that expresses PrGag in cells and its incorporation into VLPs due to the RNA packaging signal was done using quantitative real-time RT-PCR analysis. Quantitative real-time RT-PCR was carried out with the Qiagen RT-PCR Master SYBR Green kit in a 20 ul reaction volume. RNA templates were reverse transcribed at 50 C for 25 min followed by a denaturing step at 95 C for 15 min. PCR was then performed using the primers +BLV979 (5’-AACC GCCG CATCGT GCTTGGCCA-3’) and -BLV1183 (5’-CGCTTCAGCGGCGGT ATTGC-3’). These primers amplify an approximately 200 bp long fragment within the gag gene.

The PCR protocol consisted of 50 cycles of denaturation (95C for 15s), annealing (60C for 20s) and extension (72C for10s). For each step, the temperature transition rate was 20C/s. The PCR was monitored after each elongation step by SYBR Green I dye binding to amplified products using a Light Cycler (Roche Diagnostics). Quantitation was carried out using an external standard curve. Standard RNA was synthesized by in vitro transcription of a
linearized plasmid containing a BLV proviral DNA representing the 5'-end of the \textit{gag} gene. All \textit{in vitro} transcripts were Dnase I-treated and tested for DNA contamination.

Standard curves were constructed from 10-fold serial dilutions of synthetic RNA transcripts. After real-time RT-PCR was completed, logarithmic values of fluorescence for each dilution were plotted against cycle number. A baseline was set just above the fluorescence background and a crossing point was determined with amplification curves obtained during the initial exponential phase of amplification. The specificity of the amplified product was then determined by melting curve analysis. Melting curve acquisitions were done immediately after PCR was completed, by heating at 95°C for 0 s, cooling to 75°C for 15 s and heating slowly at 20°C/s until 95°C with continuous fluorescence recording.

RNA packaging efficiencies were determined by the ratio of viral RNA detected from VLPs to viral RNA detected from total RNA recovered from VLP-producing cells divided by the ratio of Gag protein detected from VLPs to Gag protein detected from VLP-producing cells. RNA packaging efficiencies of mutants were then compared relative to that of the parental pGag-HA vector.

\textbf{Confocal microscopy}

Transfected cells were grown on coverslips and fixed with 4% paraformaldehyde and permeabilized with Triton X-100, both diluted in phosphate-buffered saline (PBS). The cells were then incubated with anti-HA antibody followed by incubation with Alexa Fluor 488-conjugated goat anti-mouse
IgG (Molecular Probes). Images were collected with a Biorad MRC 600 confocal microscope.

**Results**

**Effects of mutations in the NC domain of PrGag on RNA packaging**

In order to determine the influence of NC on RNA packaging, a series of NC mutants were made in pGag-HA and tested for their ability to influence RNA packaging. Alanine residues were introduced in place of basic amino acid residues and in place of the conserved residues in each of the two zinc-finger binding domains (Fig. 3.1A). The panel of NC mutants was analyzed in parallel with the parental vector. Figure 3.1B shows the RNA packaging efficiencies of the mutants. There were 3 groups of mutant phenotypes. The first group of mutants had no RNA packaging defects compared to the parental vector. These mutants included K17A and R18A. A second group of mutants had mild RNA packaging defects (40-70 % reduction) (i.e., R12A, K17A/R18A, K59A, K66A, and K68A). The third group of mutants had severe RNA packaging defects compared to the parental vector (85-95 % reduction) (i.e., C24A, C27A, R35A, C49A, H57A, and R60A). For many of the mutants, VLP production was reduced 2- to 3-fold that of the parental vector (i.e., R12A, K17A, R18A, K17A/R18A, R35A, and C49A). This reduction is within the linear detection range for the immunoblot assay used (see below). As predicted, many of the basic amino acid residues that were mutated led to significant reductions in RNA packaging efficiencies. The mutated basic residues that resulted in the greatest reductions
were R35A and R60A. Interestingly, each of these residues lies between the conserved histidine and cysteine residues in the first (i.e., R35A) and second (i.e., R60A) zinc-finger domain.
Figure 3.1. Mutagenesis of basic residues and the zinc-fingers in the BLV nucleocapsid (NC) domain of PrGag results in RNA packaging defects. A. The BLV NC domain of PrGag. Amino acid residues in bold indicate basic residues and bolded residues that are underlined indicate basic and/or charged residues conserved among BLV, HTLV-1, and HTLV-2. The locations of the two zinc-finger (cys-his box) domains within NC are indicated. B. Relative RNA packaging efficiencies of BLV NC mutants. RNA packaging efficiencies were determined by the ratio of viral RNA to the amount of Gag from VLPs (see Materials and Methods for details). The RNA packaging efficiency with wt BLV NC was set at 100 and the values for the NC mutants (+/- SD) are relative to wt. Mutants were tested in parallel with the parental construct and each experiment was done in triplicate.
Effects of mutations in the MA domain of PrGag on RNA packaging

Previous studies with other retroviruses have provided little support for a role of the MA domain in retroviral RNA packaging (32). To test whether basic residues in the BLV MA domain influence RNA packaging, a panel of mutants that changed basic residues in MA to alanine was created (Fig 3.2A). The RNA packaging efficiencies of the mutants were then analyzed in parallel with the parental vector. Figure 3.2B shows a representative protein analysis of Gag from cells and VLPs. The K52A and K53A MA mutants led to the lowest levels of VLPs (8 % and 17%, respectively). These reductions are within 13 fold of the parental vector and are within the linear range of detection for the assay, which was at least 16 fold (Fig. 3.2C). Figure 3.2D shows the resulting RNA packaging efficiencies for the panel of MA mutants tested. As with the NC mutants, there were 3 general groups of mutant phenotypes. Several basic residues that are not conserved among BLV, HTLV-1, and HTLV-2 led to no or small changes in RNA packaging efficiencies (i.e., R31A, H48A, K53A, R69A, and R72A). A second group led to modest reductions in RNA packaging efficiencies (i.e., R27A and K52A). Finally, a third group had significant reductions in RNA packaging (66 to 92%) compared to that of the parental vector (K41A, H45A). The H45A MA mutant had a reduction in RNA packaging efficiency (92%) that was as great as any of the individual NC mutants, indicating that BLV MA plays an important role in RNA packaging. The K49 residue was not analyzed in this study.

In other retroviruses, such as HIV-1, basic amino acid residues in MA can influence PrGag membrane targeting and are proposed to form a positively
charged patch which promotes membrane binding (16, 27, 40, 41). To determine
whether MA mutants that significantly reduced RNA packaging also influenced
PrGag membrane localization, the cellular distribution of Gag was analyzed by
confocal microscopy. Figure 3.2E shows the effects of several basic amino acid
mutants on Gag distribution in cells. These mutants (i.e., R27A, K41A, H45A,
K52A, and K53A) had a cellular distribution of Gag that was comparable to that
observed with wt. Specifically, Gag expression yielded a stippled appearance
along the plasma membrane for the parental Gag and for these mutants (Fig. 3.2E).
Figure 3.2. Mutagenesis of basic residues in the BLV MA domain of PrGag results in RNA packaging defects. A. The BLV MA domain of PrGag. Amino acid residues in bold indicate basic residues and bolded residues that are underlined indicate basic and/or charged residues conserved among BLV, HTLV-1, and HTLV-2. The alpha helices in MA are indicated based upon NMR solution structure analysis of BLV MA (28). B. Quantitation of VLP production. Preparation of lysates from VLP-producing cells and VLPs along with immunoblot analysis is described in the Materials and Methods. Representative results from the immunoblot analysis is shown. C. Linear range of detection for immunoblot analysis. Protein was diluted (1:1, 1:2, 1:4, 1:5, 1:8, 1:10, and 1:16) and was subjected to immunoblot analysis. The band intensity (arbitrary units) for each dilution was determined using the Quantity One software package of the ChemiDoc 2000 Documentation System (BioRad) and plotted against the amount of protein used for immunoblot analysis (16 ul, 8 ul, 4 ul, 3.2 ul, 2 ul, 1.6 ul, or 1 ul) to determine if protein detection within the dilution range was linear. D. Relative RNA packaging efficiencies of BLV MA mutants. RNA packaging efficiencies were determined by the ratio of viral RNA to the amount of Gag from VLPs (see Materials and Methods for details). The RNA packaging efficiency with wt BLV MA was set at 100 and the values for the MA mutants (+/- SD) are relative to wt. Mutants were tested in parallel with the parental construct and each experiment was done in triplicate. E. Cellular distribution of PrGag for selected MA mutants. Cells stably transfected with VLP constructs containing mutations in MA were grown on coverslips, fixed and incubated with an anti-HA Ig followed by incubation with Alexa Fluor 488-conjugated anti-mouse Ig. Images were obtained with a confocal microscope.
A.  

\[ \begin{align*}
\alpha\text{-helix A} & \quad MGNSPSYNPPAGISPSDWNLLQSAQR\text{LP} \\
\alpha\text{-helix B} & \quad RPSPSDFTDLKNYIHWFHKTQKKPWPFTSG \\
\alpha\text{-helix C} & \quad GPTSCPPGRFGRVPLVLATLNEVLSNEGGA \\
\alpha\text{-helix D} & \quad \vdots
\end{align*} \]

B.  

<table>
<thead>
<tr>
<th>Protein</th>
<th>WT</th>
<th>R27A</th>
<th>K41A</th>
<th>H45A</th>
<th>WT</th>
<th>R31A</th>
<th>H48A</th>
<th>K52A</th>
<th>K53AR69A</th>
<th>R72AWT</th>
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C.  

Protein dilution

\[ y = 165.28x + 261.02 \quad R^2 = 0.994 \]

Signal intensity vs. Protein (ul)
D.

% RNA/VLP

<table>
<thead>
<tr>
<th>Mutant</th>
<th>WT</th>
<th>R27A</th>
<th>K41A</th>
<th>H45A</th>
<th>R31A</th>
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E.

WT     R27A     K41A

H45A    K52A    K53A
**Importance of charged amino acid residues in MA on RNA packaging**

The MA residues R27 and H45, when mutated, revealed significant reductions in RNA packaging efficiencies. To further analyze the importance of these positively charged amino acid residues on RNA packaging, mutations were made at either R27 or H45 to create either a conservative (i.e., R27H or H45R) or nonconservative (i.e., R27G or H45G) changes at these residues. Figure 3.3A shows the results of these amino acid substitutions on RNA packaging. R27G had a phenotype comparable to that of R27A, while R27H had a phenotype that was comparable to that of the parental vector. Furthermore, H45G had an RNA packaging defect similar to that of H45A, while H45R had a phenotype equivalent to that of the parental vector. These data indicate that there is a correlation between the absence of a positively charged amino acid at positions 27 or 45 and a defect in RNA packaging. The cellular distribution of Gag was not influenced by these mutations, indicating no correlation between the RNA packaging defects and Gag membrane localization (Fig. 3.3B).
Figure 3. Requirement of charged amino acid residues in BLV MA for RNA packaging. RNA packaging efficiencies were determined by the ratio of viral RNA to the amount of Gag from VLPs (see Materials and Methods for details). The RNA packaging efficiency with wt BLV MA was set at 100 and the values for the MA mutants (+/- SD) are relative to wt. Mutants were tested in parallel with the parental construct and each experiment was done in triplicate. B. Cellular distribution of PrGag. Cells stably transfected with VLP constructs were grown on coverslips, fixed and incubated with antibodies prior to confocal microscopy.
A.

![Graph showing percentage RNA/VLP for different mutants.](image)

**Mutant**

- WT
- H45A
- H45G
- H45R
- R27A
- R27G
- R27H

B.

![Images of cell cultures with different mutants.](image)

**Mutants**

- WT
- R27G
- R27H
- H45G
- H45R

92
Combined effect of MA and NC mutations on RNA packaging

To test for the potential interplay between MA and NC on RNA packaging, a mutant construct was created in which both MA and NC mutations were introduced into the same vector and then assayed for the ability to package RNA. In particular, the MA/NC double mutant MA H45A/NC C24A was tested. The combined mutant had a 10-fold reduction in VLP production and a severe defect in RNA packaging (98% reduction to that of the parental) that was significantly lower than the RNA packaging efficiency of either mutant alone (Fig. 3.4). This indicates that the combination of mutations together further reduced the efficiency of RNA packaging.

![RNA packaging efficiency graph](image)

Figure 3.4. Combined influence of NC and MA mutations on RNA packaging. RNA packaging efficiencies were determined by the ratio of viral RNA to the amount of Gag from VLPs (see Materials and Methods for details). The RNA packaging efficiency with wt BLV was set at 100 and the values for the mutants (+/- SD) are relative to wt. Mutants were tested in parallel with the parental construct and done in triplicate.
Lack of transient nuclear localization of Gag

A recent study with RSV revealed that RSV Gag protein enters the nucleus by a nuclear-targeting sequence in the MA domain and is subsequently transported to the cytoplasm by using a CRM1-mediated nuclear export pathway (35). Transient expression of a dominant-negative CRM1 or by treating cells with leptomycin B (LMB, a drug that attaches to the central domain of CRM1 to disrupt its interaction with nuclear export signals) resulted in the redistribution of Gag from the cytoplasm to the nucleus. The MA mutant, Myr1E, was insensitive to the effects of LMB treatment, apparently because it bypassed the nuclear compartment during virus assembly (35). Myr1E has a defect in RNA packaging, which implies that RSV nuclear localization of Gag might be involved in viral RNA-Gag interactions.

Since we discovered BLV MA mutants with RNA packaging defects like Myr1E, we hypothesized that the viral RNA-Gag interactions involved in BLV RNA packaging might occur in the nucleus after transient nuclear localization of Gag. To test whether BLV Gag is localized in the nucleus, 293T cells transiently transfected with the BLV VLP construct PR+ (39) were treated with either LMB or transiently cotransfected with a dominant-negative CRM1 (ΔCAN). In addition, fetal lamb kidney cells chronically infected with BLV (FLK-BLV) were either treated with LMB or transiently transfected with ΔCAN. When 293T cells were either cotransfected with a RSV Gag-GFP construct and ΔCAN, or transfected with Gag-GFP and then treated with LMB, there was a distinct nuclear localization of Gag-GFP, which is comparable to that originally reported by
Scheifele et al (35). When 293T cells were either cotransfected with the BLV vector and ΔCAN or treated with LMB after transfection with the BLV vector, no BLV Gag was observed in the nucleus of cells (Fig. 3.5). Comparable results were obtained with LMB treatment or transfection of ΔCAN into FLK-BLV cells. One interpretation of this data is that BLV Gag does not enter the nucleus, implying that the genome recognition event occurs in the cytoplasm. However, it is possible that BLV Gag does enter the nucleus and is exported by a CRM1-independent pathway.
Figure 3. Lack of transient nuclear localization of BLV Gag. 293T cells were transiently transfected alone with a Rous sarcoma virus (RSV) Gag-GFP construct or a BLV vector (PR+) and treated with leptomycin B (LMB) (10 ng/ul) or were transiently cotransfected with the RSV or BLV construct and a dominant-negative CRM1 (ΔCAN) (10 ug per approximately 5 x 10^5 cells). In parallel, fetal lamb kidney (FLK) cells chronically infected with BLV were treated with LMB (10 ng/ul) or were transiently transfected with ΔCAN ((10 ug per approximately 5 x 10^5 cells). Forty-eight hours posttransfection, cells were fixed and analyzed by confocal microscopy.

Discussion

This is the first report to provide direct genetic evidence showing that both the MA and NC domains of BLV PrGag are protein determinants of RNA packaging. The unique observation in this study is the identification of several
basic amino acid residues in MA that when mutated, led to RNA packaging defects as great, if not greater, than those observed for single amino acid changes in NC. These observations imply that BLV, and perhaps other deltaretroviruses, use distinct viral RNA-protein interactions in genome recognition and RNA packaging.

An interesting observation made with the MA mutants analyzed in this study is that the basic residues in MA that influenced RNA packaging had no effect on Gag membrane localization. This is in contrast to studies with HIV-1 that indicate a role for the basic amino residues of MA in membrane localization (by the formation of a charged patch that is thought to promote membrane binding) (11, 16, 27, 40, 41). However, our data support a previous study of the basic residues in the HTLV-1 MA which found that HTLV-1 MA basic residue mutants led to normal transport of Gag to the membrane as well as cleavage of PrGag (22). Many of these HTLV-1 mutants had reduced infectivity. Our study found that several of the BLV MA basic residue mutants affected RNA packaging and did not influence Gag membrane targeting (though they did lead to reductions in virus particle production), suggesting that the reduced infectivity observed with the HTLV-1 mutants may be (in some cases) related to a RNA packaging defect.

The locations of the basic residues that led to defects in RNA packaging help to provide some mechanistic insights into potential viral RNA-protein interactions involved in genome recognition. Among the NC mutants, the residues that lie within the first and second zinc-finger domains, including R35
and R60, had a significant impact on RNA packaging. Similar residues in the HIV-1 NC zinc finger have been implicated in interactions with SL3 RNA and have been shown important for RNA packaging by mutagenesis studies (9). The basic residues in the BLV MA that caused the greatest defects in RNA packaging (i.e., K41 and H45) are located in alpha-helix B (28). Presently, no structural data is available regarding the potential viral RNA-MA protein interactions. However, it has been previously reported that BLV MA specifically interacts with the viral RNA in a region (18, 19) that spans the location of SL1 and SL2 (26). This suggests that the contacts made by BLV MA with the viral RNA may also involve an interaction with SL1 and/or SL2. Interestingly, the HIV-1 MA protein has been found to bind to the viral RNA in the pol gene, via the basic amino acids in MA, and virus mutants that disrupted this RNA-protein interaction had delayed replication kinetics though no role in RNA packaging was reported (34). The BLV MA mutants in this study that influenced RNA packaging do not overlap with the known RNA sequence of gag known to be involved in RNA packaging (25, 26). This indicates that the mutations introduced into the VLP vector influence RNA packaging by the amino acid change in MA and not the nucleotide changes in the RNA. This conclusion is supported by the data in Figure 3, which shows the importance of charged amino acid residues (and not just nucleotide changes) in RNA packaging.

The magnitude of the defects in RNA packaging described here in this study for the most extreme MA and NC mutants (i.e., a MA/NC double mutant) are in the range of those observed previously when SL1 or SL2 was mutated (24,
26). Taken together, these observations suggest that residues in both MA and NC make direct contacts with the packaging signal or perhaps adjacent sequences that would influence genome recognition. The interaction of NC with RNA sequences outside of those RNA secondary structures of the RNA packaging signal has been shown directly or implicated with murine leukemia virus and spleen necrosis virus, and are believed to play an important role in RNA packaging (3, 10).

The data in this report provides evidence for a role of both the MA and NC domains of Gag in BLV RNA packaging. This observation implies that distinct viral RNA-protein interactions occur in BLV genome recognition and RNA packaging to that of other retroviruses. Further studies will determine the precise RNA-protein interactions involved in BLV RNA packaging, the location in the cell where genome recognition occurs, and whether both MA and NC play a role in RNA packaging of other deltaretroviruses.

References


CHAPTER 4

BOTH THE PPPY AND PTAP MOTIFS ARE INVOLVED IN HUMAN T-CELL LEUKEMIA VIRUS TYPE 1 PARTICLE RELEASE

Abstract

In retroviruses, the late (L) domain has been defined as a conserved motif in the Gag polyprotein precursor that when mutated leads to the emergence of virus particles that fail to pinch off from the plasma membrane. These domains have been observed to contain the PPXY, PTAP or YXXL motifs. The deltaretroviruses, which include bovine leukemia virus (BLV) and human T-cell leukemia virus types 1 and 2 (HTLV-1 and HTLV-2), have a conserved PPPY motif in the C-terminal region of the matrix (MA) domain of Gag, while HTLV-1 also encodes a PTAP motif in MA. In this study, we analyzed the roles of the PPPY and PTAP motifs in the C-terminus of MA in HTLV-1 particle release. Mutation of either motif (i.e., PPPY changed to APPY or PTAP changed to PTRP) reduced budding efficiencies. Particle buds and electron-dense regions of plasma membrane were observed by electron microscopy. When the locations of PPPY and PTAP were switched, particle release was eliminated. Intriguingly, the replacement of the PTAP motif with either the PPPY or YPDL motifs did not influence the release of virus particles, but the replacement of the PPPY motif with that of either PTAP or YPDL eliminated
particle production. This indicates that the role that PPPY plays in HTLV-1 budding cannot be replaced with either PTAP or YPDL. A similar observation was made with the BLV PPPY motif. Finally, HTLV-1 particle release was found to be sensitive to proteasome inhibitors, implicating a role for ubiquitin in HTLV-1 budding. In summary, our observations indicate that i) the PPPY motif plays a crucial role in virus budding and ii) the PTAP motif plays a more subtle role in HTLV-1 particle release. Each of these motifs may play an important role in virus release from specific cell types and therefore be important in efficient virus spread and transmission.

Introduction

The assembly of retrovirus particles requires the expression of the Gag polyprotein precursor (Gag), which is used as a principal building scaffold for retrovirus assembly and budding from infected cells (40, 46). During or after the process of particle release, the action of the retroviral protease cleaves Gag into mature matrix (MA), capsid (CA), and nucleocapsid (NC) proteins (13, 40, 46). There are three functional assembly domains in Gag (29); each domain is responsible for a separate function in the assembly process: the membrane-binding domain (M domain), the late domain (L domain), and the interaction domain (I domain). The M domain for most retroviruses resides primarily in the MA domain and contains a myristylation signal (3, 37) while the I domain resides in the CA and NC domains of Gag.
L domains are critical for efficient pinching off of the virus particle from the cell membrane. L domains have been identified by extensive mutational analysis in many retroviruses (7, 9, 12, 32, 45, 47, 48, 50). Different retroviruses may utilize different viral proteins and structural motifs to accomplish the same late budding function. Rous sarcoma virus (RSV), murine leukemia virus (MLV), and Mason-Pfizer monkey virus (MPMV) have L domains that consist of a highly conserved PPPY motif as the core sequence that is located near the junction of the MA and CA domains in Gag (5, 29, 44, 47-49). A PPPY motif has been also found in the matrix protein of rhabdoviruses and can function as an L domain (5). In contrast, the L domains of lentiviruses are located at the C terminus of Gag and have distinct core motifs, PTAP in HIV-1 and YXXL in equine infectious anemia virus (EIAV) (9, 32, 50). The retroviral L domains are protein interaction domains, and function by binding to specific cellular proteins that facilitate the late stages of retroviral particle release (42). It has been shown that L domains can be functionally interchanged among several different viruses (29, 32, 49).

Ubiquitin has been found to play a role in virus particle release from infected cells (27, 31, 34, 42). Specifically, cellular factors in the ubiquitin pathway have been shown to interact with L domains (8, 15, 31, 33, 41). The Nedd4-like family of E3 ubiquitin protein ligases (specifically LDI-1) has been identified as the cellular protein that interacts with the PPPY motif (11, 15). TSG101, a putative ubiquitin regulator that is involved in trafficking of endosomal proteins, has been reported to interact with the PTAP motif and therefore be involved in L domain function (6, 8, 41). The EIAV L domain, YPDL, has been
found to bind the medium chain (AP-50) subunit of the AP-2 complex (33). TSG101, and its yeast ortholog Vps23, are members of the class E family of vacuolar protein sorting (Vps) proteins and are involved in the formation of the multivesicular body (MVB)/late endosome, as well as sorting cargo into the MVB/late endosome (1, 19, 36). Recent data suggest that the PTAP, PPPY and YPDL motifs access a common pathway involving class E VPS factors (8, 25).

Proteasome inhibitors, which deplete free ubiquitin in cells, have been found to inhibit release for many different retroviruses (28, 38, 39). In particular, it has been observed that retrovirus budding is reduced by proteasome inhibitors by viruses utilizing either a PPPY- or PTAP-based L domain and that the effect does not depend on the assembly site or the presence of monoubiquitinated Gag in the virion (28). Interestingly, EIAV and mouse mammary tumor virus (MMTV) have been observed to be insensitive to proteasome inhibitors (28, 30).

The deltaretroviruses include bovine leukemia virus (BLV), human T-cell leukemia virus type 1 (HTLV-1), and human T-cell leukemia type 2 (HTLV-2). These viruses replicate to low titers in their natural hosts and are poorly infectious in cell culture. Cocultivation is typically used to infect permissive host cells. Because of these difficulties, information regarding the molecular details of their life cycles, including virus assembly and release, is limited. To date, most studies of deltaretrovirus assembly have been focused on BLV RNA encapsidation, BLV and HTLV-1 Gag myristylation, and the role of basic residues in MA of HTLV-1 Gag membrane localization and virus production (2, 14, 18, 22-24).
Two recent studies have focused on deltaretrovirus particle release. First, the PPPY motif at the C-terminus of MA was found to function as a BLV late domain. Mutations in the PPPY motif caused defects in particle budding characteristic of an L domain defect (43). The PPPY motif is conserved among all deltaretroviruses, including BLV, HTLV-1, and HTLV-2. Intriguingly, HTLV-1 encodes for both the PPPY and PTAP motifs at the C-terminus of MA. Second, the analysis of HTLV-1 PPPY-motif mutants revealed that particle release was abolished (17). An accumulation of Gag was observed at the plasma membrane by electron microscopy, but not the development of virus particle buds. These observations were interpreted to mean that the PPPY mutants caused a late assembly/early budding defect (17).

In this study, site-directed mutagenesis of the PPPY and PTAP motifs was used to study the role(s) of these motifs in HTLV-1 particle release. Mutation of either motif led to reductions of particle release and resulted in virus buds that did not release from the plasma membrane. The function of the PPPY and PTAP motifs were found to be positionally dependent, and PPPY was more sensitive to replacement by other motifs than was PTAP, indicating that PPPY plays a more important role than PTAP in virus budding. The BLV PPPY motif was also sensitive to replacement, extending observation made with HTLV-1 to BLV. HTLV-1 particle release was found to be sensitive to proteasome inhibitors, which suggests that ubiquitin is involved in the process of HTLV-1 particle release.
Material and Methods

Cell culture and transfection

293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco BRL, Gaithersburg, MD) supplemented with 10% Fetal Clone III serum (Hyclone, Logan, UT). MT-2 cells (human T-cell leukemia cells chronically infected with HTLV-1) were maintained in RPMI 1640 (Gibco BRL) supplemented with 10% Fetal Clone III. Transfection of 293T cells was performed by the calcium phosphate precipitation method. Typically, 60 mm petri dishes were used unless otherwise noted. A total of 10 µg of purified plasmid DNA was mixed with 50 ul of 2.5M CaCl2 and 2X HBS buffer and then added to cells that were split the day before transfection and at 50 to 70% confluence. Twelve to 15hr posttransfection, medium was replaced with medium containing 10mM sodium butyrate and 20 mM HEPES were incubated for 8hr at 37 C., then replaced with fresh DMEM containing 20 mM HEPES. The proteasome inhibitors MG-132 (also called zLLL) and clasto-lactacystin β-lactone were obtained from Boston Biochem (Cambridge, MA) and were dissolved in DMSO and used at a final concentration of 10 µM.

Plasmids, mutagenesis and molecular cloning

The HTLV-1 gag gene was cloned into the pMH vector (Roche, Indianapolis, IN) to construct pMH-Gag. Substitutions within PPPY or PTAP motifs were introduced into pMH-Gag vector by using the QuickChange XL kit (Stratagene) as previously described (43). The mutagenic oligonucleotides used in this study are shown in Table 1. The plasmid pCMV-HT1, graciously provided
by David Derse (NCI, Frederick), was derived from an infectious molecular clone of HTLV-1 where the 5' LTR was replaced with a CMV immediate-early promoter joined to a small fragment from the R region of the LTR which contains the major splice donor site (4). Plasmid DNAs containing the HTLV-1 proviral sequence can readily be deleted during amplification in E.coli. To limit the likelihood of deletions from occurring during plasmid amplification, pCMV-HT1 was introduced into the SCS1 strain of E.coli by transformation and plated onto Luria-Betrani agar plates containing ampicillin and incubating at 37°C for 18 h. The smaller bacterial colonies formed on plates were randomly picked with sterile toothpicks and used to inoculate 250 ml of Superbroth, grown overnight and then purified using a commercially available kit (Qiagen). To introduce the mutations into pCMV-HT1 expressing vector, a DraIII-Nhel fragment (~ 366bp) from pMH-Gag mutant constructs was cloned into the pCMV-HT1 WT vector. Nucleotide sequencing was done to verify the presence of the desired mutations. The BLV VLP vector PR+ has been previously described (43). Substitution of the BLV PPPY motif with either PTAP or YPDL was done using the QuickChange XL kit.
<table>
<thead>
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<th>Mutant</th>
<th>Primer +</th>
<th>Mutagenic oligonucleotide -</th>
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<td>APPY-PTAP</td>
<td>5'-CTGATCCAAATCCCTCCCTATGTTG-3'</td>
<td>5'-CAACATAGGGAGGGCGATTTGTTGATCG-3'</td>
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<tr>
<td>PYYY-PPTRP</td>
<td>5'-CCCTATGTTTGACCTCAGGCTCCTCCCT-3'</td>
<td>5'-GAAGACTCTGGAGCAGGGCATGAGCTGTTG-3'</td>
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<tr>
<td>AAAA-PTAP</td>
<td>5'-GTACCAATAATATGAGGGCCCTGCACTAC-3'</td>
<td>5'-ATAGCCTCAACAGCGCGGCTTTGATCG-3'</td>
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<tr>
<td>PYYY-LURL</td>
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<td>5'-GGAAGACTTTTGAAGGCCTTTCCAAAGTCCTTCC-3'</td>
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<td>PTAP-PPPY</td>
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<td>5'-GGAGGAGGCACTGGTATGCTTCCAAACGTGAC-3'</td>
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<tr>
<td>PTAP-PTAP</td>
<td>5'-GGAAGGACTCTGGAGGAGGGCATGAGCTGTTG-3'</td>
<td>5'-GCGGACAGTTGAAGGCCTTTCCAAACGTGAC-3'</td>
</tr>
<tr>
<td>YPDL-PTAP</td>
<td>5'-CCCTATGTTGAGCCTTACGCGCCCCCAAGTCCTTCC-3'</td>
<td>5'-GGCGGGGGGTGAGCCTTTCCAAACGTGAC-3'</td>
</tr>
<tr>
<td>PYYY-PYY</td>
<td>5'-CCCTATGTTGAGCCTTACGCGCCCCCAAGTCCTTCC-3'</td>
<td>5'-GGCGGGGGGTGAGCCTTTCCAAACGTGAC-3'</td>
</tr>
<tr>
<td>PYYY-YPDL</td>
<td>5'-CCCTATGTTGAGCCTTACGCGCCCCCAAGTCCTTCC-3'</td>
<td>5'-GGCGGGGGGTGAGCCTTTCCAAACGTGAC-3'</td>
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<tr>
<td>BLV PTAP</td>
<td>5'-CAGAAGAACAACCAACCACCGCTCTTGACAACCTGCC-3'</td>
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</tr>
<tr>
<td>BLV YPDL</td>
<td>5'-CAGAAGAACAATACCCCGATCTTGACAACCTGCC-3'</td>
<td>5'-GGCGGGGGGTGAGCCTTTCCAAACGTGAC-3'</td>
</tr>
</tbody>
</table>

**TABLE 4.1.** Mutagenic Oligonucleotides used to create PPPY and PTAP mutants

**Immunoprecipitation and Immunoblot analysis**

Methods for preparing cell and VLP lysates have been detailed previously (43). Briefly, 3 days after transfection, cells were lysed in RIPA buffer and immunoprecipitated with anti-HTLV1-p19 monoclonal antibody (Zeptometrix,
Buffalo, NY). Supernatant collected from transfected cells was subjected to ultracentrifugation at 40,000 x g for 1 hr to obtain the VLP pellets. HTLV immunoblot analysis was done using an anti-HTLV1-p19 monoclonal antibody as primary antibody and a horseradish peroxidase-conjugated anti-mouse Ig (Amersham, Arlington Heights, IL) as secondary antibody with the ECL western analysis kit (Amersham, Arlington Heights, IL). BLV immunoblot analysis was done using an anti-BLV CA monoclonal antibody (i.e., BLV3) (VMRD, Pullman, Wash.). The efficiency of VLP production was normalized for cell-associated Gag. Real-time quantitation of band intensities was done using the Quantity One software package with the Chemi Doc 2000 Documentation System (BioRad, Richmond, CA).

**Transmission electron microscopy**

Transmission electron microscopy (TEM) was performed as previously described (43). For thin sectioning, transiently transfected 293T cell pellets were fixed with 2.5% glutaraldehyde. After dehydration in a grade series of cold ethanol, the samples were embedded in Epon 812 resin. Ultrathin sections (90nm) were then stained with uranyl acetate. The stained sections were observed with a Philips CM 12 electron microscope.

**Confocal microscopy**

Transfected cells were grown on coverslips and fixed with 4% paraformaldehyde and permeabilized with Triton X-100, both diluted in phosphate-buffered saline (PBS). The cells were then incubated with either anti-HTLV-1 p24 polyclonal antibody (Zeptometrix, Buffalo, NY) followed by
incubation with Alexa Fluor 568-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR) (for HTLV-1) or incubated with anti-BLV p24 monoclonal antibody (VMRD, Pullman, WA) by incubation with Alexa Fluor 488-conjugated anti-mouse IgG (Molecular Probes) (for BLV). Images were collected with a Biorad MRC 600 or a Zeiss 510 META Laser Scanning confocal microscope.

**Membrane-binding assay**

The membrane-binding assay used in this study was modified from a previously published protocol (26). HeLa cells transfected with either wt or mutant VLP vectors were collected two days posttransfection and resuspended in a 10 mM Tris-HCl (pH 7.5), 4 mM EDTA solution containing a complete protease inhibitor cocktail. The cell suspensions were sonicated and spun at 2,000 x g for 3 min to obtain postnuclear supernatants. The supernatants were then mixed with 85.5% (wt/vol) sucrose and placed on the bottom of a centrifuge tube. On top of this postnuclear supernatant-sucrose mixture was layered 65% (wt/vol) sucrose, and then 10% (wt/vol) sucrose. The gradients were centrifuged at 33,200 rpm for 16 h in a Beckman SW60 rotor. Nine fractions were collected from the top of the centrifuge tube. Fractionated samples were analyzed by immuniprecipitation and Western blotting.
Results

Both the PPPY and the PTAP motifs function in HTLV-1 particle release

Unlike BLV and HTLV-2, HTLV-1 contains both PPPY and PTAP motifs at the C-terminus of MA. Both motifs have been individually identified as late domains for many retroviruses (for review see (7)) and in BLV we have shown that the PPPY motif functions in particle release (43). We hypothesized that both PPPY and PTAP motifs located at the C-terminus of the HTLV-1 MA may function in the budding process. To test this hypothesis, mutations were introduced into the gag gene of pCMV-HT1 (Fig. 4.1). To test whether the PPPY and PTAP motifs individually functioned in HTLV-1 particle release, each motif was first mutated separately. In the APPY-PTAP and AAAA-PTAP mutants, the PPPY motif was mutated and the PTAP motif remained intact. In the mutants PPPY-PTRP or PPPY-LIRL, the PTAP motif was mutated while PPPY motif remained intact.
Figure 4.1. Mutants constructed to test the role of the PPPY and PTAP motifs in HTLV-1 particle release. The wt and mutant amino acid sequences are shown for the C-terminus of the HTLV-1 MA domain of Gag. The wt and mutated PPPY and PTAP motifs are indicated by bold letters and are boxed in the wt.

Immunoblot analysis was used to measure reductions in virus particle release. To determine the linear range of detection for the assay, a protein dilution series was analyzed. Based upon this analysis, the linear range of detect for the assay is at least 16-fold (Fig. 4.2A). To ascertain how these mutations
would influence particle release, mutants were transfected into 293T cells in parallel with the parental vector. Gag expression in cells was analyzed by IP-immunoblot analysis and VLP production was analyzed by immunoblot analysis (Fig. 4.2B). In general, only Pr46Gag was readily observed from cells while only p19 was readily observed from VLP’s. A single mutation of the PPPY motif with an intact PTAP motif (i.e., APPY-PTAP) reduced particle production to about 30% that of the parental vector, while the mutant with an intact PPPY and a single mutation in the PTAP motif (i.e., PPPY-PTRP) reduced particle production to about 60% that of the parental (Fig. 4.2C). The phenotype of the PPPY-PTRP mutant is interesting because an HIV-1 mutant with the same substitution in PTAP led to a dramatic reduction in particle production (~ 5% of wt) (12). Mutating all four residues in the PPPY motif while maintaining an intact PTAP motif (i.e., AAAA-PTAP) eliminated VLP release. The mutant with the intact PPPY motif and all four residues of the PTAP motif mutated (i.e., PPPY-LIRL) was also found to produce no detectable VLPs. These observations indicate that each motif plays a role in HTLV-1 particle release and that they are not redundant in function.
Figure 4.2. Both the PPPY and PTAP motifs influence HTLV-1 particle release.  
A. Linear range of detection for immunoblot analysis. Protein was diluted (1:1, 1:2, 1:4, 1:5, 1:8, 1:10, and 1:16) and was subjected to immunoblot analysis. The band intensity (arbitrary units) for each dilution was determined with the Quantity One software package of the Chemi Doc 2000 Documentation System (Bio-Rad) and plotted against the amount of protein used for immunoblot analysis (16, 8, 4, 3.2, 2, 1.6, or 1 µl) to determine if protein detection within the dilution range was linear. 
B. Analysis of PPPY and PTAP mutants. 293T cells were transfected with wt or derivatives. Fourty-eight hours posttransfection equal volumes of supernatant medium from each culture were collected and VLPs were concentrated by ultracentrifugation. VLP production was analyzed by immunoblot analysis using an anti-HTLV-1 p19 antibody. Cell-associated material was immunoprecipitated with anti-HTLV-1 p19 prior to immunoblot analysis (see Materials and Methods). The positions of Pr46Gag and p19 (MA) are indicated. Quantitation of band intensities was determined by real-time acquisition of signals. Representative data (panel A) from at least three independent experiments is shown in panel A. Summarized data from these experiments is shown in panel B.
A.  

\[ y = 209.24x + 2043.1 \]  

\[ R^2 = 0.9873 \]

B.  

- **Cell**  
  - WT  
  - APPY, PTAP  
  - PPPY, PTRP  

- **VLP**  
  - WT  
  - AAAA, PTAP  
  - PPPY, LIRL  

- **Gag**  
- **p19**
C.

% VLP release

Mutant

WT  APPY-PTAP  PPPY-PTRP  AAAA-PTAP  PPPY-LIRL
To determine whether nascent buds accumulated on the plasma membrane, electron microscopy of cells transiently transfected with each of the mutants was done. When the parental vector was used, released particles with mature cores were readily observed (Fig. 4.3, panels A-B). Interestingly, when cells producing the APPY-PTAP mutant were analyzed, distinct phenotypes were observed along the cell surface. In particular, electron microscopy revealed the appearance of either virus particles that were in the process of pinching off from the plasma membrane but did not release, or electron-dense regions of plasma membrane that were in the early stages of forming virus particle buds (Fig. 4.3, panels C-D). Similar observations were made for the PPPY-PTRP mutant (Fig. 4.3, panels E-F). In addition, released particles were observed for both the APPY-PTAP and the PPPY-PTRP mutants. The analysis of the AAAA-PTAP and PPPY-LIRL mutants also led to interesting observations. For the AAAA-PTAP mutant, both particles in the process of pinching off from the plasma membrane and electron-dense regions of plasma membrane forming virus particle buds were observed (Fig. 4.3, panels G-K). However, the PPPY-LIRL mutant did not reveal either particle buds that had not released or regions of plasma membrane where particle buds were forming (data not shown). This observation with the PPPY-LIRL mutant suggests that the complete mutation of the PTAP motif may have resulted in an assembly defect and/or a Gag membrane targeting defect perhaps by creating a conformational change in Gag. No released particles were observed for either the AAAA-PTAP and PPPY-LIRL
mutants. Taken together, these data provide evidence for the hypothesis that both the PPPY and PTAP motifs function in virus particle release and mutation of either motif appeared to arrest VLPs at early or late stages in the budding process.

Figure 4. 3. Electron microscopy of 293T cells expressing HTLV-1 PPPY and PTAP mutants. Transiently transfected 293T cell pellets were fixed with 2.5% glutaraldehyde. After dehydration in a grade series of cold ethanol, samples were embedded, and ultrathin sections were then stained with uranyl acetate and viewed. Panels A, B – parental wt HTLV-1; Panels C, D – APPY-PTAP; Panels E, F – PPPY-PTRP; Panels G, H, I, J, and K – AAAA-PTAP. Arrows point to budding particles. Scale, 100 nm.
Influence of PPPY and PTAP motif location for function in HTLV-1 particle release

Analysis of the amino acid sequence of the HTLV-1 MA reveals that the PTAP motif lies closer to the C-terminus than PPPY (Fig. 4.1). To determine whether the specific location of the PPPY and PTAP motifs in relationship to one another was important for function in particle release, a mutant was created in which their location was switched (PTAP-PPPYY, Fig. 4.1). This mutant was then transiently transfected into 293T cells and analyzed. Comparable levels of Gag were detected in cells expressing the mutant to that of the parental vector. However, no VLP production was detected for the mutant, which was likely due to a block in the particle assembly pathway (Fig. 4.4A, D). This observation indicates that the function of the PPPY and PTAP motifs in particle release may be positionally dependent. An alternative explanation is that the defect could be due to a conformational defect in Gag. However, no defects were observed in Gag-membrane binding of the PTAP-PPPYY mutant compared to wt (Fig. 4.4E), indicating that the introduced mutations did not interfere with the ability of Gag to bind membrane. Also, the cellular distribution of this Gag mutant was similar to that of wt (see below).
Figure 4. Switching location and replacing PPPY and PTAP motifs influence HTLV-1 particle release. (A through C) Analysis of VLP production. 293T cells were transfected with wt or mutant constructs, and VLP and cell-associated Gag was analyzed as in Fig. 2. The positions of Pr46Gag and p19 (MA) are indicated. Quantitation of band intensities was determined by real-time acquisition of signals. Representative data from at least three independent experiments are shown. (D) Summarized data from panels A through C. (E) Membrane-binding assay. Parental wt and PTAP-PPPY mutant were analyzed as described in Materials and Methods.
A. & B. 

WT PPPY-PPPY PPPY-YPDL 

Mutant 

Cell 

Gag 

VLP 

p19 

C. 

WT PTAP-PPPY PTAP-PTAP YPDL-PTAP 

D. 

% VLP release 

WT PTAP-PPPY PTAP-PTAP YPDL-PTAP PPPY-PPP YPDL 

Mutant 

123
E. Membrane-bound

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<th>Fraction No.</th>
<th>Membrane-bound</th>
<th>Non-membrane-bound</th>
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WT

PTAP-PPP

Gag

Fraction No.
The PTAP motif, but not the PPPY motif, can be functionally replaced in HTLV-1 particle release

To determine whether the PPPY motif and/or the PTAP motif could be functionally replaced either by each other or with the YPDL motif, additional constructs were created (Fig. 4.1B). First, to test whether the PPPY motif could be functionally exchanged, it was swapped with either PTAP or with YPDL (i.e., the EIAV L domain motif) creating the mutants PTAP-PTAP and YPDL-PTAP, respectively. Each construct expressed wt levels of Gag in cells, but did not produce VLPs (Fig. 4.4, panels B and D). This indicates that the PPPY motif cannot be functionally replaced with either the PTAP or YPDL motifs. Second, we tested whether the PTAP motif could be functionally exchanged by creating the mutants PPPY-PPPY and PPPY-YPDL, where the PTAP motif was changed to either the PPPY motif or the YPDL motif, respectively (Fig. 4.1B). Levels of Gag detected in cells expressing the mutants were similar to that of the parental VLP vector (Fig.4.4C). Intriguingly, each of the mutants led to VLP production that was close (PPPY-PPPY) or comparable (PPPY-YPDL) to that of the parental vector (Fig. 4.4, panels C and D). This observation indicates that the PTAP motif can be functionally exchanged with either the PPPY motif or the YPDL. These data also indicate that the PPPY motif is crucial for virus particle budding, while the function of PTAP can be replaced by other motifs.
Cellular distribution of Gag for mutants with defects in HTLV-1 particle release

Several of the mutants analyzed in this study eliminated VLP production as determined by IP-immunoblot analysis. In one instance, with the PPPY-LIRL mutant, there was a suggestion of a defect in Gag distribution in cells because of the inability by electron microscopy to readily detect VLP buds or regions of plasma membrane that were in the process of forming virus buds. To further analyze the nature of why some of the mutants created in this study did not produce VLPs, we analyzed the cellular distribution of Gag in cells transiently transfected with each mutant vector by confocal microscopy. The main observation made from this analysis is that the cellular localization for the mutants was comparable to that seen with wt. In particular, a distinctive punctate staining pattern was observed throughout the cytoplasm of cells (Fig. 4.5). This punctate staining has been previously observed for HTLV-1 Gag (16). Such a staining pattern throughout the cytoplasm was also observed with the analysis of MVB localization using antibodies directed against LAMP-1 and CD63 (data not shown). This suggests a colocalization of HTLV-1 Gag with the MVB.
Figure 4.5. Cellular localization of Gag in cells. Transfected cells were grown on coverslips, fixed with 4% paraformaldehyde, and permeabilized with Triton X-100. Cells were then incubated with an anti-HTLV-1 p24 polyclonal antibody followed by incubation with Alexa Fluor 568-conjugated goat anti-mouse IgG. Images were obtained with a confocal microscope. Panel A – parental wt vector, Panel B – APPY-PTAP, Panel C – PPPY-PTRP, Panel D – AAAA-PTAP, Panel E – APPY-LIRL, Panel F – PTAP-PPPY, Panel G – PPPY-PPPYP, Panel H – PPPY-YPDL, Panel I – PTAP-PTAP, Panel J – YPDL-PTAP.

The BLV PPPY motif is crucial for virus particle release

The data presented in Fig. 4B indicate that when the HTLV-1 PPPY motif was replaced with that of either PTAP or YPDL, particle release was eliminated. Since BLV also contains a PPPY motif that was previously shown to function as
an L domain (43), we next tested whether replacement of PPPY with either PTAP or YPDL would interfere with BLV particle release. As shown in Fig. 4.6A, mutation of the BLV PPPY to either PTAP or YPDL eliminated virus particle release, similar to that observed for the HTLV-1 PPPY motif. Microscopy analysis indicated that the BLV PTAP and YPDL mutants did not alter Gag localization compared to wt (Fig. 4.6B). Together, these data indicate that the PTAP and YPDL motifs cannot replace the PPPY motif in BLV L domain function.
Figure 4. BLV PPPY motif is crucial for virus particle release. A. VLP production of BLV PPPY mutants. 293T cells were transfected with wt or derivatives. Forty-eight hours posttransfection equal volumes of supernatant medium from each culture were collected and VLPs were concentrated by ultracentrifugation. VLP production was analyzed by immunoblot analysis using an anti-BLV CA antibodies. The positions Gag and p24 (CA) are indicated. Representative data from at least three independent experiments is shown in panel A. The PTAP mutant has the PPPY motif replaced with PTAP, and the YPDL mutant has the PPPY motif replaced with YPDL. B. Cellular localization of BLV Gag mutants. Transfected cells were grown on coverslips, fixed, and permeabilized. Cells were then incubated with an anti-BLV p24 antibody followed by incubation with Alexa Fluor 488-conjugated anti-mouse IgG. Images were obtained with a confocal microscope.
Proteasome inhibitors decrease the efficiency of HTLV-1 particle release

Proteasome inhibitors have been shown to inhibit particle release for some retroviruses (encoding either a PPPY or PTAP motif), but not for others (encoding a YPDL motif). Since HTLV-1 MA contains both the PPPY and PTAP motifs, we wanted to investigate whether the two motifs together would influence the susceptibility of HTLV-1 particle release to proteasome inhibitors. To test this, MT-2 cells (human T-cell leukemia cells chronically infected with HTLV-1) were treated with 10 µM lactacystin for 11 hr, then cells and virus particles were recovered, lysed, and analyzed by immunoblot analysis. Compared to MT-2 cells not treated with proteasome inhibitors (DMSO only), lactacystin-treated cells led to reductions in the level of virus particle production (Fig. 4.7). Treatment with lactacystin led to a fourfold reduction to that from virus released from untreated cells. This fourfold reduction is within the range reported for MPMV, HIV-1 and MLV (28). Interestingly, a comparison of the ratio of p19 to total Gag protein expressed in cells for untreated and proteasome inhibitor-treated cells indicated that the inhibitor led to a mild reduction (~30% less that untreated cells) in Pr46Gag processing. MG-132, another proteasome inhibitor led to mild reductions in virus release (data not shown). Overall, these observations indicate that HTLV-1 particle release is sensitive to proteasome inhibitors.
Figure 4. HTLV-1 particle release is sensitive to proteasome inhibitors. MT-2 cells (human T-cell leukemia cells chronically infected with HTLV-1) were treated with 10 µM clasto-lactacystin β-lactone for 11h, then virus and cell-associated Gag was analyzed by immunoblot analysis. The lane marked DMSO indicates cells treated with DMSO only and no proteasome inhibitor. Representative data is shown in panel A. Summarized data from three independent experiments is shown in panel B.

Discussion

This study reports the analysis of a panel of mutants created to determine whether the PPPY and PTAP motifs in the carboxy-terminus of the MA domain of Gag function in HTLV-1 particle release. We observed that: 1) mutations into
either the PPPY or PTAP motifs reduced the budding efficiency of HTLV-1 particles; 2) the function of the PPPY and PTAP motifs was not maintained when their positions were switched; 3) the replacement of the PTAP motif with either the PPPY or YPDL motifs did not influence virus particle release, but the replacement of the PPPY motif with that of either PTAP or YPDL eliminated particle release; 4) replacement of the BLV PPPY with either PTAP or YPDL also eliminated particle release; and 5) HTLV-1 particle release was found to be sensitive to proteasome inhibitors, which is consistent with a role for ubiquitin in HTLV-1 budding.

A recent study reported that mutations of the HTLV-1 PPPY motif (i.e., LPPY, PLPY, PPLY, and PPPD) abolished particle release from 293-TSA cells (17), which is similar to what we have reported in our study with one of the mutants we had analyzed in 293T cells (i.e., AAAA-PTAP). In contrast, another PPPY mutant that we analyzed in our study (i.e., APPY-PTAP) reduced but did not eliminate virus particle release. The authors of the previous study found an accumulation of Gag in cells and by electron microscopy found an accumulation of electron-dense material at the plasma membrane but not the formation of virus buds and concluded that the PPPY mutants that they analyzed caused a late assembly/early budding defect. We observed by electron microscopy the accumulation of Gag at the plasma membrane, but also found virus buds that had not released from the plasma membrane (both for the AAAA-PTAP and the APPY-PTAP mutants). In total, our PPPY mutants display some similarities to those in the previous study with the HTLV-1 PPPY motif but have phenotypic
differences. These differences could be due in part to the different cells and the
different virus constructs used. However, our observations provide evidence
which strongly supports the conclusion that the PPPY motif functions in virus
particle release and when mutated can cause L domain defects similar to those
observed with other retroviruses.

We observed in this study for the first time that the function of the PPPY
and PTAP motifs was ablated when the locations of the motifs was swapped in
HTLV-1 Gag, as a mutant with the motifs switched in location eliminated particle
release. Previous studies have shown that L domains (consisting of single
motifs) could function when located in distal positions in Gag, indicating that the L
domain could function independently of its position (29, 32). In contrast, a more
recent study with EIAV found evidence for positional dependence (in virus
replication) when the YPDL motif in the p9 protein of Gag was replaced by YPDL
or PTAP at the C terminus of the MA domain in Gag (20). It was suggested by
these authors that full functionality of the EIAV L domain in virus replication was
dependent on its location in the context of the p9 protein of Gag, which may
suggest the influence of other p9 sequences on L domain functions.

Previous studies have indicated the interchangeable nature of the EIAV
YPDL motif, the RSV PPPY motif, and the HIV-1 PTAP motif in L domain
function (20, 29, 32). It has been suggested that these observations may
indicate that diverse motifs can utilize different entry points to common cellular
machinery for viral budding and release from the plasma membrane (20). In our
study, we found that the replacement of the PTAP motif with either the PPPY or
YPDL motifs did not influence the release of virus particles, indicating that PTAP was functionally interchangeable with either PPPY or YPDL. In contrast, we also observed that the replacement of the PPPY motif with either PTAP or YPDL eliminated the detection of released particles. These data indicate that the PPPY motif was not interchangeable in function with either the PTAP or YPDL motifs. We interpret these findings as: 1) PPPY is crucial for HTLV-1 particle release and 2) PTAP has a less prominent role in HTLV-1 particle release. We further observed that the BLV PPPY motif could not be replaced with either PTAP or YPDL, which extends the observations made with BLV to other deltaretroviruses. The overlapping Ebola virus VP40 protein PTAP and PPEY motifs have recently been reported to function independently as late budding domains (21). More recently, it was shown that both the MPMV PPPY and PSAP motifs contribute to virus release, but that PSAP requires an intact PPPY motif for function (10). Taken together, these data suggest that the PPPY and PTAP motifs play distinct roles in HTLV-1 particle release. These observations also suggest that the functional differences in the PPPY and PTAP motifs will likely provide important clues for their function in HTLV-1 particle release, in particular, and in retrovirus particle release, in general.

The punctate staining pattern of Gag seen with wt or mutants that produced VLP’s suggests colocalization with the MVB. A previous study with HTLV-1 suggested that this may not occur (16). However, the potential colocalization of HTLV-1 Gag with the MVB raises intriguing possibilities regarding HTLV-1 budding. For example, particle budding may occur in the MVB
and then particles released from cells by exocytosis. Second, localization of Gag with the MVB/late endosome would allow for the sorting of Gag into the lumen. Budding of HIV-1 particles into the lumen of the MVB has been observed in macrophages and not from the plasma membrane (35). This indicates that HIV-1 particle release can occur via different modes in different cell types. Such possibilities have not been extensively investigated with HTLV-1.

We observed that HTLV-1 particle release was sensitive to a proteasome inhibitor. Based upon previous reports with other retroviruses that encode the PPPY or PTAP motifs, this may have been expected (28, 38). However, we have found that a proteasome inhibitor can influence virus budding and release of a retrovirus containing both the PPPY and PTAP motifs. The sensitivity of HTLV-1 to proteosome inhibitors strongly suggests a role for ubiquitin in HTLV-1 particle release.

In summary, we have described mutants that have allowed for the initial characterization of the PPPY and PTAP motifs and their roles in HTLV-1 particle release. Our observations indicate that both motifs are important for HTLV-1 budding. These mutants will be extremely useful for future studies directed at understanding the cellular proteins and pathways involved in virus particle release. Furthermore, the analysis of HTLV-1 particle release should provide new insight into the specific functions of the PPPY and PTAP motifs in retrovirus budding and release. We speculate that each motif plays an important role in virus release from specific cell types, and are ultimately important in efficient virus spread and transmission. A greater understanding of how these viruses
release from cells will likely provide new targets for antiretroviral drug development.

References


CHAPTER 5

BUDDING OF HTLV-1 VIRUS PARTICLES INTO MULTI-VESICULAR BODIES

Abstract

The human T-cell leukemia virus type 1 (HTLV-1) Gag polyprotein is thought to be directly targeted to the plasma membrane for particle assembly and release. The molecular mechanism by which HTLV-1 Gag is targeted to the plasma membrane is poorly understood. Myristylation has been shown to be essential for Gag targeting to the plasma membrane. We and others have previously identified that both the PPPY and PTAP motifs are involved in HTLV-1 particle release with PTAP plays a more subtle role in virus release. These L domains function through the interaction with host cellular proteins normally involved in multi-vesicular body (MVB) morphogenesis. In this study, we demonstrate that HTLV-1 particle assembly can also take place on the limiting membrane of multi-vesicular bodies (MVBs). In HeLa cells, the plasma membrane pathway rather than the MVB pathway appears to be the primary pathway for HTLV-1 assembly. However, disruption of the PTAP motif led to a defect in the direct targeting of Gag to the plasma membrane. Mutant Gag proteins were redirected to a CD63-positive MVBs for assembly. We further
observed that the accumulation of wild-type or mutant Gag in MVBs is a result of direct assembly and budding inside MVB rather than an uptake of endocytic particles. These results indicate that in addition to its role in virus budding, the PTAP motif is also important for targeting HTLV-1 Gag to the plasma membrane. The intracellular transport of these particle-containing MVBs utilizes RILP-regulated dynein-dynactin motor complex, which is analogous to the transport of exosomes. Treatment of cells with PI3K inhibitors resulted in the restoration of plasma membrane targeting pathway of PTAP mutant Gag, suggesting that phosphoinositide PtdIns(3)P on MVBs is likely to be the Gag receptor on MVBs. These observations implicated distinct receptors for Gag targeting directly to the plasma membrane or to MVBs.

**Introduction**

Type C retroviruses are thought to initiate viral assembly and budding at the plasma membrane where Gag proteins associate with raft microdomains of the inner leaflet of the plasma membrane (14, 39). The Gag polyprotein is the only driving force for assembly and budding. Expression of Gag alone can induce the formation and release of virus-like particles. The targeting of Gag proteins to the cell membrane and stable membrane association are accomplished using a bipartite membrane binding signal consisting of the fatty acid myristate, added cotranslationally to the N terminus of Gag in concert with a patch of basic residues within MA domain (7, 35). Myristate provides a hydrophobic interaction
with the lipid membrane, while the basic residues are believed to strengthen the interaction by forming the electrostatic interactions with acidic phospholipids that are enriched at the cytoplasmic face of the plasma membrane (29, 45).

The final release of the nascent virions from the cell is mediated by late domains that function by recruiting the intracellular machinery, ESCRT complexes, to the site of assembly at the plasma membrane (12, 14, 24, 41). These ESCRT complexes are normally involved in multivesicular body (MVB) biogenesis (1, 2). The formation of MVBs, a late endosomal compartment, is characterized by the invagination of limiting membranes to bud small vesicles. Topologically, both virus budding at the plasma membrane and the vesicle budding into MVBs share striking similarities: they occur away from the cytosol, and they involve lipid microdomains, inositol derivatives and a particular cohort of proteins such as Tsg101 and ubiquitin.

We and others have investigated the role of late domains in HTLV-1 budding at the plasma membrane (6, 20, 42). Both PPPY and PTAP motifs at the N-terminus of MA domain have been demonstrated to be involved in budding of HTLV-1 virus from the plasma membrane. These two late domains function through the interaction with the Nedd4 family protein, belonging to an E3 ubiquitin ligase, and the Tsg101 protein, an E2-like ubiquitin ligase, respectively (6). The recruitment of these cellular proteins plays a key role in the efficiency of HTLV-1 virus budding and release. PPPY motif was found to play a more important role than PTAP since the PTAP motif can be replaced by other late
domains (PPPY and YPDL), but PPPY is not replaceable. However, it remains unclear why HTLV-1 needs two late domains whereas most retroviruses only require one. Also it is not known precisely what the different roles these two motifs play in HTLV-1 assembly and release.

Despite the view that type C retroviruses recruit the cellular machinery normally responsible for MVB formation to the plasma membrane for their assembly and budding, observations made a decade ago suggested that in HIV-infected macrophages virus particles accumulate in an intracellular, vacuole-like compartment (28). A number of recent reports have identified the vacuole-like compartment as CD63-positive MVB, suggesting HIV-1 may also be assembled directly at the site where this machinery is usually active, that is in MVB (25, 30, 31). Such observations were further extended to standard tissue culture cell lines previously believed to only support budding at the plasma membrane (25, 26, 36). The intracellularly assembled HIV-1 particles are likely released into the extracellular environment through the fusion of virus-containing compartments directly with the cell surface in an exocytic fashion (25, 26, 30, 31, 36). Such exosome-assisted release of proteins is best documented for the major histocompatibility complex class II (MHC class II) (32, 44). It is unclear how Gag polyprotein is targeted to MVB. Different cellular binding partners for Gag may be existent at the plasma membrane and the MVB.

To date, the use of the MVB pathway for budding has mainly been reported for HIV. Here, we have investigated the possibility that HTLV-1 directly
assembles and buds into MVBs in HeLa cells. We found that although the plasma membrane is the major site for HTLV-1 assembly and release, accumulation of HTLV-1 particles inside intracellular vacuoles was frequently observed. We demonstrated these intracellular vacuoles have characteristics similar to those described for MVBs. Interestingly, when PTAP is mutated, a large number of viral particles were found to accumulate inside MVBs rather than on the plasma membrane. Using confocal analysis and immunoelectron microscopy, we excluded the possibility that the accumulation of these HTLV-1 particles inside MVBs is the result of endocytosis of extracellular HTLV-1 particles. Thus, mutation of the PTAP motif redirected HTLV-1 Gag to MVBs rather than to the plasma membrane for assembly and release. This result suggests that in addition to its role in the virus budding process, the PTAP motif also acts as a signal for Gag targeting to the plasma membrane. When the plasma membrane pathway is disrupted, Gag proteins are retargeted to MVBs for assembly. This suggests there might be two different Gag receptors at the plasma membrane and MVBs. Further studies showed that PI3P is likely to be the receptor for Gag on MVB. Unlike HIV, basic residues in the HTLV-1 MA domain were not involved in targeting Gag to the plasma membrane since mutation on these residues did not affect the plasma membrane localization of Gag.

The intracellular traffic of HTLV-1-containing MVBs was further investigated. Our results suggest that the HTLV-1-containing MVBs are
transported along microtubules by Rab 7-regulated dynein-dynactin motor complex.

Material and Methods

Cell culture and transfection

Hela cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO BRL, Gaithersburg, MD) supplemented with 10% Fetal Clone III serum (Hyclone, Logan, UT). Transfection of cells was performed by the calcium phosphate precipitation method. Typically, 60 mm petri dishes were used in this study unless otherwise noted. A total of 10 µg of purified plasmid DNA was mixed with 50 µl 2.5M CaCl2 and 500 µl 2 x HBS buffer and then added to cells that were split the day before transfection and at 50 to 70% confluence. 12 to 15 hr after transfection, media were replaced with medium with 10 mM sodium butyrate and 20 mM HEPES. After 8-hr incubation, media were replaced by fresh DMEM with 20 mM HEPES.

Plasmids

PCMV-HT1 was derived from an infectious molecular clone of HTLV-1 by replacing the 5’ LTR with a CMV immediate early promoter joined to a small fragment from the R region of the LTR which contains the major splice donor site. Eps-DIII-GFP, and Eps-D3Δ2-GFP are kindly provided by Dr. Alexandre Benmerah (Developpement Normal et Pathologique de Systeme Immunitaire,
Paris, France). Dynamintin expression construct was kindly provided by Dr. Jacques Neefjes (Netherlands Cancer Institute, The Netherlands). Mutant constructs of matrix basic residue are a kind gift from Dr. Isabelle Le Blanc (21).

**Antibodies**

The following antibodies were used: mouse monoclonal anti-HTLV-1 p19 (Zeptometrix, Buffalo, NY), goat polyclonal anti-HTLV-1 p24 (Advanced Biotechnologies, Columbia, Maryland), mouse monoclonal anti-CD63 (BD Biosciences), mouse monoclonal anti-Lamp1 (Southern Biotechnology Associates, Inc. Birmingham, AL), mouse monoclonal anti-EEA1 (BD Biosciences), mouse monoclonal anti-GM130 (Transduction Laboratories), mouse monoclonal anti-γ-tublin (Sigma, Saint Louis, Missouri) and mouse monoclonal anti-dynamitin (BD Biosciences). Alexa Fluor 488-conjugated goat anti-mouse IgG, Alexa Fluor 568-conjugated rabbit anti-goat IgG, and Alexa Fluor 647-conjugated goat anti-mouse IgG are purchased from Molecular Probes (Molecular Probes, Eugene, OR). Nanogold conjugated rabbit anti-mouse IgG (NanoProbes Inc., Yaphank, NY) was used for EM immunolabeling.

**Transferrin uptake**

Transferrin uptake assay was performed using Alexa Fluor 546-labeled transferrin (Molecular Probes, Eugene, OR). HeLa cells were incubated with 0.1μM Alexa Fluor 546-labeled transferrin at 37°C for 15 min for binding, then washed and fixed for immunofluorescence analysis.
Wortmannin and LY treatment

24 hours after transfection, HeLa cells were treated with 50 nM Wortmannin or 100 µM LY 294002 for 12 hr. Cells were then washed and fixed for immunostaining.

Confocal microscopy

Cells were grown on coverslips and fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS, quenched in 100 mM glycine/PBS, blocked in PBS containing 3% BSA. The cells were then incubated with various antibodies diluted in the blocking buffers. Cells were washed and stained with fluorescent secondary antibodies, washed extensively, and mounted on slides. Images were collected with a Zeiss 510 confocal microscope using an attached Argon laser (Carl Zeiss, Germany).

Transmission electron microscopy

Transmission electron microscopy (TEM) was performed as before (42). For thin sectioning, transiently transfected HeLa cell pellets were fixed with 2.5% glutaraldehyde. After dehydration in a grade series of cold ethanol, the samples were embedded in Epon 812 resin. Ultrathin sections (90nm) were then stained with uranyl acetate. The stained sections were observed with a Philips CM 12 electron microscope.
EM Immunolabeling

Transfected HeLa cells were fixed by 4% paraformaldehyde in PBS for 20 minutes at room temperature. Fixed were permeabilized and blocked in PBS containing 3% BSA and 0.1% Saponin for 30 minutes. Cells were incubated with primary antibody in the blocking buffer followed by incubation with Nanogold-conjugated rabbit anti-mouse IgG. Cells were post-fixed with 1.6% glutaraldehyde in PBS followed by incubation in silver enhancement solution for 20 minutes. Cells were then postfixed with 1% OsO₄ solution for 30 min before being embedded, dehydrated and sectioned. Thin sections were examined with a Philips CM 12 electron microscope.

Results

HTLV-1 particles can assemble into multi-vesicular bodies in HeLa cells.

To visualize the morphology of the HTLV-1 particles, we transfected the Gag-expressing construct pCMV-HT1 into HeLa cells. Cells were fixed and ultrathin sections were prepared for transmission electron microscopy 48 hr post-transfection. We observed large amounts of HTLV-1 particles budding at the plasma membrane (42). Surprisingly, we also found that the transfected cells appeared to accumulate intracellular compartments containing HTLV-1 virus particles. Similar to the plasma membrane particle, the intracellular particle contains spherical shaped electron-dense core and is of 100-110 nm in diameter, which corresponds to the size of HTLV-1 virus particles (Figure 5.1A).
To identify the nature of the intracellular compartments where the HTLV-1 virus particles were observed, HeLa cells transfected with pCMV-HT1 were prepared for confocal analysis. Forty-eight hr post transfection, Gag proteins were detected primarily on the plasma membrane. However, a very small portion of Gag accumulated in the perinuclear region (Figure 5.1B). To determine whether the intracellular vacuoles into which the HTLV-1 virus particles were budding were MVBs, we co-labeled the transfected Hela cells with antibodies against CD63, a tetraspanin found in late endosomes and MVBs, particularly in the membranes of the internal vesicles. The peri-nuclearly accumulated Gag was found to colocalize with CD63 (Figure 5.1C and 5.1D). Gag was also found to colocalize with another MVB marker LAMP-1 (data not shown). However, the punctate staining pattern observed at the cell surface did not colocalize with CD63.

The accumulation of HTLV-1 virus particles inside the lumen of MVBs could be explained by two possible mechanisms. First, newly assembled virions could enter the lumen of MVBs by a budding process from the cytosol. Alternatively, viral particles could be released by the cells on the plasma membrane and then reinternalized and targeted to these compartments via endocytosis, a phenomenon reported previously for HTLV-1. (33). Further examination of ultrathin sections of preparations of transfected Hela cells revealed immature ring-like particles inside the lumen and the budding profiles of immature particles, such as those depicted in Figure 5.1E, F and G. This
observation supports the hypothesis that Gag protein assembled at the limiting membranes of MVBs.
Figure 5. Accumulation of Intracellular HTLV-1 particles in HeLa cells. HeLa cells transfected with wild-type HTLV-1 Gag-expressing construct were fixed, embedded and examined by transmission electron microscopy. The intracellular compartment containing HTLV-1 particles is shown in A. Arrows in E, F, and G identify assembling (E), budding (F) or immature particles (G). Bars, 100nm. Transfected cells were labeled with anti-p24 (B) and anti-CD63 (C) followed by secondary antibody Alexa Fluor 568 rabbit anti-goat IgG and Alexa Fluor 488 goat anti-mouse IgG. Merged image of Gag and CD63, with colocalization indicated in yellow, are shown in D.
PTAP motif plays a role in targeting Gag to plasma membrane.

Previously we and others (6, 20, 42) have reported both PPPY and PTAP are critical for HTLV-1 budding. Mutations in either motif arrested particle release in an early budding step. However, it appears PTAP plays a less important role than PPPY since mutation of PTAP did not reduce the particle dramatically (60% of wild type level) and it can be replaced by PPPY or YPDL without affecting efficiency of particle production (42). It remains unknown why HTLV-1 possesses two late domain motifs and how these two motifs act differently in HTLV-1 assembly and budding. Here we continued to investigate the function of both motifs in HTLV-1 assembly and release. A close look at the Gag localization pattern was obtained on HeLa cells transfected with the mutants made previously (42). Interestingly, we found that when PPPY is mutated, mutant APPY-PTAP Gag proteins display plasma membrane localization (Figure 5.2B) similar to the wild type Gag (Figure 5.2A). However, when PTAP is mutated, mutants PPPY-PTRP, PPPY-PPPY and PPPY-YPDL display a clear peri-nuclear localization pattern (Figure 5.2C, 5.2D, and 5.2E).
Figure 5.2. Different localization pattern of wild-type and L domain mutant Gag. Wild type (A), PPPY mutant constructs APPY-PTAP (B), or PTAP mutant construct PPPY-PTRP (C), PPPY-PPP (D) and PPPY-YPDL (E) were transfected into HeLa cells. Cells were fixed and stained with anti-p19 antibody and Alexa Fluor 488 goat anti-mouse IgG as detailed in Material and Methods. Gag localization was indicated as green.
To test if Gag from these mutants accumulates inside MVBs, transfected HeLa cells were labeled for MVB marker CD63. An extremely high degree of colocalization of mutant PPPY-PTRP or PPPY-PPPY Gag and CD63 was observed (Figure 5.3A and 5.3D). The mutant Gag also showed perfect colocalization with LAMP-1 (data not shown).

No obvious colocalization was observed between the Gag of PPPY-PTRP or PPPY-PPPY and ER marker (data not shown), Golgi marker GM130 (Figure 5.3B and 3E) and an early endosome marker EEA1 (Figure 5.3C and 5.3F). It is generally believed that the endocytic particles pass through early endosomes before they are transported to MVBs. However, if the particles bud directly into MVBs, early endosome would not be a necessary step. Thus, the fact that Gag and EEA1 accumulated in different compartments lends support to the hypothesis that the particles found inside MVBs are not endocytic virions.
Figure 5.3. PTAP mutant Gag proteins colocalize with marker for the MVB. HeLa cells were transfected with PPPY-PTRP (A, B and C) or PPPY-PPPY (D, E and F). Cells were stained with anti-p24 antibody (left columns) and three organellar markers (middle columns). The markers used were MVB marker CD63 (A and D), Golgi marker anti-GM 130 (B and E) and early endosomal marker anti-EEA1 (C and F). Merged images of Gag and organellar signals, with colocalization indicated in yellow, are shown in the right columns.
Transmission EM revealed a large number of MVBs filled with a high number of virus particles inside (Figure 5.4A) in contrast to very few found in the wild type or APPY-PTAP transfected cells (data not shown). Immunoelectron microscopic analysis was performed to test the origin of these intracellular HTLV-1 particles. Strong labeling with p19 was observed (Figure 5.4B). The membrane of these particles was also labeled with CD63 (Figure 5.4C), suggesting that these particles incorporated CD63 into their membrane as they assembled at the limiting membrane of MVBs.
Figure 5. Intracellular PTAP mutant particles are associated with CD63. (A) Overview of a large amount of intracellular vacuoles (arrowheads) filled with HTLV-1 particles is shown in PPPY-PTRP transfected HeLa cells. The transfected HeLa cells were fixed and incubated with anti-p19 (B) or anti-CD63 (C) antibody followed by incubation with Nanogold-conjugated rabbit anti-mouse IgG. Cells were then postfixed and sectioned for examination with electron microscopy as detailed in Material and Methods. Small arrowheads indicate virus particles incorporated CD63 into their membrane (C). Bars, 100nm.
The possibility of endocytosis was further excluded by examining the effect of overexpression of an endocytic mutant on the distribution of PTAP mutant Gag. The rationale behind this is that if the Gag ended up in the MVBs through the endocytic pathway, blocking of endocytosis will prevent the accumulation of Gag inside MVBs. Eps15 (EGFR pathway substrate clone 15) is a constituent of plasma membrane clathrin-coated pits. Overexpression of a dominant negative mutant of Eps15, Eps-DIII, but not another mutant Eps-D3Δ2, was shown to inhibit the clathrin-dependent endocytosis by inhibiting the clathrin-coated pit assembly (3, 4). The inhibitory effect of Eps-DIII on endocytosis was tested in HeLa cells. HeLa cells were transfected with the Eps-DIII-GFP or Esp-D3Δ2-GFP construct. Transfected cells were incubated with Alexa Fluro-547-labeled transferrin (Tf), a small molecule that is normally taken up into cells through clathrin-mediated endocytosis. Cell were fixed and then subjected to confocal analysis. The distribution of DIII-GFP is mainly cytosolic as reported before (3). The punctate staining of Tf indicates the Tf molecules were taken into cells through endocytosis and located in intracellular vesicles in the cells that did not express DIII-GFP (Figure 5A, right panel, red). However, the cells expressing DIII-GFP (Figure 5.5A, left panel, green) reduces the uptake of Tf dramatically. In contrast, overexpression of Esp-D3Δ2-GFP did not inhibit the endocytosis of Tf into HeLa cells (Figure 5.5B). The construct Eps-DIII-GFP was then used to test the effect of DIII overexpression on the Gag localization. HeLa cells were co-transfected with PPPY-PTRP or PPPY-PPPYP and DIII-GFP. Cells were then labeled for Gag and CD63 and subjected to confocal microscopy analysis. Figure
5.5C depicts the localization of three proteins: DIII-GFP, PPPY-PTRP Gag and CD63. This figure clearly shows that in the cell expressing DIII-GFP (green), Gag still colocalized with CD63 as indicated by the merged pinkish color. Similar observations were made with PPPY-PPPYP Gag (Figure 5.5D). These results indicate that Gag did not reach the MVBs through endocytosis. The accumulation of PTAP mutant particles inside MVBs is a result of direct targeting and assembly of Gag.
Figure 5. Overexpression of endocytic mutant did not affect accumulation of PTAP mutant Gag into MVBs. HeLa cells were transfected with endocytic mutant constructs DIII-GFP (A) or D3Δ2-GFP (B) followed by incubation with transferrin. The uptake of transferrin by the cells was examined by confocal microscopy. The expression of DIII or D3Δ2 is shown in left column (green) and the distribution of transferrin is shown in right column (red). The inhibition of transferrin uptake by DIII was observed. The effect of DIII overexpression on mutant Gag localization is shown in C and D. HeLa cells were co-transfected with DIII-GFP and PPPY-PTRP (C) or PPPY-PPPY (D) and stained with anti-p24 and anti-CD63 antibodies followed by secondary antibody Alexa Fluor 568 rabbit anti-goat IgG and Alexa Fluor 688 goat anti-mouse IgG. Localization of DIII (green), Gag protein (red) and CD63 (blue) were examined by confocal microscopy. Merged images of DIII-GFP, Gag and CD63, with colocalization indicated in pink, are shown in the lower right panel.
The intracellular transport of HTLV-1 MVB particles

We have previously shown that the amount of virus particles produced by PPPY-PPPY and PPPY-YPDL is comparable to wild type (42). Here we have found that the majority of particles accumulated inside MVBs with very few observed to form at the plasma membrane. A legitimate thought is that the MVB particles produced intracellularly by these mutants can be released outside the cells. How are the MVB particles transported to the plasma membrane and released? Many intracellular vesicles are transported along polarized cytoskeletal elements, microtubules, by utilizing molecular motor proteins (15). Kinesin moves towards the fast growing end (or plus end), away from the microtubule organizing center (MTOC), and cytoplasmic dynein moves toward the minus end. In the case of dynein, vesicle binding is thought to be mediated by the interaction of the dynein intermediate chains (ICs) with the 150 KD Glued subunit of a linker complex, dynactin. Overexpression of the 50 kDa ‘dynamitin’ subunit of the dynactin complex was found to dissociate the complex. Under these conditions dynein was released from its cargo sites, resulting in inhibition of minus end-directed vesicle transport. The dynein-dynactin motor complex has been reported to be involved in the intracellular transport of the MIIC-containing MVBs and these MIIC can be released as exosomes (32, 43, 44). To study the role of dynein-dynactin complex in the intracellular transport of MVB particles, dynamintin p50 was overexpressed together with PPPY-PTRP or PPPY-PPPY Gag construct. The effect of p50 on the distribution of Gag was analyzed by confocal microscopy. Overexpression of dynamitin resulted in a major
redistribution of Gag. Without the co-transfection of p50 the Gag was found to accumulate mainly in a perinuclear area around the microtubule organizing center (MTOC) stained by anti-γ-tublin antibody (Figure 5.6A). However, in co-transfected cells, Gag proteins no longer accumulated in the MTOC region but were relocated to the cell peripheral region (Figure 5.6B and C) and still associated with CD63-positive MVBs (data not shown). Apparently, the intracellular transport of these MVB particles is analogous to MIIC transport riding the MVB as a vehicle to reach the plasma membrane.

To further confirm the idea that the transport of HTLV-1 MVB particles depends on the dynein-dynactin motor complex, we examined the effect of overexpression of RILP on Gag localization. RILP is required for recruiting the dynein-dynactin motor proteins to specific membranes (9). RILP expression was shown to induce the recruitment of functional dynein-dynactin motor complexes to Rab7-containing late endosomes and lysosomes. Consequently, these compartments are transported by these motors toward the minus end of microtubules, effectively inhibiting their transport toward the cell periphery (17). HeLa cells were co-transfected with RILP-GFP and PPPY-PTRP or PPPY-PPPY. The localization of Gag protein was examined by confocal analysis. Figure 5.6 D and E demonstrate that RILP-GFP induced a collapse of HTLV-1-containing MVBs. In control cells that did not express RILP-GFP (indicated by yellow asterisk), CD63 positive MVBs were located at some distance from the MTOC. In cells expressing RILP (indicated by white asterisk), CD63 was densely clustered around the MTOC. In cells that co-express both Gag and RILP-GFP proteins, all
three proteins, i.e. Gag, CD63 and RILP-GFP, were found to localize to the same area within a very close distance to the MTOC (This colocalization is indicated by whitish color). These results further support the idea that the traffic of intracellular HTLV-1 particles is associated with MVB and is mediated through dynein-dyncatin complexes which are recruited to the MVBs by RILP.
Figure 5.6. The intracellular transport of HTLV-1 MVB particles using RILP-regulated dynein-dynactin motor complex. (A). Accumulation of particle-containing MVBs in the peri-nuclear MTOC region. HeLa cell were transfected with PPPY-PPPY construct and stained with anti-p24 (left panel) and anti-γ-tublin (middle panel). Arrowhead indicates the location of MTOC. Right panel shows the merged image. The effect of overexpression of dynamintin p50 on Gag localization is shown in B and C. HeLa cells were co-transfected with p50 and mutant Gag construct PPPY-PTRP (B) or PPPY-PPPY (C). Cells were stained with anti-p50 and anti-p24 antibodies and subjected to confocal analysis. P50 is localized diffusively in the cytoplasm (left column, green) is. Localization of Gag protein (middle column, red) is dispersed to the cell peripheral region. The merged images are shown in right column. The effect of overexpression of RILP on Gag distribution is shown in D and E. HeLa cells were co-transfected with RILP-GFP and PPPY-PTRP (D) or PPPY-PPPY (E) constructs. Transfected cells were stained with anti-p24 and anti-CD63 antibodies followed by secondary antibody Alexa Fluor 568 rabbit anti-goat IgG and Alexa Fluor 688 goat anti-mouse IgG. Localization of RILP (green), Gag protein (red) and CD63 (blue) were examined by confocal microscopy. Merged images of RILP-GFP, Gag and CD63, with colocalization indicated in whitish color, are shown in the lower right panel.
Targeting of Gag to MVB is mediated by PI3P

The fact that Gag can assemble at both the plasma membrane and in MVBs implies that Gag polyproteins can be targeted to both places. Two different cellular binding partners for Gag might exist at the different assembly sites and distinct targeting pathways for Gag may also exist. Gag receptors on cellular membranes have never been identified. An in vitro study suggested that HIV-1 Gag may interact with phosphorylated derivatives of phosphatidylinositol (8). The phosphatidylinositol 3-phosphate, PI3P, a product of phosphatidylinositol 3-kinase (PI3K), has been reported to localize on the endosomal membranes and target a variety of proteins to these membranes through specific interaction (13, 22, 38). To test if the PI3P is involved in targeting Gag to MVB, HeLa cells were transfected with a PTAP mutant and treated with Wortmannin (WM) or LY 294002 (LY), inhibitors of phosphatidylinositol-3Kinase. Confocal analysis revealed that the treatment of Gag-expressing cells with WM or LY obviously caused the Gag to be redirected to the plasma membrane (Figure 5.7). The localization of Gag to MVBs was dramatically reduced. This result indicates that PI3P could be the putative receptor for Gag on MVB.
Figure 5. Treatment of PI3K inhibitors retargeted mutant Gag protein to plasma membrane. HeLa cells transfected with mutant construct PPPY-PTRP (A) or PPPY-PPPY (B) were incubated with DMSO (left column) or PI3K inhibitors WM (middle column) or LY (right column) and stained with anti-p19 antibody as detailed in Materials and Methods. Localization of Gag proteins was examined by confocal analysis.
Basic residues of MA Play no Role in Gag Plasma Membrane Targeting.

In addition to the N-terminal myristylation signal, basic residues within MA domain of many retroviruses are thought to be important for Gag targeting and binding to the plasma membrane (27, 29, 34, 45). Mutations of these residues could cause defective Gag membrane targeting. Interesting, a recent study showed that amino acid substitution of certain HIV MA basic residues retargeted the Gag to MVBs for assembly (26). Single amino acid substitutions of HTLV-1 MA were shown to have no effect on the intracellular punctate staining pattern of Gag. This punctate staining pattern could be a result of accumulation of particles inside MVBs or aggregation of Gag proteins in the cytosol which was suggested to proceed the particle assembly at plasma membrane. To distinguish between these two possibilities and to test if any of the mutations in the HTLV MA basic residues redirected Gag proteins to MVBs, a panel of basic residue mutants were transfected into HeLa cells and analyzed for Gag localization. Surprisingly, the distribution of Gag at the plasma membrane was readily detected in all mutants (Figure 5.8). Some cells showed intracellular punctate staining of Gag but no colocalization with CD63 was observed (data not show). This observation suggests that plasma membrane targeting pathway is intact in these mutants and mutant Gag proteins were not redirected to MVBs.
Figure 5. Mutation of HTLV-1 MA basic residues did not lead to Gag retargeting. HeLa cell were transfected with Wild type (A) or a series of mutant constructs of HTLV-1 MA basic residues: Arg3-Leu (B); Arg7-Leu (C); Arg14-Leu (D); Arg17-Leu (E); Arg33-Leu (F); Lys47-Ile (G); Lys48-Ile (H); Lys51-Ile (I); Lys74-Ile (J); Arg79-Leu (K); Arg97-Leu (L). Cells were stained with anti-p19 antibody. Gag localization was examined by confocal microscopy and shown in green.
Discussion

For most retroviruses, the Gag polyproteins are thought to be targeted to the plasma membrane where they assemble into virions and bud off the cell membrane. This view has prevailed until recent years. We have previously observed the assembly and budding of HTLV-1 at the plasma membrane in standard culture cell lines including HeLa and 293T. In this study, we demonstrate the existence of an alternative intracellular location for HTLV-1 assembly and budding. With wild type or PTAP mutant transfected HeLa cells HTLV-1 particles were observed to accumulate into certain intracellular compartments. Using both confocal microscopy and electron microscopy, we have identified these intracellular compartments as CD63- or Lamp1-positive MVBs. Two possible mechanisms could explain the observation. First, HTLV-1 particle could be secreted by the cells and then reinternalized and targeted to MVB via the endocytic pathway, a phenomenon that has been reported previously (33). Alternatively, HTLV-1 Gag polyproteins could be targeted to MVBs and assembled into viral particles at the limiting membrane in a manner similar to the biogenesis of the internal vesicles of MVB. Several lines of evidence argue against the former view. First, we have observed immature ring-like particles inside the lumen of MVB and the electron-dense Gag aggregates that are in the process of assembly or budding along with mature particles. These observations suggest that Gag were targeted to MVB and assembled directly into virions. Second, The intracellularly accumulated Gag showed no obvious colocalization with an early endosomal marker EEA1, suggesting that Gag
proteins are not transported to the MVB from early or recycling endosomes which will be the case if the particles are internalized through endocytosis. Third, immunogold labeling of pre-embedded cells showed that the HTLV-1 particles inside the MVB associated with CD63, a protein that is normally found in MVB membrane, suggesting the viral particles budded into the MVB, incorporating CD63 into their membrane envelope as they assembled at the limiting membrane. Collectively, the data strongly suggest that the accumulation of HTLV-1 particle inside MVB is the consequence of direct assembly and bud of virions into these intracellular compartments rather than a result of internalization of released viral particles. The discovery of the MVB pathway suggests that, like HIV, not only HTLV-1 can hijack the MVB machinery to the plasma membrane for its assembly, but under certain conditions, the assembly and budding can take place at the site where the machinery is usually active. In HeLa cells, the plasma membrane appears to be the primary site for wild type HTLV-1 Gag assembly. The majority of virus particles were observed to form at the plasma membrane and confocal analysis revealed very little or no colocalization between Gag and CD63.

The assembly and budding of retroviruses have been an extensively studied area in recent years. Many studies support the view that late domains are critical for retroviral budding at the plasma membrane (reviewed in (12)). These small motifs are thought to accomplish their function by recruiting the host cellular machinery for MVB biogenesis to the site of assembly and budding at plasma membrane. Striking similarities between the retrovirus budding
machinery and the biogenesis of internal vesicles in MVB have been noted: topologically, both processes occur by membrane invagination away from, rather than into the cytosol.

Previously, we and others have identified both PPPY and PTAP as HTLV-1 late domains (6, 20, 42). In particular we showed that when PPPY is mutated to APPY or AAAA, large amount of electron-dense patches were observed on the plasma membrane indicating particle assembly is arrested at the late assembly and early budding stage. This type of arrested particle phenotype was also observed for PTAP mutant PPPY-PTRP. However, several observations suggested that PTAP plays a minor role in HTLV-1 budding at the plasma membrane. First, the late domain phenotype is much less pronounced in PTAP mutant than in PPPY mutant. Arrested early assembly intermediate was not seen as often as in the PPPY mutants. Second, the particle production of PPPY-PTRP was only reduced slightly (about 60% of the wild type level). Third, the PTAP motif can be replaced by PPPY or YPDL motif without affecting the particle production. It raised an intriguing possibility that PTAP might have other roles in HTLV-1 Gag assembly. Here in this study the role of PPPY and PTAP were further investigated. Interestingly we found that majority of PPPY-PTRP Gag proteins was actually redirected to MVBs for assembly. This observation suggests an important role of PTAP motif in targeting Gag to plasma membrane. The idea is further supported by the exclusive MVB localization of PPPY-PPPY or PPPY-YPDL mutant. In contrast, PPPY motif does not appear to be involved in Gag targeting since Gag of mutant APPY-PTAP was primarily targeted to
plasma membrane for assembly. Our results shed a light on the differential role that PPPY or PTAP play in HTLV-1 assembly which was previously unnoticed. This suggests that during the assembly process PTAP first acts to promote the targeting of Gag to plasma membrane and then it may function synergistically with PPPY in the viral assembly and budding by recruiting host cellular machinery. It is very likely that PTAP executes its targeting function through its interaction with TSG101 since Bouamr et al (6) reported that overexpression of N-terminal fragment of TSG101 redirected the particle assembly inside cytoplasmic vacuoles. Consistent with our finding, the role of PTAP-Tsg101 in recruiting matrix protein VP40 to plasma membrane for assembly has been suggested for Ebola virus (40). Interestingly, both HTLV-1 and Ebola virus contain two late domains: PPPY and PTAP.

The involvement of a late domain motif in targeting Gag to plasma membrane is very interesting, suggesting a unique mechanism underlying HTLV-1 assembly and budding. For many retroviruses, a bipartite signal comprising a myristylation signal and a patch of basic residues in MA domain is thought to be critical for Gag targeting to the plasma membrane (45). Previous studies showed that myristylation was essential for HTLV-1 Gag membrane targeting (5, 21). Destruction of the N-terminal glycine of HTLV-1 MA, which prevented myristylation, resulted in a diffusive cytoplasmic distribution of Gag because they are no longer anchored to the cellular membrane. The fact that disruption of myristylation did not retarget Gag to MVBs suggests that myristylation is also a prerequisite for MVB targeting. It appears that the myristylation signal is critical
for Gag to be targeted to the appropriate cellular membrane. When it is altered, Gag can no longer be targeted to either the plasma membrane or to MVBs thus remains cytoplasmic. If the myristylation signal is intact, Gag can be targeted to either the plasma membrane or to MVBs. The plasma membrane is the primary destination in HeLa cells. When plasma membrane targeting signal is disrupted, Gag is directed to MVB for assembly and budding.

The importance of the basic residues of HTLV-1 MA in Gag membrane targeting was examined. The role of these residues in HTLV-1 assembly and transmission has been investigated previously (21). These investigators showed that alteration of basic residues scattered along the HTLV-1 MA did not change the punctate localization pattern of Gag (21). Our data suggest that the punctate localization pattern does not indicate the location of assembly. Both the plasma membrane pathway and MVB pathway could give rise to an intracellular punctate localization pattern. In the former case the punctate pattern was thought to be a reflection of pre-assembled Gag aggregates inside the cytosol (19). In the latter case we noticed the intensity of the punctate spots observed under confocal microscopy is very high and these spots have a large size reflecting the accumulation of a large number of particles inside MVBs. A closer examination of the Gag localization suggested the single mutation of basic residues did not retarget the Gag to MVB pathway. The majority of Gag proteins were localized on the plasma membrane with some accumulated intracellularly but they did not co-localize with MVB marker CD63. Therefore, the basic amino acids of HTLV-1 MA do not appear to play an essential role in Gag plasma membrane targeting
despite the fact that the HTLV-1 MA domain was thought to adopt similar structure as HIV MA domain and basic residues are exposed on the surface of the MA trimer (10). Other roles of HTLV-1 MA in viral transmission was suggested (21).

It is not clear what cellular factor(s) determine the destination site to which Gag is targeted. A variety of proteins are known to be targeted to the plasma membrane or endosomal membranes through specific interactions with phosphorylated derivatives of phosphatidylinositol (PI), known as phosphoinositides which play a key role in spatial regulation of crucial cellular processes such as signal transduction and membrane trafficking by recruiting cytosolic proteins to specific membrane compartments (11, 22). An in vitro study has suggested a role for PI in HIV-like particle assembly (8), raising the possibility that these lipids may play a role in Gag targeting.

Among all phosphoinositides PtdIns(3)P is a key phosphoinositide in membrane trafficking. It is found primarily in membranes of early endosomes and multivesicular bodies but not on plasma membrane (13) raising the possibility that PI(3)P is the receptor for Gag on MVBs. In this study, we showed that treatment with PI3K inhibitors, Wortmannin (WM) and LY294002 (LY) disrupted MVB pathway and the plasma membrane targeting of Gag was restored. This result indicates that PI3P is important for Gag targeting to MVBs. Two distinct protein domains have been found to bind to PI3P with high affinity and specificity. One is Phox homology (PX) domain. The other is the cystein-rich domain
reminiscent of a zinc finger, FYVE finger (zinc finger originally observed in Fab1p, YOTB, Vac1p and EEA1) (11, 22, 37). Retroviral Gag proteins do not appear to possess the characteristic PX or FYVE domain; therefore, the recruitment of Gag to MVBs may be mediated by an intermediate binding partner that possesses one of these two binding domains. Another phosphoinositide PtdIns(4,5)P2 which accounts for approximately 1% of lipid molecules in the plasma membrane of a typical mammalian cell and recruits a wide array of different membrane-association domains (23) could serve as a good candidate as the Gag receptor on plasma membrane. In HeLa cells, the plasma membrane receptor (possibly PI(4,5)P2) may play a dominant role if the targeting signal (i.e. the PTAP motif) is intact. If this signal is altered, Gag would be targeted to PI3P-enriched MVB and assembly of virus particles would occur in the MVB. The abundance and strength of the Gag receptor on either plasma membrane or MVB could be cell-type dependent. A cell-type dependent assembly pathway has been reported for HIV (25, 26). It would be interesting to find out if HTLV-1 Gag assembly pathway is cell type-dependent.

What is the fate of the intracellular MVB viral particles? Can they be released into the extracellular environment in a fashion of exocytosis instead of being trapped in MVBs for degradation? Such an exocytic pathway has been reported in a variety of hematopoietic cell types such as B lymphocytes and dendritic cells (18, 32). In these cells, exocytosis results in the secretion of the intra-lumenal membrane vesicles, called exosomes. The exocytic process is best documented for the MHC class-II containing compartments (MIIC). These
compartments identified as late endosomes/MVBs have been previously shown to serve as transport organelles, which guide MHC class II to the cell surface (43, 44). Microtubules are involved in the intracellular transport of MIIC. Two molecular motor proteins, kinesin and dynein, are in control of the movement of MIIC away or toward the MTOC region (43). The recruitment of the dynein-dynactin motor complex to MVBs are mediated by RILP (17). Several of our observations support the view that these virus-containing MVBs can traffic to the cell surface and release the inside content by fusion with plasma membrane (Stoorvogel, W. and G. Raposo, 2002). (I) Our finding that overexpression of p50 and RILP severely affects intracellular distribution of Gag suggested that analogous to the fate of MIIC, transport of these virus-containing MVBs occurs along microtubules using RILP recruited dynein-dynactin complex. (II) Despite the defect in plasma membrane pathway, the mutant PPPY-PPPY, PPPY-YPDL can still efficiently release viral particles into extracellular environment (42). (III) In addition to the classical perinuclear localization of the Gag-containing MVBs, large vacuoles stained with both Gag and CD63 were frequently observed on the surface of the cell (Figure 3D insets). (IV) Although not as many intracellular virus-containing vacuoles were observed in chronically HTLV-1 infected MT-2 cells, we constantly observed virion-filled deep invaginations of the plasma membrane (data not shown). These invaginations most likely represent the fusion of virus-containing vacuoles with plasma membrane for the subsequent release of particles because they were mainly found on cells producing virions.
The release of the MVB particles may be triggered by certain unknown stimuli. Ca2+ was tested and found not to be able to trigger the release (data not shown).

HTLV-1 infection produces very few cell-free virions and only one in $10^5$ to $10^6$ of these virions is infectious. It is believed that cell-to-cell spread is the major route for HTLV-1 transmission. A recent study reported that HTLV-1 might spread across the ‘viral synapse’ formed between cell-cell junction (16). Gag proteins and viral genomes were shown to transfer from the infected to the uninfected cell across the viral synapse. Released cell-free virions are not needed for this type of cell-cell spread. However, the existence of the MVB pathway raises an interesting possibility of HTLV-1 transmission. If the MVB pathway is the primary assembly pathway in HTLV-1 infected cells under certain condition, the pocket of MVB particles could be directly transferred to a susceptible cell at the cell junction. Studies to investigate the existence of MVB assembly pathway in human T cells are under way.

References


CHAPTER 6

DISCUSSION AND PERSPECTIVES

The data presented in this study are the first detailed investigation of late events occurring during Deltaretrovirus replication cycle. For other retroviruses, the molecular interactions involved in assembling the virus particles have been extensively studied (10), whereas very little is yet known about Deltaretrovirus genus in this regard primarily due to the difficulty of growing these viruses in cell culture system. Human T-cell leukemia virus and bovine leukemia virus are notorious for their extremely low particle productivity and low infectivity of cell-free particles. In contrast, other retroviruses can be more readily transmitted. In my dissertation, I have explored several aspects of Deltaretrovirus life cycle mainly focusing on how Gag protein drives viral assembly and release.

Development of a Virus-like System for Analysis of BLV Assembly and Release

An effective assay system was needed to study the various aspects of BLV virus assembly process. BLV expression in cells infected with BLV or transfected with a cloned provirus was very limited, making it difficult for the molecular genetic studies (7). Thus a VLP system expressing BLV gag alone
was developed. Expression of gag, in a number of expression systems such as bacteria (18, 26), yeast (36), insect cells (19, 22, 36, 42) and mammalian cells (13, 16, 38) has led to the synthesis of VLPs that mimic the immature form of the authentic virus. Thus, the VLP system has provides a tool to identify Gag determinants that are important for virion formation. Especially valuable is the simplification of the complex assembly process. Particularly wide-spread use has been made of the expression of Gag in insect cells using recombinant baculoviruses, with VLP assembly being reported for HIV-1 and 2 (11, 23), SIV (42), BIV (33), BLV (19), HTLV-II (40), RSV (17), and M-PMV (39). However, it has become evident that host cell factors are involved in retrovirus assembly and release; results obtained from insect cells may be different than those from mammalian cells. Such a discrepancy has been reported for HTLV-1 (2). The expression system that I developed has at least two advantages. First, BLV gag was under the driving of CMV promoter, allowing high expression of Gag protein in mammalian cells. Indeed, the expression of BLV gag in such a system led to the efficient production of BLV virus like-particles. Second, the exclusion of other viral proteins allows dissection of the role of only Gag in BLV assembly without much complication.

It should be pointed out that although Gag is the central player in assembly of virions, other retroviral components might also have an effect. For instance, Env protein was shown to influence the cytoplasmic transport of Gag (1, 37). Our study in Chapter 2 also showed the BLV late domain defect is enhanced by the presence of viral protease. Thus observations made in a Gag-
only expression system may not apply to systems using full-length, infectious molecular clones. Further studies of assembly that allow for expression of other BLV genes may be important in understanding how these genes may influence particle assembly and release.

**Myristylation of MA is an Important Determinant for BLV Gag Targeting**

Using the BLV VLP expression system, I studied the role of myristylation in Gag targeting to the plasma membrane. As expected, myristylation of Gag was shown to be important for Gag targeting and binding to the plasma membrane. Similar results were also obtained in the insect cells using baculavirus expression system (19).

The importance of myristylation of Gag has been recognized for most retroviruses (5, 12, 34, 35). In HIV, in addition to the N-terminal myristate, a conserved basic region within the MA is thought to contribute to the targeting of Gag to the plasma membrane by forming electrostatic contacts with acidic phospholipids (43). My data showed that myristylation is essential but may not be the only determinant to the Gag membrane targeting. Additional elements may be needed for this process. However, data presented in Chapter 3 indicate basic residues of BLV MA play no role in membrane targeting because mutations of these residues had no obvious effect on Gag membrane localization. A previous study of the basic residues in the HTLV-1 MA also found that HTLV-1 MA basic residue mutants led to normal transport of Gag to the plasma membrane (21). Collectively, these observations suggest that other determinants may exist and
future studies will identify other residues involved in BLV Gag membrane targeting. It should be noted that data presented in Chapter 5 revealed an interesting finding that one of the HTLV-1 late domain motifs, PTAP, may play a role in targeting HTLV-1 Gag to the plasma membrane in addition to its late domain function. When this motif was mutated, Gag was redirected to intracellular organelle MVB for assembly.

The second application of this expression system is the identification of BLV late domain. A well-conserved PPPY motif was shown to function in BLV late budding step. Late domain will be discussed in more detail below.

**BLV MA is Involved in RNA Packaging**

Study of RNA packaging is another successful application of the BLV VLP expression system. It is based on the unique location of BLV encapsidation signal. In most retroviruses the location of the packaged signal is in the untranslated leader region between the major subgenomic splice donor and the start of the *gag* codon. Therefore, *gag* transcripts generated by a *gag*-only VLP expression system would lack the entire package signal and thus could not be packaged into VLPs. However, the primary packaging signal region of BLV was mapped just downstream of the *gag* start codon in the MA domain and a secondary packaging signal also was located in the CA domain of Gag (24). This allows the Gag RNAs the ability to compete with cellular RNA and to be specifically packaged into the VLPs through the interaction with Gag proteins.
Very little effort has been given to identify the protein element that is involved in BLV genomic RNA packaging despite the numerous reports on other retroviruses. In Chapter 3 using quantitative real time PCR analysis we showed that like other retroviruses, the NC domain of BLV plays an essential role in genomic RNA packaging. In addition, we also found that some basic residues of MA are important for RNA packaging, although these residues did not appear to be involved in Gag membrane binding.

These observations imply that distinct mechanisms of viral RNA-protein interactions and Gag membrane binding are used by BLV compared to other retroviruses. Future studies should be directed at addressing the following questions: (i) Could MA and NC be interacting with the same RNA region that spans the location of SL1 and SL2? Which domain confers the specificity of viral RNA binding? (ii) Does the HTLV-1 MA influence RNA packaging of genomic RNA? (iii) Where in the cell does the genome recognition occur? In the end of Chapter 3 presented data suggested that genome recognition of BLV did not appear to occur inside the nucleus. It is not clear how Gag and viral genomic RNA reach common sites at the plasma membrane to assemble, bud and form virion. A recent report brought up an interesting model that Env and Gag tether the RNA onto endosomal vesicles, allowing the resulting complex to be routed to the cell surface (1). Novel technique for visualization of single mRNA molecule would allow the study of intracellular traffic of retroviral genomic RNA and identification of the location where the genome recognition event occurs.
Figure 6.1. Gag-genomic RNA interaction of BLV. The Gag-RNA interaction is likely to occur in the cytoplasm after the full-length viral RNA is transported out the nucleus. Both NC (gray oval) and MA (green square) could interact with BLV primary packaging signal that consists of two step-loop structures (SL1 and SL2). The interaction allows the transport of the viral genomic RNA to site of the virion assembly at the plasma membrane.

**Identification of BLV/HTLV-1 Late Domains**

Virion budding is presently an intense area of study. Late domains for different retroviruses have been promptly identified. My data in Chapter 4 and 5 are among the few to focus on deltaretrovirus release (3, 20, 41). Two powerful tools, electron microscopy and immunocytochemistry were combined in these
studies. I found that PPPY functions as BLV late domain and same motif was also found to function in HTLV-1 release. Interestingly HTLV-1 possesses a second late domain PTAP. I also found that although PTAP plays a minor role in late assembly/early budding step, it plays an important role in targeting HTLV-1 Gag to the plasma membrane. PTAP is a classical late domain and the use of it as a plasma membrane targeting signal has never been reported. Surprisingly, disruption of PTAP redirected Gag to multi-vesicular bodies (MVBs) for assembly. This is the first study to demonstrate the existence of two targeting destinations for HTLV-1 Gag, i.e. plasma membrane and MVBs. I hypothesized two different Gag receptors are present at the plasma membrane and the MVB. My data suggested the Gag receptor on MVBs is likely to be phosphoinositides PtdIns(3)P.
HTLV-1 is not the only retrovirus using alternative targeting pathway. While this study was under way, several reports showed that the MVB is the primary assembly site of HIV-1 in the macrophage whereas in HeLa or Jurkat cells, Gag is mainly targeted to the plasma membrane (27-30, 32). The possible advantage
of intracellular budding is that the concentration of viral glycoproteins exposed on
the surface of the infected cell is reduced because these proteins accumulate in
intracellular membranes. This property would decrease the likelihood that the
infected cell would be recognized by host antibodies and other components of
the immune system before the maximal number of progeny virions were
assembled and released.

It remains unknown whether HTLV-1 Gag targeting and assembly is cell-
type dependent. There is a need to extend such studies to a more biologically
relevant system, i.e. the primary T-cell model. If the MVB pathway is the primary
assembly pathway in primary T-cells, these MVBs could serve as secretory
organelles and direct release of virions onto the target cell may be induced by
specific stimuli initiated by cell-cell contacts. This hypothesis could be tested in
an in vitro model using fluorescence and electron microscopy.

HTLV-1 virions are produced only by certain continuous in vitro T cell
lines: fresh, naturally infected lymphocytes do not produce cell-free particles.
Furthermore, of the cell-free HTLV-1 virions that are produced by transfected T
cells or continuous producer T cell lines, only one in $10^5$ or $10^6$ is infections (9).
Its infectivity ratio is considerably lower even than that of other RNA viruses. It
has been known for many years that cell-cell contact is required for efficient
transmission of HTLV-1 both in vivo and in vitro. A major mystery regarding the
natural history of HTLV-1 infection has been how it moves from cell to cell. A
recent study (15) proposed that HTLV-1 exploits the normal physiology of the T
cell to enable efficient cell-to-cell transmission by forming a close contact (viral
with the recipient cell using the cytoskeleton to propel viral material into the recipient cell (Figure 6.3A). In such a model, HTLV-1 has lost the need to release cell-free virions in order to spread from cell to cell.

The cell-to-cell transmission is not unique to HTLV-1. Many animal viruses are able to spread both in vitro and in vivo by inducing cell-cell fusion, which is a more efficient than by release of virus particles. Free virus particles must travel within the host until they encounter a susceptible cell. The new host cell may be an immediate neighbor of the originally infected cell or a distant cell reached via the circulatory or nervous systems of the host. Virions are designed to withstand such intercellular passage, but they are susceptible to several host defense mechanisms that can destroy virus particles. Localized release of virus particles only at points of cell-cell contact can minimize exposure to these host defense mechanisms.

Possible roles and mechanisms of cell-to-cell transmission of HIV have been studied in in vitro models between various cell types involving T cells, epithelia, macrophages and dendritic cells (4, 6, 8, 25, 31). In most cases, cell-cell transmission of HIV-1 is thought to involve the formation of enveloped extracellular particles. The formation of cell-cell contact results in a rapid polar budding of viral particles into an enclosed space formed by interdigitating microvilli of the contacting cells. Released HIV-1 virions were then taken up into the recipient cell via phagocytosis, coated pits and direct fusion (4, 8). The virions-containing MVBs could act as an intracellular virus stores which can be transported to the site of cell-cell contact and release the virions to the recipient
cell upon stimuli from the cell-fusion (Figure 6.3B). Such a transmission mode could apply to cells in which the MVB is the primary site for virion formation such as HIV-1-infected macrophage.

Figure 6.3. Models of cell-cell transmission of virus. (A) A proposed model suggests that HTLV-1 viral material (Gag, Env and viral genome) concentrated at the site of cell-cell contact and released to the recipient cell through a “viral synapse”. (B) A possible mechanism used to spread MVB virions through cell-cell contact. Virion-containing MVBs transport to the site of cell-fusion and release the particles to recipient cell.

The assembly of even the simplest virus is a complex process in which multiple reactions must be completed in the correct sequence and coordinated in
such a way that the overall pathway is irreversible. The development of new structural methods, coupled with the experimental power and flexibility provided by modern molecular biology, has revitalized investigation of the crucial processes of assembly, release, and maturation of retrovirus particles. These discoveries will stimulate the design of new therapeutic agents inhibiting virus-specific reactions crucial for the production of infectious particles.

Most of our previous knowledge on Delta-retrovirus assembly is from other retroviruses based on the assumption that replication cycle of most retroviruses share great similarities. Work from my dissertation revealed that although basic principles probably apply to many retroviruses, the deltaretoviruses do display many unique features. More effort should be given to the study of molecular aspects of deltaretrovirus replication cycle.

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