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A Functional Analysis Of The Human Immunodeficiency Virus

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A FUNCTIONAL ANALYSIS OF THE HUMAN IMMUNODEFICIENCY VIRUS ENVELOPE GLYCOPROTEIN CYTOPLASMIC DOMAIN

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ABSTRACT

The retroviral life cycle is separated into two distinct phases of infection. In the first phase, viral enzymes and proteins allow the virus to establish infection. The virus usurps host machinery in the second stage to produce infectious virus. In both phases of the replication cycle, the envelope (Env) glycoprotein (gp) is a key component of infectivity.

Env plays a pivotal role in viral entry, by mediating attachment and facilitating fusion of the viral and cellular membranes, and during assembly, where Env must be incorporated into budding virions in order to produce infectious virions. The Env cytoplasmic domain (CD) interacts with Gag facilitating incorporation. Human immunodeficiency virus type I (HIV-1) gp41 CD is unusually long and characterized by multiple highly conserved motifs that resemble classic trafficking and internalization motifs. The membrane-

proximal tyrosine motif (Y_{712}) has been characterized as the major determinant for internalization, however, there are additional Y (tyrosine) and LL (dileucine) determinants distal to this membrane-proximal signal. The caveat in analyzing the conserved motifs within the CD include the trimeric nature of the Env complex, the interdependence of the gp120 and gp41 ectodomain on the gp41 CD, and the presence of potentially more dominant signals. Considering these limitations, we employed a progressive mutagenesis strategy to comparatively analyze the motifs conserved within the CD.

The results presented within this body of work suggest that mutagenesis of the tyrosine (Y) and dileucine (LL)-motifs in the Env CD can influence the biological function of the Env glycoprotein. Evidence supporting the contribution of the conserved motifs in the processes of Env-mediated cell-cell fusion, viral infectivity, viral entry, and incorporation of Env into budding virions is presented. A threshold of virus defect resulting from progressive substitutions in the CD was correlated to motifs overlapping a region of the CD described to play a role in the fusion process. This work brings perspective to the role of the highly conserved motifs of the Env CD in the HIV-1 life cycle.

Keywords: Retrovirus, HIV-1, Envelope, cytoplasmic tail, fusion, infectivity.

DEDICATION

I dedicate the work described in this dissertation to my loving parents, Jyoti and Hasmukh Bhakta. Thank you for your love, support, and understanding.

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DISUSSION

ABBREVIATIONS

α	alpha
A	adenine
AA	di-alanine
aa	amino acid
att	attachment
Ab	antibody
AC	acidic cluster
AIDS	Acquired Immundeficiency Syndrome
ALP	alkaline phosphatase
ALSV	avian leukosis sarcoma virus
AP	adaptin protein complex
APC	antigen presenting cell
ATP	adenosine triphosphate
Arg	arginine
β	beta

bp	base pair
BlaM	β-lactamase
CA	capsid protein
CD	cytoplasmic domain
CD3	cluster of differentiation 3
CD34	cluster of differentiation 34
CD4	cluster of differentiation 4
CD8	cluster of differentiation 8
CDK	cyclin-dependent kinase
CRF	circulating recombinant forms
CTD	C-terminal domain
CTE	Constitutive Transport Element
C-terminal	carboxy terminal
CycT	cyclin T
CypA	cyclophilin A
DC	dendritic cells

DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DIS	dimer initiation site
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dsDNA	double stranded deoxyribonucleic acid
dUTPase	deoxyuridine-triphosphatase
E	encapsidation signal
EBFP	enhanced blue fluorescent protein
EIAV	equine infectious anemia virus
eIF	eukaryotic initiation factor
ELISA	enzyme-linked immunosorbent assay
env	envelope (gene)
Env	envelope protein
ER	endoplasmic reticulum
ESCRT	endosomal sorting complex required for transport

ϕ	residue with bulky hydrophobic side chain
FBS	Fetal Bovine Serum
FI	Phenylalanine Isoleucine
FLV	feline leukemia virus
G	guanine
g/mL	grams/ milliliter
gag	group specific antigen (gene)
Gag	group specific antigen (protein)
GDP	guanine diphosphate
GHR	growth hormone receptor
Glu	glutamic acid
GLUT4	glucose transporter
gly	glycine
gp	glycoprotein
GTP	guanine triphosphate
HA	hemagglutinin protein
HIV	human immunodeficiency virus

ABBREVIATIONS (Continued)

HIV-1	human immunodeficiency virus type I
HIV-2	human immunodeficiency virus type II
HR	heptad repeat
HTLV	human T-cell leukemia virus-1
I	Interaction
IgG	Immunoglobulin G
Iip31	invariant chain
IL	interleukin
IN	integrase
INF	interferon
IRES	internal ribosome entry segment
is	inhibitory sequence
kb	kilobase
kDa	kilodalton
“L”	late domain
LL	dileucine
LLP	lentiviral lytic peptide

LTR	long terminal repeat
lys	lysine
Lys	lysine
mRNA	messenger ribonucleic acid
M	myristic acid
μ	middle subunit of AP complex
M subtype	main
MA	matrix domain
mAb	monoclonal antibody
MDCK	Madin-Darby Canine Kidney epithelial
Me	methylated
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MPMV	Mason Pfizer Monkey Virus
MPR	mannose-6-phosphate receptor
mRNA	messenger ribonucleic acid
MVB	multi-vesicular bodies

ABBREVIATIONS (Continued)

N subtype	non-M and non-O
NC	nucleocapsid
Nef	negative factor (protein)
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanograms
nm	nanometer
NMR	nuclear magnetic resonance
NRS	negative regulator of splicing
nt	nucleotide
NTD	N-terminal domain
N-terminal	amino terminal
O subtype	outlier
OH	hydroxyl group
ORF	open reading frame
PBS	primer binding site
PCR	polymerase chain reaction
PI(4,5)P2	phosphoinositide phosphatidylinositol (4,5) bisphosphate

ABBREVIATIONS (Continued)

PIC	pre-integration complex
PKR	protein kinase R
PM	plasma membrane
Pol	polymerase
PPT	poly purine tract
PR	protease
pre-mRNA	precursor mRNA
pro	proline
Pro	proline
P-site	peptidyl tRNA binding site
R	Arginine
R	repeat region
Rev	regulation of expression of viral protein (protein)
RNA	ribonucleic acid
RNAP	RNA polymerase
RNase H	ribonuclease H
rpm	revolutions per minute

ABBREVIATIONS (Continued)

RRE	rev-response element
RT	reverse transcriptase
S	Serine
SD	splice donor
SDS	sodium dodecyl sulfate
SIV	simian immunodeficiency virus
SIV_{cpz}	simian immunodeficiency virus in chimpanzee
SIV_{sm}	simian immunodeficiency virus in simian monkey
SL	stemloop
SP	spacer protein
ss	single stranded
SU	surface protein
T cells	subgroup of immune cells that mature in the **thymus**
TAC	T cell antigen receptor lacking zeta chains
TAK	Tat-associated kinase
TAR	transactivation response RNA
Tat	trans-activating (protein)

TFR	*S*-transferase-transframe region
TIP47	tail interacting protein of 47 kDa
TM	transmembrane
TRBP	TAR-binding protein
TRIM5α	**TRI**partite **M**otif
tRNA	transfer RNA
trp	tryptophan
TSG101	tumor susceptibility gene 101
U/ml	Units/milliliter
UTR	untranslated region
V3	variable 3
Vif	virion infectivity factor (protein)
VLP	viral like particles
Vpr	viral protein R (protein)
Vps4	vacuolar protein sorting 4
Vpu	viral protein U (protein)
W	tryptophan

ABBREVIATIONS (Continued)

WT	wildtype
x	any residue
Y	tyrosine
ψ	psi element
Yxxϕ	tyrosine-based motif
-	negative
+	positive

INTRODUCTION

Acquired Immunodeficiency Syndrome (AIDS) currently affects the lives of tens of millions of people worldwide. This devastating pandemic has the potential to wipe out an entire generation of people within a sub-population of Africa, and the causative agent of this disease is the Human Immunodeficiency Virus (HIV). The efficiency of viral transmission and the ability of HIV to usurp the host machinery to propagate and to evade the host immune system are quite extraordinary. Despite global efforts towards prevention of HIV transmission and the development of a cure, HIV maintains its catastrophic grip on mankind. Therefore, a clear understanding of the basic properties of the viral life cycle and the factors essential to those processes are necessary to define new therapeutic targets and a hope for a cure.

The HIV life cycle consists of two major phases: the early phase where viral enzymes conduct unique functions and the late stage in which the host machinery and enzymes are used in their normal capacity to complete the viral replication cycle. There are a number of viral components critical to viral pathogenesis, but HIV infectivity is dependent on the

integrity and functionality of the envelope (Env) glycoprotein (gp). The components of the mature Env complex play a critical role in viral life cycle and viral pathogenesis. Both the surface (SU) and trans-membrane (TM) components of the Env complex are important during HIV entry into target host cells. The SU component mediates viral attachment to the host cell. The cytoplasmic domain (CD) of the Env TM glycoprotein plays several important roles in viral replication and viral pathogenesis, including interaction with Gag during assembly (129, 269, 397), interaction with cellular components during intracellular transport (27, 30, 385), and it appears to modulate the fusogenicity of the Env complex both in the cell and within the virion during viral entry (27, 327, 386). *In vivo* studies have shown that mutations made in the CD attenuate viral pathogenicity (134). However, which signals mediate these phenotypically critical roles remains to be elucidated.

A number of highly conserved motifs, which classify as classic internalization and trafficking motifs, are located within the unusually long CD of the HIV-1 (Human Immunodeficiency Virus type I) Env. Studies reveal that these motifs overlap with other functional regions within the long CD, further complicating the functional roles that each of the motifs plays in the HIV-1 life cycle. The focus of this dissertation is to elucidate

the contribution(s) made by these trafficking motifs in the various steps of the HIV-1 life cycle.

RETROVIRIDAE FAMILY

The *Retroviridae* family is a large and diverse group of viruses defined by common taxonomic characteristics, including structure, composition, and replication strategy (73, 75). Retrovirus virions, which are slightly pleomorphic to spherical, range in diameter from 80-100 nanometers (nm). An outer envelope, containing glycoprotein spikes of 8nm in length that are embedded and presented in an even distribution, distinguishes these viruses. The capsid (CA) paracrystalline core of these virions ranges in internal shape from spherical, cylindrical, or cone-shaped and is specific to each genus. A buoyant density of 1.13-1.18 grams/milliliter (g/mL) in sucrose and a virion sedimentation rate of 600S characterize retrovirus virions, which are also sensitive to heat, detergent, and formaldehyde. Virion composition includes 1-2% ribonucleic acid (RNA), about 35% lipids consisting of sphingomyelin & cholesterol (6, 310), roughly 3% carbohydrates, and approximately 60% proteins. Retroviruses contain virions of only one kind within a preparation, and their target hosts are vertebrates (403).

The retrovirus genome is uniquely organized by dimerization of two single strands of positive sense RNA at their self-complimentary 5' ends. The non-segmented RNA monomers range in size from 7 kilobases (kb) to 11kb. The retroviral genome associated with the nucleocapsid (NC) protein is highly condensed, and the complex is encased within the CA protein core. The virion core is encircled by a spherical matrix (MA)-containing shell, which is then enveloped by the virion lipid bilayer (403).

Retroviruses are the only viruses to initiate replication using a + (positive) sense strand RNA synthesized by normal host transcriptional machinery, thus containing many features of normal mRNA. A number of physical components distinguish the retrovirus RNA genome including a 5'terminal cap m7G5'ppp5'G_mp structure, which is important for translation of messenger RNA (mRNA), and post-transcriptional methylation, which occurs in cell mRNA. The genome undergoes polyadenylation the length of 200 bases at its 3' end, another post-transcriptional modification that occurs in eukaryotic mRNA. Host transfer RNA (tRNA) targets the primer binding site, a segment of 18 nucleotides (nt), for hybridization; and this junction serves as the start site for reverse transcription initiated deoxynucleic acid (DNA) synthesis. The specific tRNA primer varies among the genera of retroviruses, although it is highly conserved for each genus, including

tryptophan (trp), proline (pro), glycine (gly), lysine^{3} (lys^{3}), or lysine^{1,2} (lys^{1,2}) (261, 307, 349, 350).

5’ cap—R---U_5---[PBS]---ψ---*gag*---*pol*---*env*---[PPT]---U_3---R---A_n 3’

Fig. 1 Retrovirus RNA genome. The retrovirus genome is organized from 5’ to 3’ beginning with the 5’ cap, the R (repeat region), the 5’ UTR (untranslated region), PBS (primer binding site for reverse transcription), the psi element, the genes for *gag, pol,* and *env*, the polypurine tract (PPT), the 3’ UTR, the second R region, and the polyadenylate (A) tail.

The retrovirus genome is also characterized by a number of cis-acting regions. The repetitive sequence element R (repeat region) is terminally redundant and located after the 5’ cap and again before the 200 base pair (bp) long 3’ poly adenylate (A) tail, and it permits the transfer of nascent DNA from one end of the RNA genome to another during reverse transcription. The R sequence varies in length from 12 to 235 bases. The 5’ untranslated region (UTR), which contains one of the *att* (attachment) sites required for integration (36, 111, 294), is the first region of the genome copied into DNA during reverse transcription, thus, becoming the 3’ end of the long terminal repeat (LTR). Studies of this region of the genome indicate that it is essential for reverse transcription initiation (2, 3, 272). A feature shared by other RNA viruses, the leader sequence is an

unusually long sequence that precedes the first translated region of the genome. The leader sequence functionally serves as the donor site for all spliced subgenomic mRNAs encoded by the virus, and it contains a signal, described as the psi (ψ) element, which is a major recognition site for the packaging of genomic RNA into virion particles (227). Another cis-acting region of the retrovirus genome is the polypurine tract (PPT), which includes a run of at least 9 A (Adenine) and G (Guanine) residues immediately preceding the 3' UTR. The PPT region, containing the initiation site for positive strand DNA synthesis, is resistant to the ribonucleaic activity of ribonuclease H (RNase H) digestion and subsequently serves as the primer for the second strand synthesis (149). A 3' UTR, which is short and variable, exists in the genome of complex retroviruses and most simple retroviruses. In simple retroviruses, its function varies from signaling incorporation of the genome into virions to regulating transport of unspliced mRNA, which is facilitated by virus encoded proteins of complex viruses like HIV. The 3' UTR contains the other *att* site necessary for integration, and it defines the 5' end of the LTR. In DNA form, it contains a number of cis-acting elements required for replication. Because the 3' UTR is located at the 5' end of the provirus, it includes signals recognizable to the host transcriptional machinery and it modulates almost all of the transcriptional control of virus expression. The 3' UTR also contains a canonical

consensus sequence, which is representative of most eukaryotic promoters, and is located at the 3' end of the 3' UTR. The R, 5' UTR, 3' UTR, and PPT components of the retrovirus genome are critical for reverse transcription.

The hallmark of the *Retroviridae* family is its reverse transcription replication strategy. The reverse transcriptase (RT) enzymatic activity has forced the revision of the central dogma of replication, and it facilitates the reverse transcription of viral RNA into double stranded DNA (dsDNA), which can subsequently be integrated into the cell genome. This process will be described in detail in a following section.

Retroviruses can be further categorized by whether they have simple or complex genomes. Replication competent retroviruses contain at least the major coding regions for *gag*, *pol*, *pro*, and *env*, whereas complex retroviruses encode for additional regulatory proteins (73, 273). The Gag, group-specific antigen, protein contains the information for the three invariant proteins that form the MA, the CA, and the NC structures inside the virion. The polymerase, *pol*, region guides the synthesis of the RT and the integrase (IN) enzymes, both of which are required early in infection. The *pro* gene encodes the virion protease (PR), which is responsible for the cleavage of the *gag* and *pol* encoded

polyproteins. The Env protein is the precursor from which the SU and TM components of the viral envelope complex are processed. The TM component anchors the complex into the virion envelope layer, and it contains domains that facilitate fusion of the viral and cellular membranes. The additional regulatory non-virion proteins encoded by complex retroviruses are derived from multiply spliced messages.

The International Committee on Taxonomy of Viruses has recently updated classifications for the genera of the *Retroviridae* family. Retroviruses containing simple genomes encode the Gag, PR, Pol, and Env proteins, and they include Alpharetrovirus, Betaretrovirus, and Gammaretrovirus. Alpharetroviruses are recognized by their C-type morphology, which is described as assembling at the plasma membrane and containing a central, symmetrical spherical inner core. They also use the primer $tRNA^{Trp}$ in reverse transcription and express PR from the same reading frame as Gag. Alpharetroviruses are exemplified by the Avian leukosis sarcoma virus (ALSV), and their host range consists of many avian species. Both exogenous and endogenous chicken alpharetroviruses have been found, as well as endogenous alpharetroviruses of pheasants.

The Betaretrovirus genus is signified by either a B-type morphology with a round but eccentric core or a D-type structure, which includes assembly in the cytoplasm and a defined cylindrical core. These retroviruses are also known for their usage of the genomic primers $tRNA^{Lys3}$ or $tRNA^{Lys1-2}$, and different reading frames are necessary to express *gag*, *pro*, and *pol*. Additionally, betaretroviruses encode for a deoxyuridine-triphosphatases (dUTPase). The Mason Pfizer Monkey Virus (MPMV) is a prototypical betaretrovirus that assembles immature capsids in the cytoplasm, after which the completed immature particle is transported to the plasma membrane where budding occurs. This genus contains endogenous as well as exogenous viruses representing a variety of hosts such as mice, primates, and sheep.

The retrovirus genus containing the largest number of members is the Gammaretroviruses, which posses a C-type morphology. These viruses express *gag*, *pro*, and *pol* from the same reading frame and utilize the $tRNA^{Pro}$ or $tRNA^{Glu}$ primer. The Feline leukemia virus (FLV) is a member of the Gammaretrovirus genus. The murine virus members of this genus are grouped by their receptor requirements, and both endogenous and exogenous gammaretroviruses have been found in a range of hosts including mammals, reptiles, and birds.

Complex retroviruses differ from simple retroviruses in that they express a variety of regulatory proteins with diverse functions in addition to Gag, PR, Pol, and Env. The complex retroviruses include the following genera: Deltaretrovirus, Epsilonretrovirus, Spumavirus, and Lentivirus. Deltaretroviruses, exemplified by human T-cell leukemia virus-1 (HTLV-1), are characterized by their C-type morphology and usage of the $tRNA^{Pro}$ primer. These viruses contain three different reading frames for the expression of *gag*, *pro*, and *pol*; and they require two frameshifts to synthesize the Gag and Pro-Pol polyproteins. The regulatory genes encoded by the deltaretroviruses are rex and tax, which are synthesized from alternatively spliced mRNA. Exogenous deltaretroviruses are rare and found in a few mammals.

Epsilonretroviruses are also represented by a C-type morphology, and are tyified by the walleye dermal sarcoma virus. These retroviruses express the *gag*, *pro*, and *pol* genes from the same reading frame; and they contain one to three additional genes, including the open reading frame (ORF) A, B, and C. Epsilonviruses utilize either $tRNA^{His}$ or $tRNA^{Arg}$ as primers during reverse transcription. Members of this genus are exogenous viruses in reptiles and fish.

The spumaviruses (previously known as Foamy viruses) have a distinct morphology including prominent spikes protruding on the surface of the virion with a central uncondensed core. No major morphological change occurs in these viruses during maturation. Spumaviruses assemble in the cytoplasm followed by viral budding into the endoplasmic reticulum (ER) and the plasma membrane (PM). These viruses prefer priming with the $tRNA^{Lys1,2}$, and they encode the tas/bel-1 and bet regulatory proteins (124, 256). Other unique features of this genus of viruses are that Pol is expressed from spliced mRNA and their virions contain a large amount of reverse transcribed DNA (260). Furthermore, exogenous spumaviruses have been found in mammals and distantly related endogenous spumaviruses have been located in humans.

The last genus of the *Retroviridae* family is Lentivirus. These viruses are distinguished by their unique morphology that includes cylindrical or conical cores. HIV-1 (Fig. 2) is the most recognizable of the lentiviruses. These viruses contain a reading frame for *gag* and another for *pro-pol*, requiring a single frameshift to produce the Gag-Pro-Pol polyprotein. The accessory genes encoded by lentiviruses include *vif*, *vpr*, *vpu*, *tat* , *rev*, and *nef* which function to control transcription, the processing of RNA, virion assembly, expression of host genes, and a number of other replicative roles. These viruses also

utilize the $tRNA^{Lys3}$. A large number of exogenous lentiviruses are found in a variety of mammals, and the only endogenous virus in this genus is distantly related.

HUMAN IMMUNODEFICIENCY VIRUS TYPE I

HIV-1 Infection and Its Global Prevalence

HIV-1 was first introduced to the medical community and identified as the causative agent of AIDS in the early 1980's (17, 137). Impaired immune systems and T lymphocytes in affected patients became associated with the transmission of an unknown pathogen through contaminated blood or sexual transmission. It was soon discovered that the causative pathogen was HIV-1. HIV-1 causes a chronic infection in its host that usually leads to AIDS, through a slow loss of immune cells, called cluster of differentiation 4 (CD4) helper T cells, a subgroup of immune cells that mature in the thymus. AIDS eventually results in death from an (opportunistic) infection or cancer.

There are four main stages of HIV infection that lead to the development of AIDS: window period, seroconversion, asymptomatic period, and symptomatic period. The window period reflects the time between initial HIV infection and the development of

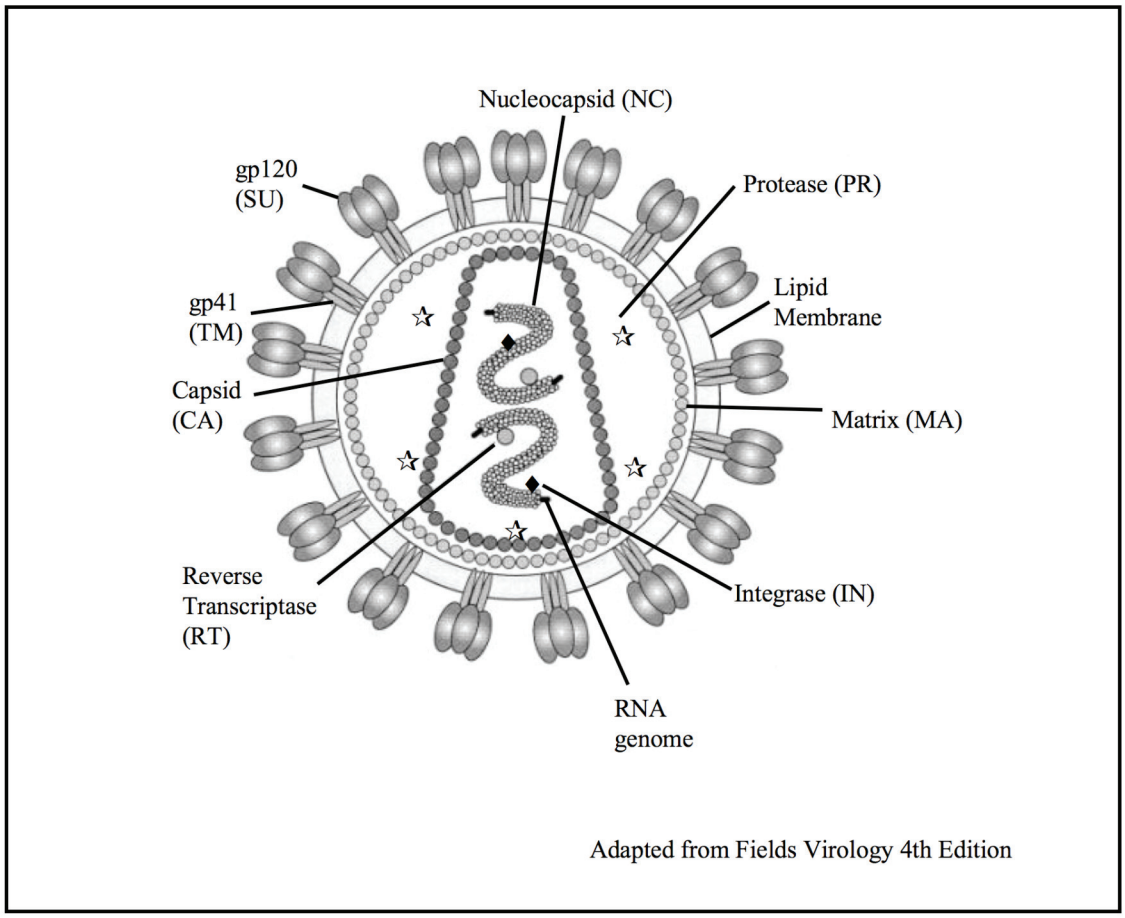


Fig. 2 Schematic of the mature Human Immunodeficiency Virus type I (HIV-1).

HIV antibodies in the bloodstream. During this time, HIV testing on an infected individual may result in false negatives, but individuals usually test positive within six months of the initial infection. Seroconversion describes the change in status of an individual from HIV(-) to HIV(+), as the body produces antibodies as protection against the virus. The window period and early antibody positive period is a highly infectious stage of HIV-1 infection, since high levels of virus circulate in the blood and genital

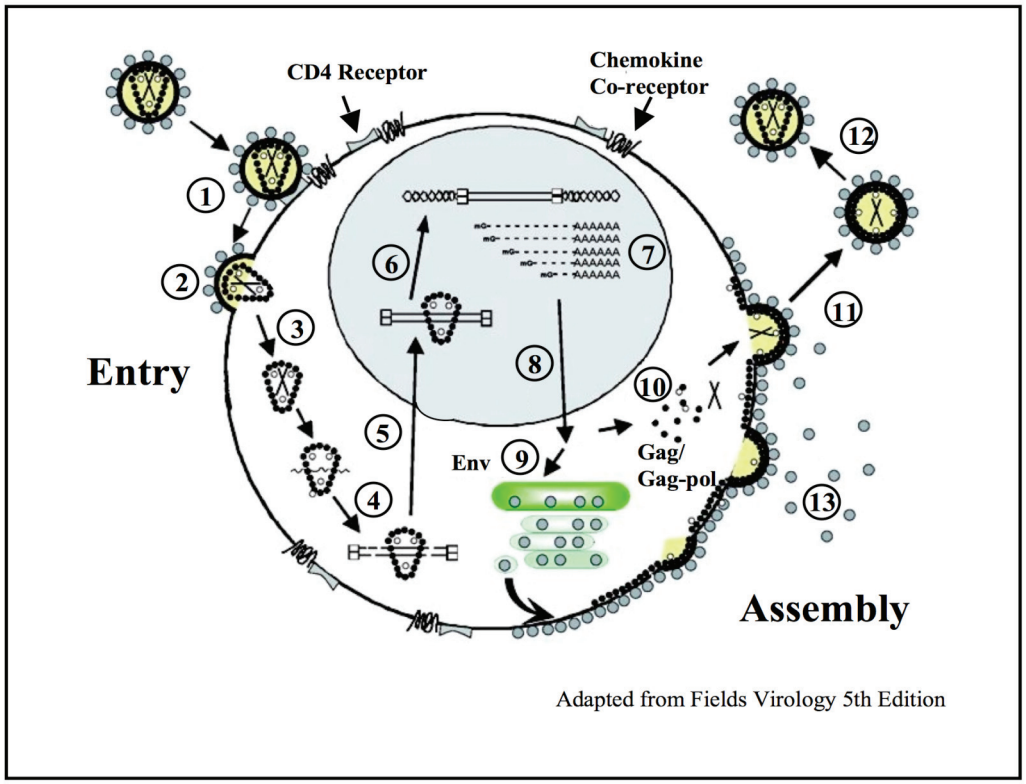


Fig. 3 Human Immunodeficiency Virus type I (HIV-1) replication cycle. (1) Productive infection by cell-free HIV-1 virions is initiated by virion attachment to the CD4 receptor and chemokine co-receptors. (2) HIV-1 entry is facilitated by fusion of the viral and cellular membranes and (3) the subsequent partial uncoating of virions and the release of the nucleocapsid core into the host cell cytoplasm. (4) Within the subviral particle, reverse transcriptase (RT) mediates reverse transcription of the viral RNA genome into dsDNA, which is associated with the pre-integration complex (PIC). (5) The PIC is then transported to the nucleus where (6) dsDNA is integrated into host cell chromosome by the virus-encoded integrase (IN) enzyme (depicted by open circles). (7) Integrated viral DNA is then transcribed into viral mRNA by DNA-dependent RNA polymerase (pol II). Some of the viral mRNAs are spliced and (8) exported to the cytoplasm, where they are translated into viral proteins. (9) Env and (10) Gag and Gag-Pol polyproteins are transported to the PM of the infected cell through independent pathways. (11) These proteins are transported to the site of assembly and particle budding, and virions are released as immature particles. Subsequent to viral release is (12) protease (PR)- mediated protealysis, resulting in virion maturation and containing the characteristic condensed core. (13) The gp120 component of Env that is not associated with virions is also released from the cell.

secretions, and it is can be characterized by flu-like symptoms and swollen lymph nodes in infected individuals. The asymptomatic stage, or symptom-free stage, usually occurs from three months post infection to ten years later, although this varies from person to person. Despite the lack of symptoms, the virus continues to replicate inside the body and weakens the immune system. The symptomatic stage results from severe loss of CD4+ T cells, is frequently characterized by opportunistic infections, and it is the stage referred to as AIDS. AIDS is the final stage of HIV-1 infection, and in untreated individuals, it eventually leads to death.

Upon its discovery, HIV was classified in the *Retroviridae* family because of its notable particle-associated RT activity. The HIV genome is organized like a replication-competent retrovirus, and its taxonomic classification as a lentivirus is indicative of its ability to cause a slow, persistent disease targeting different lineages of immune cells (312). Upon isolation and discovery of distinct primate lentiviruses, their genetic relationship to HIV was determined. These viruses were isolated from different species of monkey, from captivity and from the wild, in Africa. Due to the close contact between humans and monkeys, which are hunted for food and kept as pets in West Africa, it is suggested that a cross-species transmission of the simian immunodeficiency virus (SIV_{sm})

in simian monkeys to humans produced the human immunodeficiency virus type 2 (HIV-2) (140, 141) and the transmission of simian immunodeficiency virus (SIV_{cpz}) in chimpanzees in Central Africa to humans resulted in HIV-1 (139). As blood samples from HIV-infected individuals from different continents became available for analysis, the genetic heterogeneity of HIV became quite evident (24), even within a single patient. Sample comparisons between patients and within individual patients revealed the most variability in the Env glycoprotein.

The unusually high heterogeneity of HIV-1 is in part due to the high error frequency in DNA synthesized during reverse transcription, the high rate of recombination during reverse transcription, the considerable number of progeny virus produced during an HIV-1 infection *in vivo*, and the significant prevalenc of HIV-1 infection around the world (74, 189, 242, 300). This is best represented by the genetic diversity of virus within an HIV-1 infected individual, which increases about 1% annually compared to the founder strain during the early stage of infection (340). The infection of a single host cell with distinctly different viral strains and template switching during reverse transcription has generated a significant amount genetic recombination that has become associated with these viruses.

The diverse strains of HIV are genetically grouped into three subtypes: M (main), O (outlier), or N (non-M and non-O). Subtype M consists of over 95% of the isolate sequences globally, and these are further grouped into nine subgroups or clades, including A, B, C, D, F, G, H, J, and K, and 15 circulating recombinant forms (CRFs). These clades are phylogenetically equidistant from each other, and all are found in Africa. Within a given clade, there is a 3-10% variability in *gag*, a 5-12% diversity in *env*, and a sequence diversity of up to 10% in *env* within an infected individual (212). There is a 65% sequence identity of viruses between Group O and Group M.

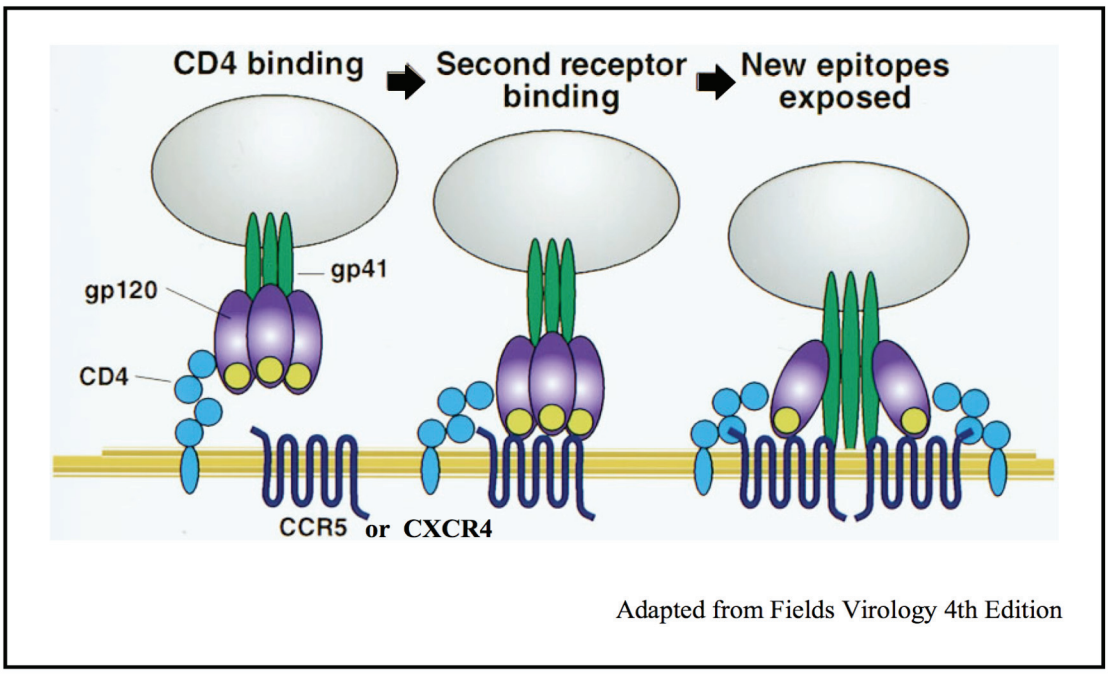


Fig. 4 HIV-1 attachment to target host cell. Env gp120 binds to the CD4 receptor on the target cell surface, which causes a change in gp120 and subsequently allows gp120 to interact with the co-receptor, CCR5 or CXCR4. Co-receptor binding results in a conformational change in Env that mediates the fusion process.

The diverse strains of HIV are genetically grouped into three subtypes: M (main), O (outlier), or N (non-M and non-O). Subtype M consists of over 95% of the isolate sequences globally, and these are further grouped into nine subgroups or clades, including A, B, C, D, F, G, H, J, and K, and 15 circulating recombinant forms (CRFs). These clades are phylogenetically equidistant from each other, and all are found in Africa. Within a given clade, there is a 3-10% variability in *gag*, a 5-12% diversity in *env*, and a sequence diversity of up to 10% in *env* within an infected individual (212). There is a 65% sequence identity of viruses between Group O and Group M.

HIV-1 Viral Life Cycle

Viral Entry

The HIV-1 replication cycle (Fig. 3) begins with viral entry (Fig. 4) into target CD4-expressing cells, including a subset of T-lymphocytes, monocytes and macrophages, and dendritic cells (331). HIV-1 entry into target cells requires viral binding to both the CD4 molecule and a chemokine receptor, which is usually CXCR4 or CCR5, and is mediated by the mature Env glycoprotein complex (Fig. 3, step 1) (26, 176, 228, 331). Upon binding of the gp120 surface component of the Env complex to CD4, a conformational change occurs within Env thus exposing a highly conserved region of gp120 to attach to

the co-receptor, CXCR4 or CCR5 (26, 176, 228, 331). The HIV-1 gp41 then facilitates fusion of the viral and targets cell membranes by bringing them within close proximity to each other through formation of a six-helix bundle (Refer to Fig. 3, step 2). Following viral fusion, the viral core containing the genomic RNA is released into the host cell cytoplasm where the availability of deoxynucleotide triphosphates (dNTPs) allow reverse transcription to be initiated (Fig. 3, step 3-4).

Reverse Transcription

Synthesis of viral DNA from RNA presents two problems for HIV-1: (1) the requirement for an RNA primer to initiate RT prevents precise copying of the genome from one end to the other, and (2) the need for signals directing the cell enzyme RNAP (RNA polymerase) II-mediated RNA synthesis to be located upstream of the initiation site and outside of the region to be copied. HIV-1 resolves this issue by including both ends of the RNA molecule in its replication strategy to produce an additional copy of certain sequences that appear only once in the viral RNA genome. The additional sequences, repeated at both ends of the DNA, together are called the LTR, which encodes most of the cis-acting sequences critical for integration of viral DNA and expression of the

Fig. 5

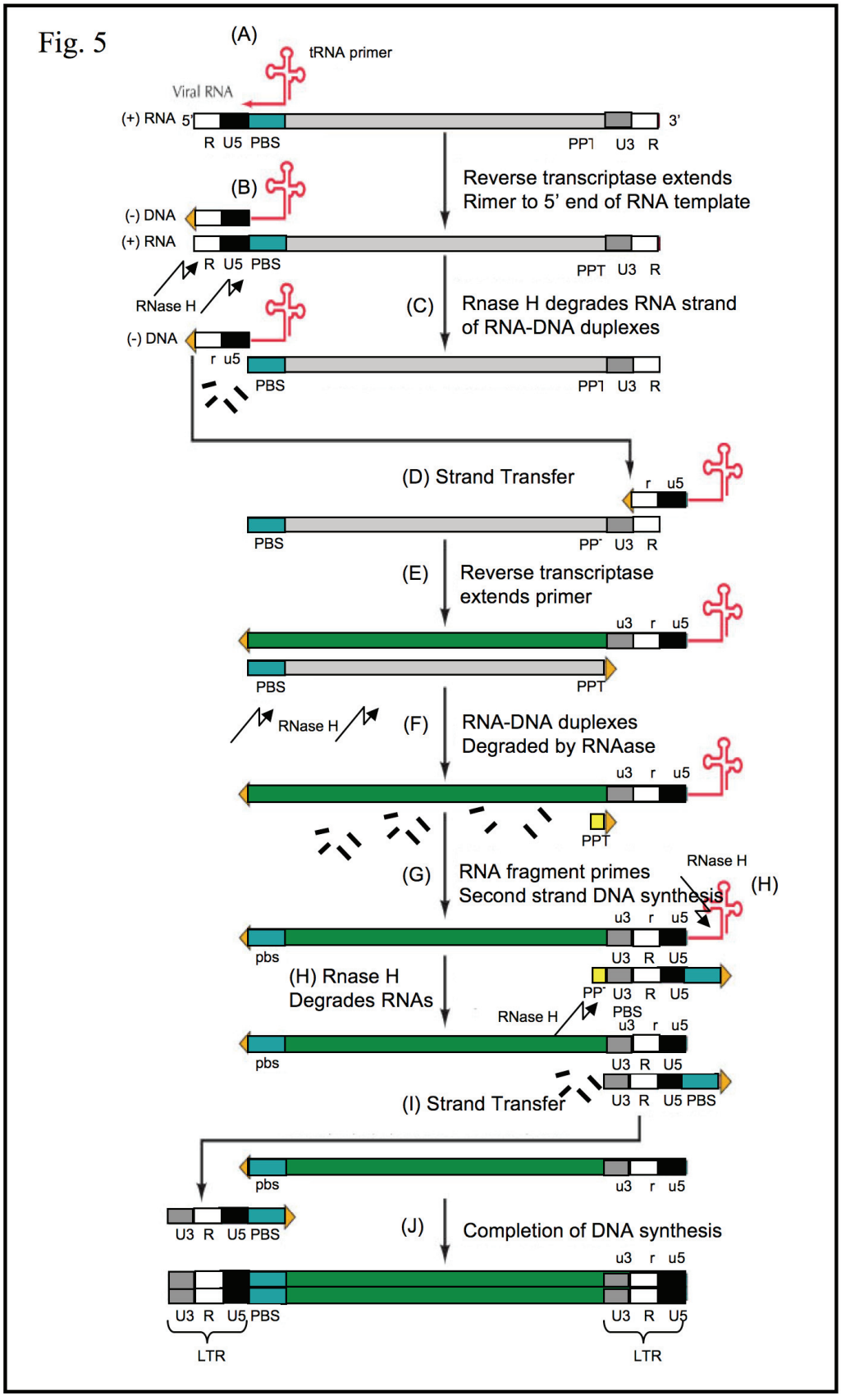

(A)
tRNA primer
Viral RNA
(+) RNA 5'
3'
R U5 PBS
PPT U3 R
Reverse transcriptase extends
Rimer to 5' end of RNA template
(B)
(-) DNA
(+) RNA
R U5 PBS
PPT U3 R
RNase H
(C)
Rnase H degrades RNA strand
of RNA-DNA duplexes
(-) DNA
r u5
PBS
PPT U3 R
(D) Strand Transfer
r u5
PBS
PPT U3 R
(E)
Reverse transcriptase
extends primer
u3 r u5
PBS
PPT
RNase H
(F)
RNA-DNA duplexes
Degraded by RNAase
u3 r u5
PPT
(G)
RNA fragment primes
Second strand DNA synthesis
RNase H
(H)
u3 r u5
pbs
U3 R U5
(H) Rnase H
Degrades RNAs
PPT U3 R U5
PBS
RNase H
u3 r u5
pbs
U3 R U5
U3 R U5 PBS
(I) Strand Transfer
pbs
u3 r u5
U3 R U5 PBS
(J)
Completion of DNA synthesis
u3 r u5
U3 R U5 PBS
U3 R U5
LTR
LTR

Fig. 5 Reverse transcription of single-stranded RNA genome into double-stranded proviral DNA. (A) Cellular tRNA hybridization to the primer binding site (PBS) initiates (B) the synthesis of (-) strand DNA, continues to the 5' terminus of the viral RNA genome, and (C) the RNA-DNA duplex is hydrolyzed by the synthesis-dependent activity of RNase H. Synthesis-independent RNase H hydrolysis of the replicative intermediate, which occurs within 8-10 nt of the template 5' terminus allows a (D) strand transfer of nascent (-) strand DNA to the 3' end of the RNA genome, which is facilitated through homology between the repeat sequences (R and r) at the 5' and 3' ends. (E) Synthesis of the (-) strand of DNA continues along the RNA genome. (F) RNase H activity hydrolyzes the RNA of the RNA-DNA duplex, (G) except for the polypurine tract (PPT), which is resistant to RNase digestion and serves as the primer for the (+) strand synthesis. Synthesis of the (+) strand of DNA proceeds along the (-) strand DNA template and over the 18nt long tRNA primer, where a methylated base causes transient pausing of RT that leads to (H) RNase H-mediated excision of the tRNA primer. (I) Homology between the pbs and PBS regions of the (-) and (+) strands of DNA permit a second strand transfer which relocates the nascent (+) strand DNA to the 3' terminus of the completely elongated (-) strand of DNA. Following the second strand transfer, (J) DNA-dependent DNA polymerase completes the synthesis of both (-) and (+) strands thus producing a double-stranded pre-integration intermediate.

provirus, which is the integrated form of the dsDNA viral genome. This genomic transformation occurs through the activity of one enzyme, reverse transcriptase, which enters the host cell encased in the virion and reverse transcribes viral RNA into DNA during the HIV-1 replication cycle (Fig. 5) (154, 375).

The first step in reverse transcription is negative (-) sense strand DNA synthesis. The signal stimulating viral DNA synthesis is not known. However, conformational changes

in the viral RNA genome at the tRNA primer site may function to initiate DNA synthesis (20-22). The positive (+) sense single strand (ss) RNA of the virus serves as the template for reverse transcription. The $tRNA^{Lys3}$ (405) primer binds to the primer binding site (PBS) on the (+) RNA template, which serves as the initiation site for reverse transcription, and synthesizes (-) strand DNA thus forming an RNA-DNA duplex. The RT enzyme contains an RNase H activity, which degrades the (+) strand RNA in the RNA-DNA replication intermediate (405). The primer terminus, now consisting of DNA, extends through the catalytic center of the polymerase, and RNA-dependent DNA synthesis proceeds towards the 5' terminus of the viral RNA genome. RT then relocates this strong stop (-) sense DNA to the 3' terminus of the viral RNA template to continue with elongation, also described as a strand transfer step.

The (-) strand DNA transfer from the 5' terminus to the 3' end of the (+) strand RNA template is facilitated by the homology of the R and r regions at the 5' and 3' ends (406) and it forms the first copy of the LTR by moving a copy of the U5 region flanking the R sequence to the 3' end of the RNA and continuing synthesis of the (-) strand DNA through the U3 region. The RNA of the RNA-DNA replication intermediate continues to be degraded by the RNase H domain of RT, at about 18 bases behind the point of

synthesis (147, 160). Synthesis of a complete dsDNA molecule requires a primer for generating the (+) sense strand of DNA; and the PPT sequence, located at the 5' end of the U_3 region of the RNA genome, serves that function. The PPT sequence is resistant to RNase H digestion (407), remaining base-paired to the nascent (-) strand DNA where (+) strand DNA synthesis can be initiated and proceed towards the 3' end of the genome. The (-) strand DNA retains the tRNA primer and (+) strand synthesis extends into this RNA only 18 nts, (corresponding to the PBS) after which the replication complex stalls upon encountering a methylated (M) template base.

After the replication complex stalls at the $Me^{58}A$, the nascent (+) strand strong stop DNA, from which the tRNA primer has been released by RT-associated RNase H activity, is subsequently relocated in a second strand transfer step to the 5' end of the genome via PBS homology of the (-) and (+) DNA strands. DNA-dependent DNA synthesis continues at the 5' terminus, producing full-length proviral DNA, flanked at both the 3' and 5' terminal ends by the LTR promoter sequence. The final product of reverse transcription is a double-stranded pre-integration intermediate, which contains both (-) and (+) strands of DNA. This pre-integration intermediate is the form of the viral genomic material that is inserted into the host chromatin in the nucleus (44).

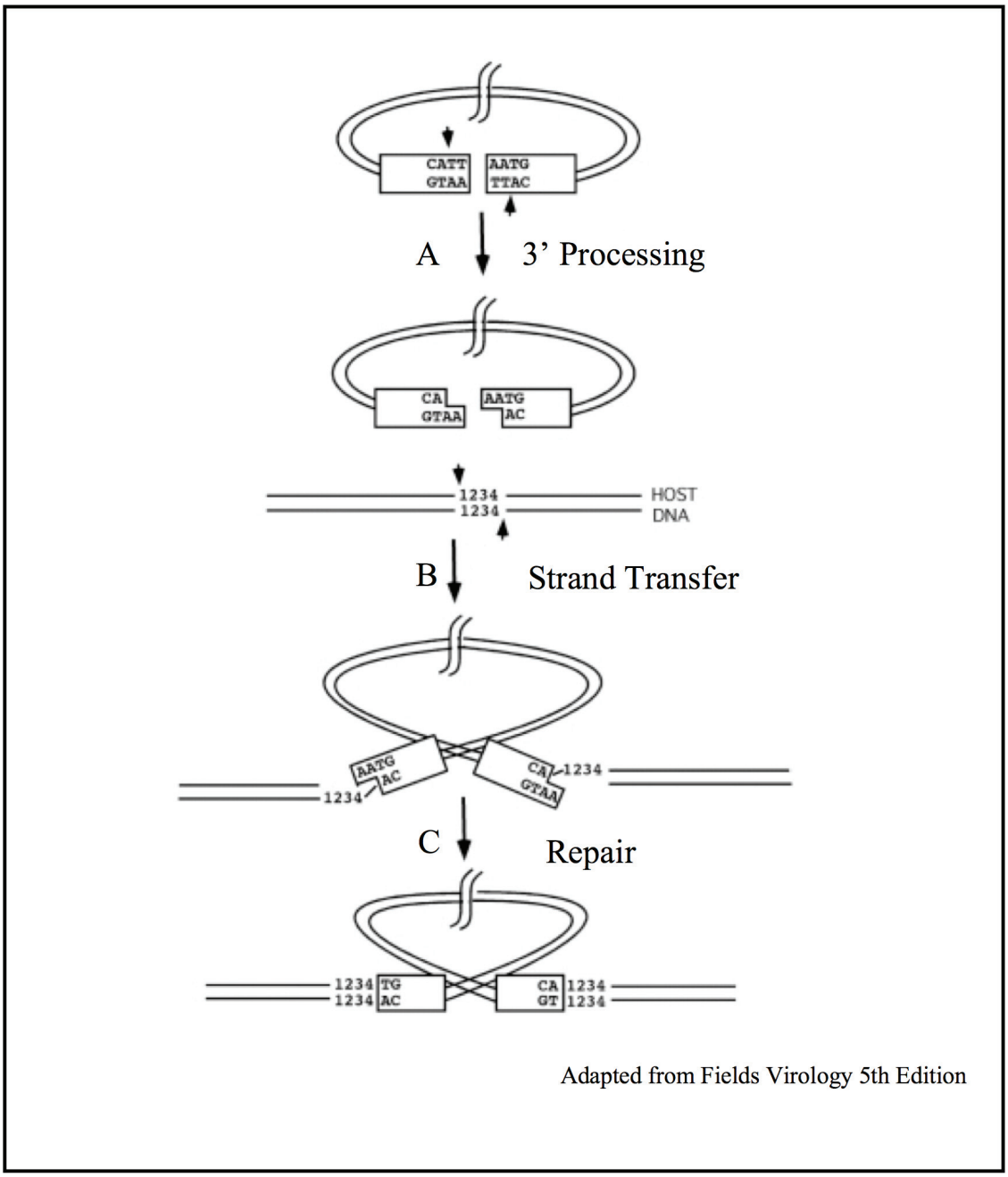


Fig. 6 Integration of viral genome. (A) Full-length viral dsDNA, associated with the pre-integration complex (PIC), undergoes 3' processing or endonucleolytic removal of dinucleotides. (B) A strand transfer reaction is then mediated by the 3' OH, located on the resulting cleaved viral DNA, which makes staggered breaks in both strands of the host target site DNA. (C) The resulting gap in the DNA intermediate is presumably repaired by host cell enzymes.

Integration

The newly translated DNA in the pre-integration intermediate must then be transported across the nuclear membrane into the nucleus for integration (Fig. 3, step 5). Although Vpr (viral protein R) (169, 179) or MA (50, 380) have been implicated in facilitating this transport from the cytoplasm to the nucleus in an adenosine triphosphate (ATP)-dependent manner (58), this area remains controversial. Recent publications, however, suggest that the integrase enzyme may contain the most important signals for nuclear transport of the pre-integration intermediate (103, 220, 388, 392, 403). A combination of proteins and nucleic acids comprises the large pre-integration complex (PIC), which is responsible for producing provirus *in vivo* (56, 82). In complex retroviruses like HIV-1, the PIC components include MA, NC, Vpr, RT, and IN (255), and it requires both the IN enzyme and the correct sequences at the terminal ends of the DNA to hold the PIC together (398). The IN enzyme also recognizes the *att* sites located within the 5' and 3' LTR regions of the viral DNA, which are important for integration (66, 89, 92, 389) and intolerable to small changes in their sequence (93).

Integration is a process unique to retroviral replication (47, 162, 343, 344, 375). Although other viruses may incorporate reverse transcription in their replication strategy,

the retrovirus possesses the unique ability to form a stable association between its genome and the host DNA. The integrase enzyme mediates integration, or insertion of the HIV-1 dsDNA into the host chromatin, in a two-step process (Fig. 6). The first step in the integration process includes IN-mediated cleavage of the 3' terminus of the dsDNA in the PIC, resulting in the removal of a dinucleotide and the formation of a 3' hydroxyl group (OH) ends. In the second step of integration, the 3' OH ends are used in the strand transfer reaction targeted at attaching the phosphodiester bonds at the insertion site (93), through Sn2-type reactions (123). The strand transfer step results in an intermediate containing a small gap, which is repaired by the cellular DNA repair system (337). Mutational analysis suggests that the cleavage and strand transfer activities of the integration process take place at the same active site residues (118, 205, 221, 370) and target similar chemical mechanisms (117, 123, 370). Both reactions involve a hydroxyl group attack on a phosphate group resulting in the exchange of an internucleotide bond. Although integration does not require a specific site within the host genome, HIV-1 prefers to integrate its DNA into transcribed regions (156, 171, 406).

Successful integration of the viral DNA into the host cell chromatin (Fig 3, step 6), which is structured in nucleosomes, and could result in latent infection of the host cell, since the

LTR promoter of the integrated viral DNA, or provirus, is transcriptionally inactive when associated with nucleosomes. The major function of the LTR is to provide the host machinery with signals for expressing provirus efficiently (250). Nucleosomes require unfolding to initiate transcription, which is mediated by chromatin-remodeling complexes that modify histone-DNA interactions. The chromatin-remodeling complexes are activated by cytokines, chemokines, phorbolesters, or various cellular events, enhancing basal transcription of the HIV-1 LTR promoter (83, 170, 194, 237, 309, 371). In the first step of integration, the chromatin-remodeling complex acts on integrated provirus to destabilize the nucleosomal structure and to allow basal transcription. Basal transcription of the HIV-1 provirus based on cellular factors results in very low levels of viral mRNA production, necessitating the functions of the trans-activating protein, Tat, to enhance transcription of the viral genome. After a threshold amount of mRNA is produced, spliced, and exported to the cytoplasm, the viral proteins Tat, Rev, the regulation of expression of viral protein, and negative factor protein, Nef, are translated using host translational machinery. Tat and Rev are then transported back to the nucleus, where Tat exerts its function on the chromatin-modifying complexes and its transactivating activities in the nucleus. Integration marks the end of the early stage and the start of the late phase of HIV-1 infection. Whereas the early stage is characterized by unusual

activities catalyzed by viral enzymes, host enzymes performing normal processes and functions mediate the late phase of infection.

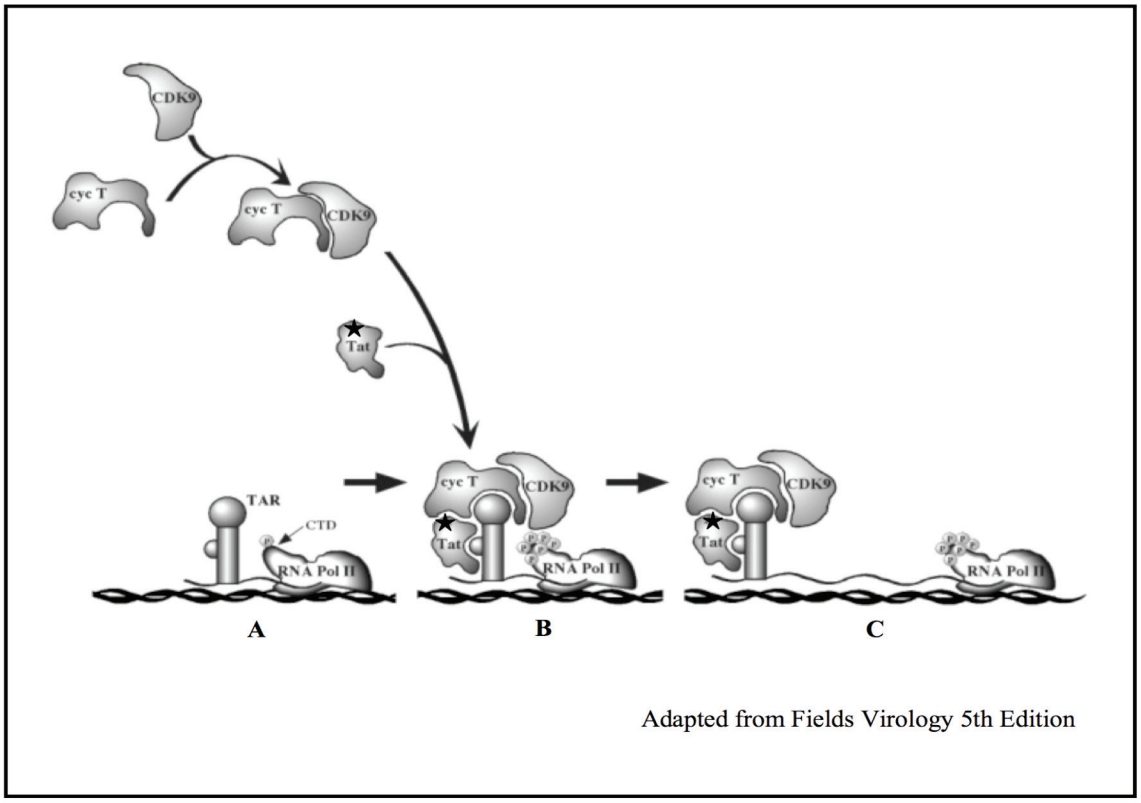


Fig. 7 Tat-mediated transcriptional trans-activation. (A) The RNA polymerase II (RNAP II) RNA processivity is impaired in the absence of Tat binding to the transactivation response region (TAR). (B) Upon interaction of the Tat activation domain (★) with the cyclin T (cyc T)- Tat-associated kinase (CDK9) complex, Tat undergoes a conformational change resulting in a stronger and more specific binding for TAR RNA. (C) Tat recruitment of cyc T and CDK9 within close proximity to the promoter mediates the hyperphosphorylation of the CTD of RNAP II and elongation, or promoter clearance, of the transcriptional complex.

Transcriptional Transactivation

Tat, the HIV-1 transactivator factor, targets the transactivation response RNA, or TAR, located within the R portion of the LTR and present in the transcript where it folds into a

characteristic secondary stemloop structure (107, 109, 110, 133, 338, 358). The novelty of this reaction is that it reads through the transcriptional termination site and it upregulates transcriptional initiation by concurrently interacting with nascent RNA and transcription factors (59, 60, 76, 77, 200, 202, 356, 390). The TAR element is a stable 60 nt stemloop structure located at the 5'end of the HIV-1 transcript that binds to Tat with high affinity (165, 266, 317). The organized activities of the TAR, the host transcriptional machinery, and the viral Tat protein yield result in effective production of viral mRNA.

Through Tat recruitment of a cyclin-dependent kinase, CDK9, associated with transcriptional elongation factors, and phosphorylation of RNAPII, the pre-initiation complex is competent to trigger polymerase release, promoter clearance, and efficient elongation (as shown in Fig. 7) (14, 16, 37, 40, 174). Tat also undergoes posttranslational modification by enzymes. Acetylation of Tat is important for its transactivation activity (39, 100, 203, 290, 316), and methylation inhibits the activity of Tat (33). Studies reveal that cells infected with HIV-1 can release Tat, which can efficiently be taken up by uninfected cells (120, 252). The latter properties may stimulate

abnormal growth in nearby cells, and are further implicated as a possible source of the Kaposi's sarcoma complication of AIDS (13, 119, 379).

Viral RNA Synthesis

Transcription of proviral DNA results in the synthesis of long primary transcripts, which upon processing and splicing yield a small number of stable transcripts. There are a number of viral genomic features that contribute to transcription. The viral LTR mediates synthesis of the viral genomic RNA by the cellular RNAPII machinery (Fig. 3, step 7). The primary viral transcriptional products are processed by cellular machinery in a similar manner to cellular precursor mRNA (pre-mRNA), which is spliced, exported to the cytoplasm from the nucleus, and translated by host protein synthesis machinery (Fig. 3, step 8). Some of the viral pre-mRNA undergoes normal RNA processing and interacts with viral and cellular proteins for active export out of the nucleus. On the other hand, a fraction of the viral pre-mRNA remains unspliced and genome length, and it is packaged into assembling virions in the form of genomic RNA. In the case of complex retroviruses, like HIV-1, there is a single population of RNA that interchangeably functions as mRNA and genomic RNA (199).

Transcription of the HIV-1 provirus is initiated at the cap site, at the junction of the 3' UTR and R region, and proceeds through the 3' LTR into the flanking cellular DNA, with the 3' terminus defined by cleavage following the R sequence and polyadenylation processing (166). Cellular RNAPII synthesizes the genomes of all retroviruses, in addition to cellular mRNA. Therefore, the LTR sequence contains signals that are located on cellular genes. The complex combination of these signals located within the HIV-1 LTR serve the role of facilitating and modulating virus expression. The specific signals located in the HIV-1 LTR 3' UTR relevant to transcription initiation and in order of increasing distance from the cap site are as follows: TATA BOX, multiple binding sites for the SP1 ubiquitous cellular transcription factor, site receptive towards T-cell activation through NF-κB, the nuclear factor kappa-light-chain-enhancer of activated B cells activity, and a negative regulation region.

The viral RNA contains a number of regions significant in transcription. The R region corresponds to the +1 site of transcription and maps the cap site. The leader sequence, or the 5' untranslated region, contains numerous cis-acting RNA structural elements that function in transcriptional regulation. These elements include the TAR element that transactivates viral transcription and modulates translation of viral mRNA, a

polyadenylation loop structure responsible for the addition of a 3' poly (A) tail, the PBS which mediates reverse transcription initiation via $tRNA^{Lys3}$ binding, the SD (splice donor) site which facilitates generation of subgenomic RNA, the DIS (dimer initiation site) that mediates dimerization of the viral RNA genome, and a core packaging signal for directional encasing of the viral genome into assembling virions. Additional regions of the viral RNA include the viral ORF, the 3' UTR, and the 3' poly (A) tail. The 5' cap, 5' UTR, 3' UTR, and 3' poly (A) tail are characteristics exhibited on viral mRNA resulting from the use of similar cellular mechanisms as eukaryotic mRNA.

Viral transcripts experience posttranscriptional processing, including polyadenylation and splicing. Although retroviruses have conserved the addition of a poly (A) signal, it is not absolutely necessary for the replication cycle. The single primary polyadenylated transcript provides both genomes and most mRNA species. All retroviruses use full-length transcripts for their genome, as mRNA for *gag* and *pol* genes (Fig. 3, step 10), and a spliced mRNA encoding *env* (Fig. 3, step 9). Complex retroviruses, like HIV-1, contain elaborate splicing schemes, generating subgenomic species of mRNA encoding a variety of accessory and regulatory proteins in addition to Env (80, 95, 130, 143, 279, 369). The location of the full-length transcript splice start site for HIV-1 is within the

leader region upstream of *gag*; and the corresponding sites for the subgenomic species of mRNA contain their own initiation codons. Regulation of the splicing process exerts balance between the relative amounts of different proteins and genomes maintained for an optimal rate of replication.

Transport of Viral RNA from Nucleus to Cytoplasm

RRE/Rev interaction. Under normal conditions, only completely spliced RNA is transported to the cytoplasm. Rev (regulation of expression of viral protein) is a 116 aa protein that facilitates the transport of incompletely spliced viral RNA from the nucleus to the cytoplasm (72, 107, 159, 404). The *env* gene has conserved a highly ordered RNA stemloop structure of 35 nt called the rev-response element, RRE (72, 107, 159, 241, 404). Rev binds to the head of the central stem of the RRE with great affinity, and recruits additional Rev proteins through cooperative binding to form protein-protein and protein-RNA interactions (18, 168, 186, 206, 365). This results in differential compartmentalization of transcripts containing the cis-acting RRE by amplifying their association with cytoskeleton ribosomes (11, 87, 204). Rev facilitates and regulates the export of viral RNA out the nucleus and is responsible for viral RNA localization with ribosomes, thereby enhancing their translation by cellular machinery (155).

Translational Control

HIV-1 utilizes several mechanisms for translation control. First, it contains a "negative regulator of splicing" (NRS) (158, 259), which is capable of reducing the efficiency of splicing by interacting with the components of the splicing machinery (158). In the context of the complex HIV-1 virus, this additional signal is provided by interaction of the target RNA with the Rev regulator.

Modulation of the levels of completely and incompletely spliced RNA is an active process. In the presence of Rev, full complements of viral mRNA are generated, whereas only multiply spliced mRNAs accumulate in the cytoplasm in the absence of Rev, which results in location of unspliced and singly spliced *env* mRNA in the nucleus. Because the transactivating Tat and regulatory Rev proteins are expressed from multiply spliced RNAs, they accumulate early in the replication cycle in the absence of *gag*, *pol*, and *env* expression. Upon buildup of the *gag*, *gag-pol*, and *env* transcripts, more virion proteins are synthesized, thus creating a temporal regulation of expression.

Translation

The complex HIV-1 genome encodes the minimal retroviral proteins Gag, PR, Pol, and

Env, as well as the regulatory proteins viral infectivity factor (Vif), viral protein R (Vpr), Tat, Rev, viral protein U (Vpu), and Nef (refer to Fig. 8). The three primary translation products, which include Gag ($Pr55^{Gag}$), Gag-Pol polyprotein ($Pr160^{Gag\text{-}Pol}$), and Env (gp160), are synthesized in the form of polyprotein precursors, which then undergo maturation by viral or cellular protease processing. The mature forms of these polyproteins are associated with the mature virion. Gag is synthesized as a 55 kilodalton (kDa) precursor, upon maturation consists of the MA, CA, NC, and the p6 domains. The Gag-Pol polyprotein, or $Pr160^{Gag\text{-}Pol}$, is composed of PR, RT, and IN. The third primary translation product is the Env glycoprotein precursor, gp160, which is cleaved into the gp120 and gp41 Env complex subunits by cellular proteinase. The remaining proteins are the result of primary translation products of splicing mRNA (shown in Fig. 8).

Following nuclear export, the structural proteins and enzymes are synthesized from unspliced mRNA (Gag and Gag-Pol) on free ribosomes (Fig. 3, step 10), whereas the Env precursor is expressed from incompletely spliced viral mRNA on membrane-bound ribosomes (Fig. 3, step 9). There are strong implications that three different mechanisms are used to translate full-length Gag polyprotein, and these include the following:

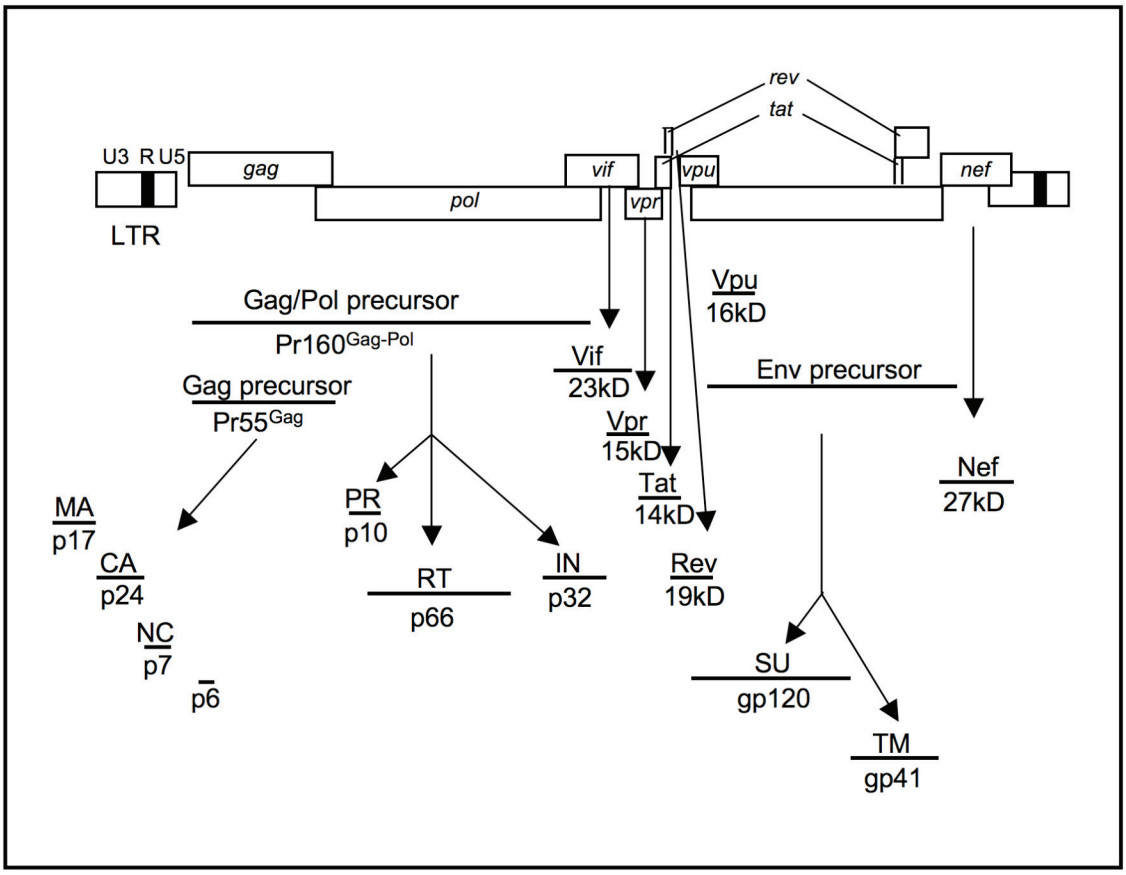


Fig. 8 Genome organization and viral proteins of HIV-1. The HIV-1 genes are depicted in their location within the proviral genome. The corresponding sizes of the primary translation products and mature viral proteins are indicated by arrows below the complex retroviral genome.

ribosomal assembly, entry, and scanning initiated at the RNA 5'cap; internal ribosome entry segment (IRES)-mediated internal entry of the ribosome from a 5' UTR location; and internal ribosomal entry through the IRES located in the coding region of the RNA. The viral RNA-containing segment called the IRES is located in the 5' UTR and in part of the coding region of mRNA (38, 43, 172, 278, 282). This segment of the mRNA

facilitates translation initiation by directly binding the ribosome to the 5' UTR of viral mRNA (188, 231, 329). There is growing evidence that the IRES may actually be located within the *gag* region, as evident by the presence of isoforms of the Gag polyprotein precursor observed for HIV-1, HIV-2, and SIV (43, 172, 278); and further investigation revealed that these isoforms result from an IRES-mediated alternative translation initiation. Mutational studies abrogating the synthesis of these isoforms of Gag resulted in a significant reduction in viral infectivity, implicating a role in viral pathogenesis of full-length polyprotein from the upstream authentic AUG codon (43, 172).

In additional to transactivation of transcription of viral mRNA, Tat and the TAR element play a role in the translational control of protein synthesis. The TAR element also controls viral protein translation both in *cis* and in *trans*. This regulation occurs through an interferon (INF)-induced, dsRNA-dependent serine/threonine protein kinase R (PKR) phosphorylation of the alpha (α) subunit of the eukaryotic initiation factor (eIF)-2 (239). The phosphorylation of eIF2 almost completely halts protein synthesis by inhibiting the recycling of eIF2B-GDP (Guanine diphosphate) to eIF2B-GTP (Guanine triphosphate) (eIF2B GDP-GTP exchange factor//translation initiation factor eIF2B delta subunit)

(293). Tat functions to inhibit PKR by competing with eIF2 (239, 321, 336, 346), the natural substrate for PKR, and by blocking PKR autophosphorylation (53, 254, 321) through a direct interaction with PKR (254, 321). Further, a cellular TAR-binding protein (TRBP) has been shown to bind strongly to TAR, transactivate the HIV-1 LTR with Tat in a synergistic manner (145), and inhibit PKR activity through protein binding.

During translation, a delicate regulation of Gag and Pol protein expression is required. The first reading frame encoded on the viral genome is the *gag* gene, which is located upstream from *pol*. Ribosomal synthesis of protein continues until it encounters a stop codon in *gag*, after which the synthesis strategy changes by means of a -1 nt frameshift mechanism to just before the stop codon (187, 243). This strategy creates a structural change in the peptidyl tRNA binding site, or P-site, of the tRNA and impedes ribosomal translocation (274), resulting in the ribosome leaving *gag* and translating *pol*. HIV-1 conserves the synthesis ratio of 20 to 1 Gag to Gag-Pol polyproteins; and disruption of this ratio significantly reduces the infectivity of the virus (342).

The structural proteins are synthesized as two polyproteins, including Gag and Gag-Pol. The Gag polyprotein is composed of MA-CA-p2-NC-p1-p6, and MA-CA-p2-NC-[*S*-

transferase-transframe region (TFR)]-PR-RT-IN represents the Gag-Pol precursor protein (218, 289). These precursors consist of subgroups connected by peptide bonds, which are targeted by PR for cleavage during maturation.

Following the synthesis of the Gag-Pol polyprotein, the protease domain exhibits a monomer fold that spans at least 10-90 residues. Release and maturation of PR, a 99 aa aspartic acid protease (289, 297), from the Gag-Pol precursor occurs by means of two distinct steps. The first cleavage step occurs at the TFR-$p6^{Pol}$ junction to generate the intermediate precursor $p6^{Pol}$-PR through hydrolysis of the flanking peptide bonds, which is mediated by the folding and dimerization of PR in the Gag-Pol precursor. The intramolecular cleavage of the $p6^{Pol}$-PR, the rate-limiting second step in protease release, and generation of mature protease is concurrent with a large increase in catalytic activity, which will be discussed further in a following section. (233, 234) The increase in proteolytic activity and formation of stable PR dimers occurs concurrent to the rate-limiting step involving the second cleavage reaction, and subsequent processing of the Gag-Pol precursor downstream of the PR domain occurs via intermolecular reactions (391). Because the relative stability of the precursor dimer is much lower than the mature

dimer protease, this may facilitate the initial recruitment of assembling components of the virion to occur before the proteolytic activity is initiated (391).

The key player during assembly of the HIV-1 virion is the Gag polyprotein precursor $Pr55^{Gag}$, which drives virion formation through protein-protein, protein-RNA, and protein-lipid interactions. Gag organizes the incorporation of the major HIV-1 components into the assembling particle. The Gag precursor, translated from unspliced viral mRNA on free ribosomes, encodes the structural components of the virion including MA, CA, NC, p6 domain, and the SP1 and the SP2 spacer proteins (Fig. 9).

The structural framework of the immature particle requires the multimerization of Gag. The MA protein plays a structural role in assembly and is significant in Gag trafficking, and Env incorporation during assembly (57, 65, 132, 263, 264, 399). The major determinants of Gag multimerization are the CA C-terminal domain (CTD) and SP1 (CA-SP1). CA is composed of two individual helical domains, the NTD and CTD, connected by a short, flexible linker (29). The CA CTD mediates Gag multimerization by means of dimerization; these dimers are formed by parallel packaging of the helix 9 component of the CA CTD against its symmetrical counterpart (393). The CA-SP1 junction is a strong

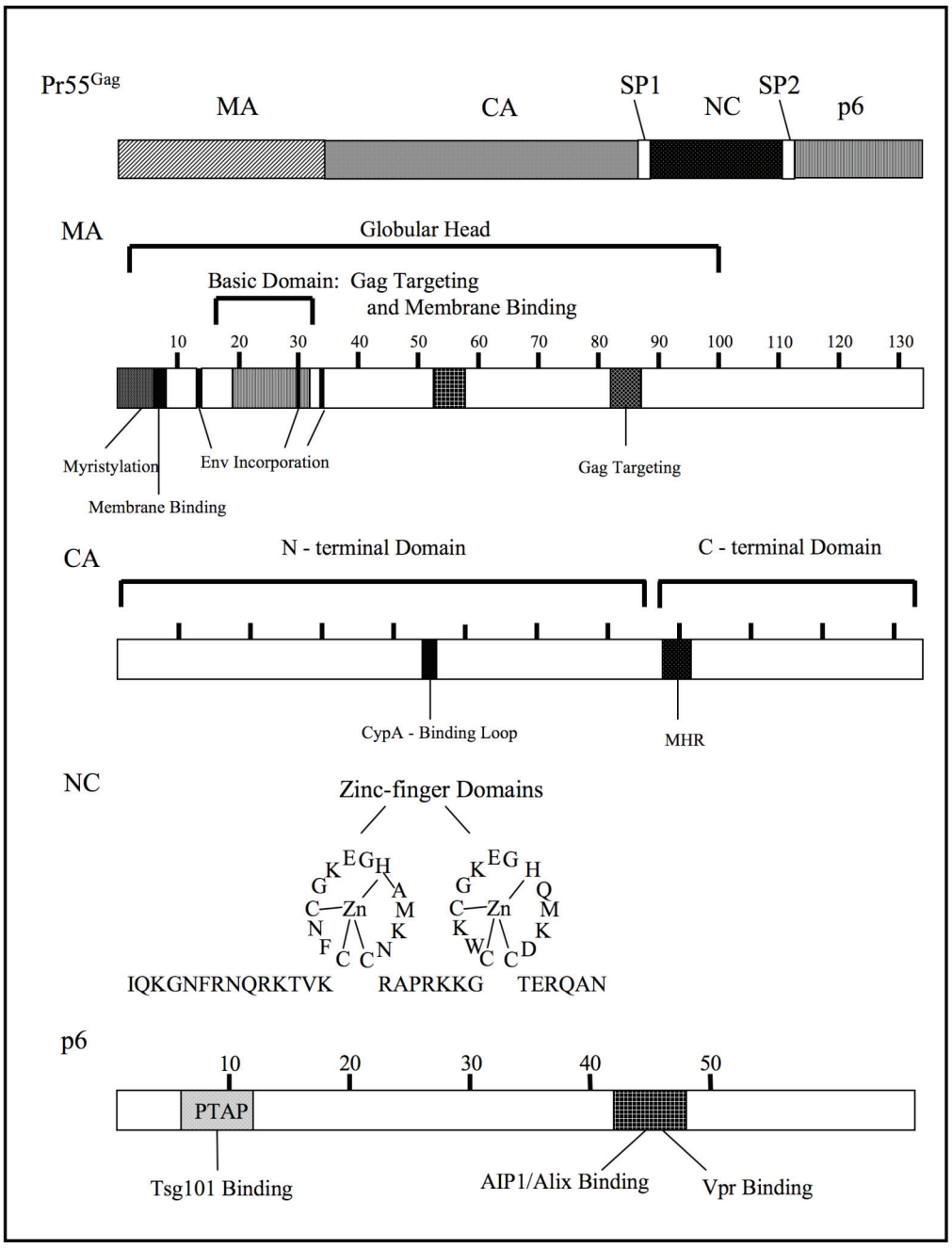


Fig. 9 Linear representation of the major HIV-1 Gag structural proteins. (A) Gag precursor protein, $Pr55^{Gag}$, with the location of its domain groups include matrix (MA), capsid (CA), nucleocapsid (NC) and p6. The spacer peptides SP1 and SP2 are also indicated. (B) MA domain. (C) CA domain. (D) NC domain. (E) p6 domain. The numbers correspond to aa position and the major functional domains are shown.

interaction within the Gag dimer, and this higher order multimerization is essential to particle formation (157, 223, 224, 263, 285). During particle assembly, CA has also been shown to bind to the cellular protein cyclophilin A (CypA) through a proline-rich loop in the NTD (138). High amounts of CypA have been found incorporated into assembled virions (125, 236, 363); and the relationship between CA and CypA has been suggested to provide some protection against the host restriction factor TRIM5α (**TRI**partite **M**otif), which is a protein found in most primate cells and a novel, innate immune response against retroviruses (164, 280, 334, 348). NC also contributes to Gag multimerization and assembly via its I (interaction) domain.

The I domain primarily consists of two motifs resembling zinc fingers that are flanked by highly basic sequences (262, 357). The zinc-like fingers contribute to the specific encapsidation of the viral genomic RNA. The basic residues actually contribute to the function of the I domain, which appears to promote the multimerization of $Pr55^{Gag}$ through its high affinity for full-length RNA, which brings Gag molecules within a close proximity to align and to interact (55, 56, 70, 271).

The Gag polyprotein undergoes multimerization, forming contact points between multiple domains of $Pr55^{Gag}$. The assembly of the viral components occurs at the site of

budding in most cell types. Assembly occurs at the PM in most cell types, including T cells. In certain other cell types, including primary monocyte derived macrophages, there still seems to be some controversy of whether assembly occurs at intracellular compartments defined as multivesicular bodies (323). The MA domain mediates Gag binding to targeted membrane. During virion budding, the immature virion becomes wrapped in a host cell-derived lipid bilayer. Release of HIV-1 from the host cell is catalyzed by elements of the ESCRT-1 complex (endosomal sorting complex required for transport) machinery as well as other factors recruited to the assembly site released by P(T/S)AP late domain motif. Although $Pr55^{Gag}$ is the only virion encoded protein necessary for the assembly and release of immature VLP (viral like particle), production of mature infectious virions requires the encapsidation of the viral genomic RNA and incorporation of both the mature Env complex and the Gag-Pol precursor, which are actively recruited for assembly by $Pr55^{Gag}$. The interaction of the MA domain with the mature Env complex is necessary for incorporation of Env embedded in the cellular membrane into the lipid bilayer of the particle. The NC domain of $Pr55^{Gag}$ is responsible for encapsidation of full-length, unspliced RNA genome of virus into assembling virions. The remaining components, including PR, RT, and IN, are incorporated into assembling particles via the interaction of $Pr55^{Gag}$and the Gag component of $Pr160^{Gag\text{-}Pol}$.

Encapsidation of Viral RNA Genome

Encapsidation of two copies of full-length viral genomic RNA into immature viral particles is essential to virion infectivity. HIV-1 genomic RNA is specifically selected for packaging from the cytosol, containing an excess of cellular and spliced viral mRNA. NC mediates the packaging of the viral RNA through interaction with a region in the 5' end of the genome variously termed the ψ element, encapsidation element (E), or viral genome packaging signal (88, 190, 313). The functional role of NC is to mediate the efficient and specific packaging of the viral RNA genome. The two zinc finger-like structures mediate the specificity of the reactions by binding zinc to the NC, which promotes folding of the structure and such that the "fingers" are brought into close proximity because of the flexible linker (262, 351, 357). The basic sequences flanking the zinc finger like structures are implicated in bonding with nucleic acids (70, 90, 94, 304, 335), along with SP1 of $Pr55^{Gag}$ (199).

Newman and colleagues used an NMR, nuclear magnetic resonance, relaxation and sedimentation equilibrium (SE) study to show that the CA CTD and NC domains are folded as previously described, however, the p2 segment and the connected thirteen residues at the CTD of CA and the thirteen residues at the N-terminus of NC are flexible

(275). Further analysis of these forty residues also suggests a more helical than coiled coil conformation, suggested to explain the slow rate of proteolysis of the CA-p2 junction (275). Further, this group also described that CA CTD-p2-NC forms dimers and at a self-assembly rate similar to intact CA protein, suggesting that p2 des not significantly influence Gag dimerization (275).

The ψ element consists of about 120 nt segment of genomic RNA, which participates in packaging and contains the entire 5' UTR domain and the 5' half of the *gag* gene (88, 190, 249). The ψ signal mediates genomic RNA packaging via a stable secondary structure composed of four hairpin stemloops or SL (SL1, SL2, SL3, SL4), spaced close to each other (19, 71, 163, 167, 326). The SL1 promotes RNA dimerization and may be linked to RNA packaging (324). SL4, which like SL1 does not bind very well to NC (8, 216), is also thought to contribute to the RNA-RNA interactions that stabilize the tertiary structure of the ψ site (8). The key determinants driving the interaction between NC and the zinc-fingers via high affinity binding are SL2 and SL3 (7, 94). SL2 also contains the major splice donor suggested to function as a possible mechanism for encapsidation of specific full-length genomic viral RNA (7, 88, 190, 313). The ψ site packaging signal

maps to the 5' end of the genomic RNA, along with three other structural elements: the TAR, polyA hairpin, and the PBS.

Transcription of the integrated proviral DNA produces genome length RNA. Accumulation of Gag molecules in the cytoplasm facilitates a switch from translation to packaging as Gag binds to the genomic RNA at the 5' IRES-ψ-signal. Genomic RNA is continuously translated, allowing newly formed Gag molecules to bind to the pool of non-translated genomic RNA, virus formation follows (217).

Trafficking of Virion Components

All virion components must converge at the site of viral assembly, which occurs at the PM in most cell types including T cells and in intracellular compartments or MVBs (multi-vesicular bodies) of other cell types such as monocyte-derived macrophages (148, 277, 286, 288, 298, 311). $Pr55^{Gag}$ is directed to the PM via a PM-binding signal M located within the N-terminus of the MA domain. The PM-binding signal M consists of a "myristic acid" moiety covalently attached to the N-terminal Gly (Glycine) of the MA domain (132, 152). The second component of this membrane-binding signal is a basic region from residues 17-30 (401, 407). The p6 domain interacts with the MVB

machinery, although its abrogation does not alter the targeting site of Gag (286, 322). Trafficking of Gag to different locations in different cell types may result from a differential presence of host cell factors and their regulation of the assembly site. One such example is the PI(4,5)P2 (phosphoinositide phosphatidylinositol (4,5) bisphosphate), a lipid that regulates the localization of different proteins to the PM (253). PI(4,5)P2 interacts directly with MA (127, 284, 325, 345). Adaptin protein (AP)-3, another example of a cellular factor, has also been shown to facilitate Gag intracellular trafficking to MVBs (95, 319).

Envelope. Briefly, synthesis of the Env precursor, gp160, occurs on ribosomes associated with the ER. A type I integral membrane protein, Env is anchored into the membrane by a hydrophobic stop-transfer signal. The gp160 precursor undergoes folding and oligomerization, after which it is transported to the TGN, trans-golgi network, where it undergoes extensive glycosylation and protease cleavage into gp120 SU and gp41 TM components. The SU and TM components of the mature Env complex are non-covalently linked. The mature Env complex is then transported to the PM, where it is either incorporated into budding virions or internalized into intracellular compartments. The cytoplasmic domain of gp41 mediates the endocytosis of Env, and the membrane-proximal Yxxϕ (where Y is tyrosine, x is any aa, and ϕ represents an aliphatic or

hydrophobic residue) motif has been established as the major determinant of endocytosis within the HIV-1 Env CD. This tyrosine-based motif in gp41regulates Env endocytosis through its association with the μ2 chain of AP-2 (31, 283). Env has also been shown to localize in MVBs (283, 320). Retrograde transport of Env from MVBs to the TGN has been reported to be facilitated by the interaction between the tail interacting protein of 47 kDa (TIP47), a mannose-6-phosphate receptor (MPR) known to transport lysosomal hydrolases from the endosome to the Golgi, and the gp41 CD. This association appears to be important for Env incorporation into assembling virions (30, 232). Env and $Pr55^{Gag}$ independently traffic to both of the HIV-1 assembly sites at the PM and at the MVBs.

Acquisition of Lipid Bilayer and Associated Env Glycoproteins

Assembly and release of the HIV-1 virion is driven by a highly ordered multimerization of Gag at the plasma membrane. Upon $Pr55^{Gag}$ binding to the plasma membrane, which is critical to assembly and Gag multimerization, the membrane is curved outward and forms a spherical-like particle. The M domain mediates Gag binding to the PM. The M domain consists of "myristic acid" and basic residues, which are clustered in a face of the MA trimer (175). The basic residues may be positioned towards the membrane, thereby creating a membrane binding surface, and oriented to interact electrostatically with the

negatively charged phospholipid head groups on the inner surface of the PM (175). For example, PI(4,5)P2, which is normally located on the inner side of the PM (23, 127, 325), anchors MA to the PM by nestling its inositol head group and the 2' fatty acid chain lipid into a hydrophobic pocket in MA and by the interaction of negatively charged phosphates of salt bridges with basic residues in MA. A myristyl switch (9, 10), which describes a change between an exposed versus sequestered conformation of the myristic acid moiety (361), regulates MA binding to the plasma membrane (173, 314, 352, 361, 408). The myristyl switch may be catalyzed by MA-PI(4,5)P2 interaction which facilitates membrane binding (325).

Particle Release

The HIV-1 replication cycle requires virus release following assembly and budding of the virion in order to infect the next target cell. As the viral particle buds from the surface of the producer cell, the virion becomes enveloped with the cellular lipid membrane. As the membrane wraps around the virion, a neck-like structure appears linking the virion envelope to the producer cell. The neck of the budding HIV-1 virion must pinch off to release the virion. HIV-1, as well as other enveloped RNA viruses, usurp host factors and a cellular budding machinery to mediate their release (96, 128, 265, 305).

The viral assembly site directs how the virions are released: the PM assembly site directs the release of progeny virus into the extracellular space, and for MVB assembly the release of virions into the extracellular space occurs through an exosomal pathway, which includes MVB fusion with the PM (277, 298, 299, 311). The interaction between the virion and the host release factors is through the "late" or "L" domain of $Pr55^{Gag}$ at its PT/SAP motif near the N-terminal of the p6 domain (151, 181). Mutagenesis of this motif results in the accumulation of virions attached to the cell membrane by a thin stalk that are unable to be released, (97, 151). A similar arrest is observed at the MVBs in Macrophages (97). Three different late domains have been classified, including PT/SAP, PPXY, $YP(X)_nL$, all of which have been identified in retroviruses (96, 128, 265, 305). Functional late domains have been identified in other enveloped RNA viruses, such as filo, rhabdo, arena, orthomyxo, and paramyxo, suggesting that they may also utilize a similar mechanism in viral release. Some viruses contain two "L" domains, the exact function of which remains to be explained. The late domain provides binding sites for certain host cell factors.

L domains are small regions of the protein that are highly conserved; however, they maintain their function upon relocation within Gag or between different viruses that

utilize a late domain (222, 295, 400). Host proteins associated with mediating viral release link to the cellular ubiquitylation and the endosomal (MVB) protein-sorting pathway (described in Fig. 10) (96) (128, 265, 305). Multi-protein complexes, including ESCRT-I, -II, and –III, sequentially recognize the recruited cellular proteins within the virion, and in the final step of viral release the Vps 4 (vacuolar protein sorting 4) protein catalyzes their disassembly from the cellular membrane.

TSG101, the tumor susceptibility gene 101, is a member of the ESCRT-I complex that interacts with the HIV-1 p6 PT/SAP motif (144, 305, 373), and this interaction has been determined to be a primary requirement for HIV-1 release (98, 144, 246). The PPXY and $YP(X)_nL$ late domains are also linked to the ubiquitylation and endosomal MVB protein sorting pathways. The $YP(X)_nL$ domain in HIV-1 and in EIAV (Equine Infectious Anemia Virus) has been shown to bind to the ALIX (AIP-1) protein (247, 267, 354, 376), providing a link between ESCRT-I and ESCRT-III (98, 151, 247, 267, 308, 354, 355, 376). The PPXY domain has been shown to bind to the Nedd4 family of E3 ubiquitin ligases, but the mechanism by which this interaction facilitates viral release remains to be explained (96, 265). The Vps4 protein catalyzes viral release, and interruption of its function inhibits viral release (144, 245, 265, 341, 362, 376).

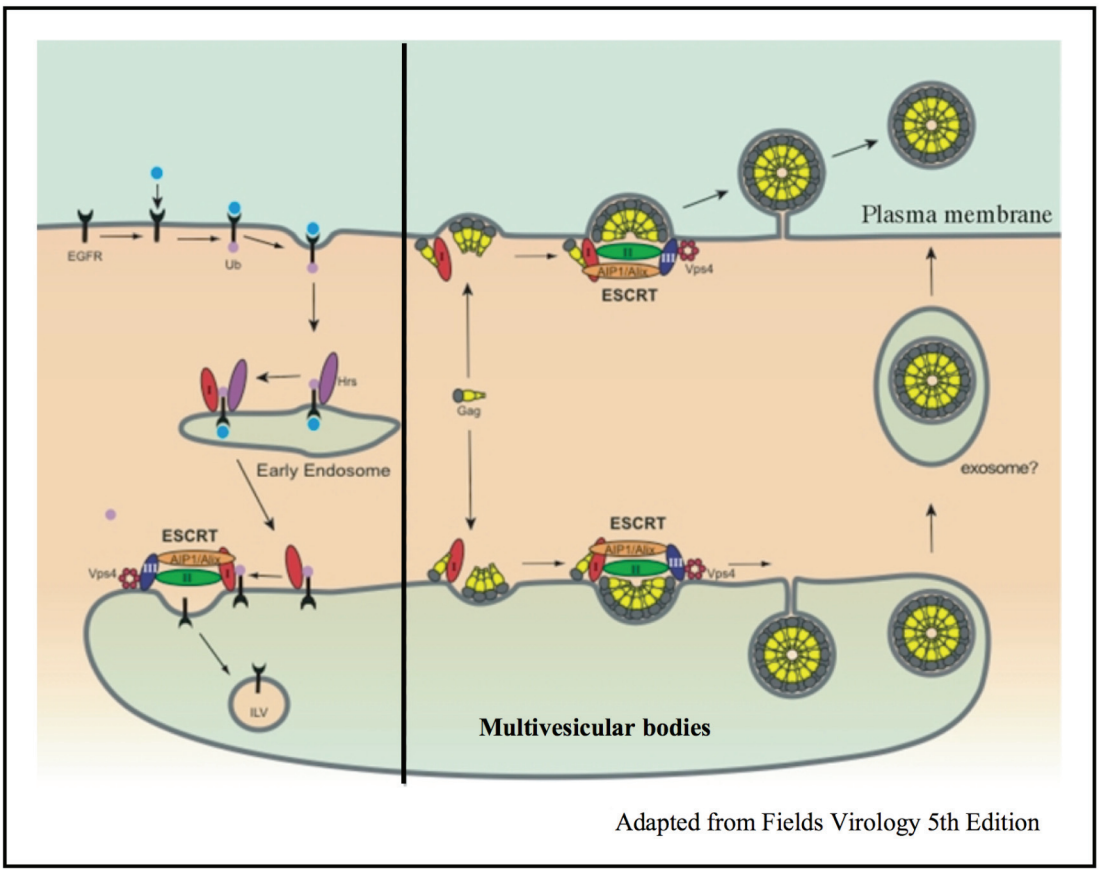


Fig. 10 Model for retrovirus release. On left. The Hrs protein recognizes and sequesters ubiquinated cargo (Ub) at the early endosomal membrane. Hrs recruitment of the endosomal sorting complexes required for transport, ESCRT-I, by directly interacting with Tsg101. ESCRT-I thereby recruits ESCRT-II and –III, additional components of the MVB pathway. AIP-1/Alix is recruited to the assembled complex and, in the context of mammalian cells, links ESCRT-I and –III. Vps4, the AAA adenosine triphosphatase (ATPase), is essential in the disassembly of the complex after intralumenal vesicles (ILV) inward budding into the lumen of the endosome. On right. Depiction of viral hijacking of the MVB sorting machinery for particle release. Virion assembly and budding is shown at the PM, and virion release (in macrophages) is described to occur through the exosome pathway following virion assembly at the MVB.

Maturation

Following virion release, the immature particle undergoes maturation, which involves PR-mediated cleavage of $Pr55^{Gag}$ and $Pr160^{Gag\text{-}Pol}$ polyproteins into their respective individual components. During this process, the MA domain continues to interact with the viral membrane, the NC condenses with the viral genome, and CA morphologically reassembles to produce a closed conical capsid shell that encases the NC-RNA complex. Maturation of the released virion is required for entry and for viral disassembly upon entry into a new target cell, a requirement for viral replication. (402, 338)

Thus far, this dissertation has addressed the major components in each step of the viral life cycle. The description of the viral life cycle initiated with the attachment of virion to the host target cell and has come full circle to virion budding, release, and maturation. The mature virion is competent and necessary to begin the next round of infection that commences with viral entry into the host target cell. The focal point of this body of work will now transition from the HIV-1 life cycle to the Env glycoprotein and its role in HIV-1 infectivity.

The mature Env complex has been introduced in this thesis as an essential component of HIV-1 infectivity, requiring both its incorporation into assembling and budding virions as well as the ability of the Env glycoproteins to facilitate attachment, fusion, and entry of the virion into host target cells. Env trafficking and co-localization with Gag is essential

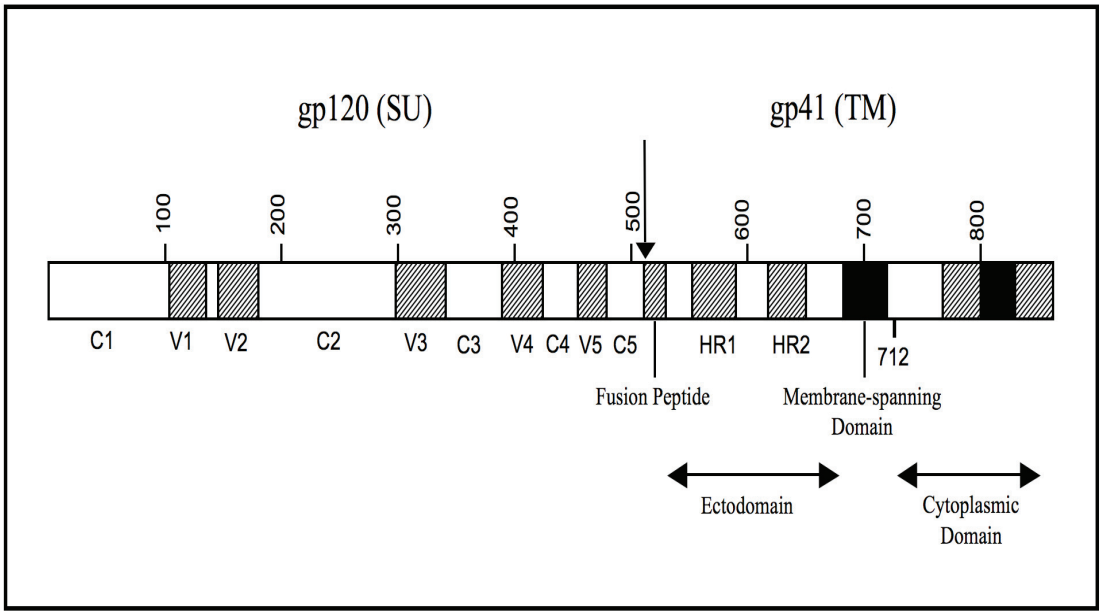


Fig. 11 Linear organization of the HIV-1 Env glycoprotein. The vertical arrow indicates the location of the gp160 cleavage site to gp120 and gp41. For gp120, the textured boxes represent the variable domains (V1-V5) whereas the open boxes depict conserved sequences (C1-C5). The gp41 component of Env is composed of the ectodomain, the membrane-spanning domain (MSD), and the cytoplasmic domain (CD). The gp41 ectodomain consists of the N-terminal fusion peptide and two heptad repeats (HR1 and HR2). The MSD is represented by a solid black box. The CD depicts the location of the membrane-proximal tyrosine motif at residue 712 and the two predicted helical domains (lytic peptides 1 and 2). The numbers correspond to the aa location.

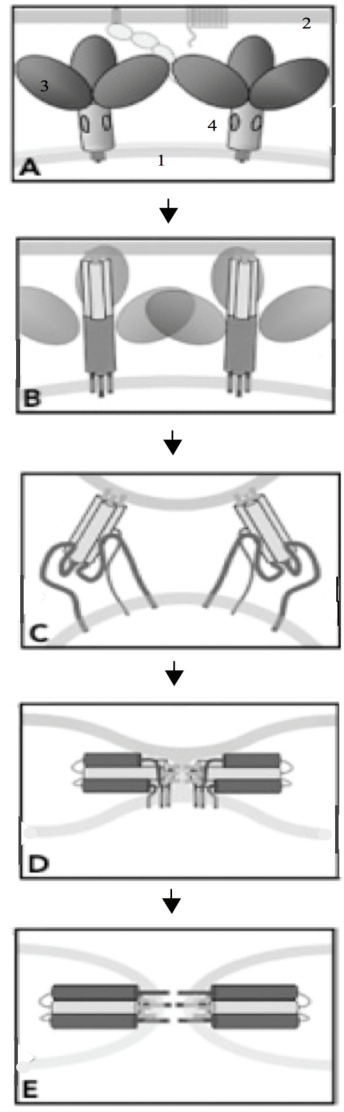
to glycoprotein incorporation into virus particles, as described previously, and is directed by the Env CD. The following section is dedicated to the importance of Env in attachment to the host cell, fusion of the viral and cellular membranes, and entry of the viral core into the host cytoplasm, leading to the crux of this dissertation: a functional analysis of the HIV-1 envelope cytoplasmic domain.

Role of the Envelope Glycoprotein in HIV-1 Infectivity

Contributions of Envelope during Viral Entry

In the context of the virion, Env (Fig. 11) exists in a resting state. Binding of gp120 to the CD4 receptor induces a conformational change in Env, which facilitates gp120 binding of co-receptor on the target cell. The formation of a ternary gp120-CD4-co-receptor complex is followed by gp41 adoption of a major conformational change, which enables the fusion peptide to insert into the lipid bilayer of the target cell. Formation of a six-helix bundle, composed of antiparallel interactions between the gp41 heptad repeats (HR), HR1 and HR2, bring the viral and cellular membranes together in close proximity to facilitate the fusion process (Fig. 12).

Fig. 12 Envelope-mediated Fusion



Adapted from Fields Virology 5th Edition

A. Receptor binding. HIV or SIV Env binding of CD4 receptor and CXCR4 or CCR5 co-receptor. Viral membrane at bottom (1); host cellular membrane at top (2); receptor- and co-receptor-binding gp120 component of Env; fusion-mediating gp41 component of Env.

B. Dissociation of gp120 component from receptor and projection of gp41 fusion peptide toward the target host cell membrane. This state is called the "prehairpin intermediate".

C. Rearrangement of C-terminal end of Env. The helical region 1, HR1, forms the inner core of trimer and the helical region 2, HR2, folds back and brings the two membranes together. The N-terminal fusion peptide of gp41, which is inserted into the target cell membrane, and the C-terminal transmembrane domain, which anchors Env into the viral membrane, are brought within close proximity to each other.

D. Formation of hemifusion intermediate.
E. Fusion pore formation.

Attachment

The first step in the viral life cycle is the interaction between gp120 component of Env and CD4, the major cell surface receptor for the primate lentiviruses (34). CD4, a 55

kDa trans-membrane protein associated with the superfamily of immunoglobulins (Ig), is composed of four distinct extracellular domains (D1-4), a hydrophobic trans-membrane domain, and a highly charged CD (238). CD4 provides stability between MHC II (major histocompatibility complex type II) on antigen presenting cells (APC) and T cell receptors on the cell surface of T lymphocytes. The gp120 component of Env binds to CD4 with high affinity. The CD4 binding site on gp120 is located at the C3 and C4 domains of gp120, which are conformation-dependent and discontinuous, and interacts with the N-terminus of the extracellular domain of CD4. Binding of CD4 induces a conformational change in gp120 and gp41, which activates the fusogenicity of the Env complex (330, 364). CD4 is the major receptor for both HIV and SIV, however, HIV-induced fusion and viral entry require additional factors.

A number of studies reveal that HIV-1 entry requires co-receptors from the G protein-coupled receptor superfamily of 7-transmembrane domain proteins (4, 69, 99, 121). CCR5 and CXCR4, two major co-receptors required for HIV and SIV infectivity, are also important in determining HIV-1 tropism. Co-receptor requirement and HIV-1 tropism also provide a new nomenclature for HIV-1 isolates, including M-tropic for CCR5 or R5 isolates, T-tropic for X4 or CXCR4 utilizing isolates, and R5/X4 for dual

tropic isolates (25). In some cases, certain isolates are able to enter into target cells in a CD4-independent manner (115, 244, 306).

The SU component of Env, gp120, consists of an inner and outer domain connected by a "bridging sheet", composed of segments derived from the C1, C2, and part of the C4 region organized into a four-stranded, anti-parallel beta (β)-sheet. The conformational change that occurs in Env upon CD4 binding of gp120 results in exposure of highly conserved regions of gp120 that interact with the co-receptor, and antibody (Ab) binding studies reveal that the interaction between gp120 and CCR5 are located within the bridging sheet (213, 395). In the case of isolates that replicate in a CD4-independent manner, these epitopes are constitutively exposed (177, 211) and tend to be hypersensitive to neutralization.

CD4 receptor and chemokine co-receptors promote the infection of T lymphocytes and macrophages, but HIV-1 also utilizes dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), a C-type lectin expressed in dendritic cells (DC), to enhance infection *in trans*. Following internalization of HIV-1 particles into dendritic cells, the virions localize to the virological synapse, junctions between

virus infected DCs and susceptible T cells, and their concentration at those junctions facilitates the transmission of virus (142, 251).

Membrane Fusion

The fusion peptide, a hydrophobic domain located at the N-terminus of gp41, has been shown to facilitate fusion of HIV-1 virion and target cell membranes (Fig. 12) (130). Two amphipathic HR domains, which are located at the carboxy (C)-terminus of gp41, are also required for the fusion process, as evident by impaired infectivity resulting from mutation of HR1 (112). X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy (52, 64, 360, 383) revealed a trimeric structure of the fusion peptide, in which HR1 and HR2 are stacked in an anti-parallel manner to generate the six-helix bundle. HR1 forms a coiled-coil in the center of the bundle, while HR2 is packed into hydrophobic grooves on the exterior of the bundle. The structure of the gp41 ectodomain is similar to the influenza HA2 fusion peptide (activated by low pH), suggesting that the mature Env complex of HIV and SIV may also activate membrane fusion by a "spring-loaded" mechanism analogous to that described for influenza (45).

Binding studies reveal that gp41, in the native form, interacts with HR2 only after CD4 binding, suggesting that a conformational change in HR1 and HR2 is required for the fusion process (135). The gp41 CD contributes to fusion modulation by regulating the cell surface expression of the mature Env complex and by the conformational dependence of gp120 and the gp41 ectodomain on the gp41 CD (35, 398). A direct interaction between gp41 and the lipid bilayer catalyzes the fusion reaction of viral and cellular membranes, however, a number of domains in gp120 facilitate the activation of Env fusogenicity.

The variable 3 (V3) loop, shown to elicit specific neutralizing Ab, is also critical to the membrane fusion process. Mutation of the V3 loop of HIV-1 Env has been shown to block viral infectivity and the formation of syncitia (131). The V3 loop physically projects out from gp120 toward the target cell membrane during the gp120 interaction with coreceptor expressed on the target cell (180). The V3 crystal structure reveals a close association between the base of V3 and the conserved gp120 coreceptor binding site, and the regions distal to the base projecting towards the cell surface, which may mediate the interaction with the coreceptor extracellular loops (281). The V3 loop is essential for gp120 binding co-receptor and primarily determines tropism for the CCR5

and CXCR4 coreceptors (281). The V3-coreceptor interactions and the basis for CCR5 and CXCR4 specificity, however, are not fully understood.

The lipid composition of the target cell membrane may also play a role in membrane fusion by directionally targeting infection, which has been suggested to occur in microdomains or lipid rafts highly concentrated with glycosphingolipids and cholesterol, which appear to mediate entry of a variety of enveloped viruses into host target cells, including HIV-1 (287). Studies of cholesterol (287) or specific glycosphingolipid (41, 182) depletion from virions or cellular lipid bilayer interrupt the fusion process. Lipid rafts may also contribute to virion transmission between T cells (193) and DC (54).

Envelope Glycoprotein Cytoplasmic Domain

Lentiviruses, such as HIV-1 and SIV, that encode TM proteins with unusually long CD (183), have additional determinants distal to the membrane-proximal tyrosine signal. The multiple motifs conserved within the gp41 CD resemble three major classes of internalization or trafficking motifs: tyrosine-based (Yxxϕ) motif, dileucine-based (LL) motif, or di-aromatic motif (32, 78, 104, 184, 368).

The gp41 CD has been shown to direct the basolateral targeting of Env in polarized epithelial cells (230, 318). This is of particular interest considering that when tested in both epithelial and mononuclear cells chronically infected with HIV-1, the gp41 CD was shown to mediate polarized budding of the virus, through an Env-mediated intracellular redirection of Gag (102) This targeted expression of Env and polarized budding of virus may facilitate cell-cell transmission (102) and/or direct the spread of virus to key anatomic areas. Although Env incorporation into budding virions requires interaction of its CD with Gag (126, 129, 269), it is believed that there is competition between Gag and components of the endocytic pathway for interaction with gp41 CD. Thus, if Env is not incorporated into a budding viral particle upon cell surface expression, it undergoes rapid clathrin-mediated endocytosis (320).

Sequences in the CD of gp41 have been shown to mediate the internalization of Env, with the membrane-proximal Yxxϕ motif being established as the major endocytosis signal (31, 283, 320). Following internalization, Env has been shown to localize in discrete intracellular compartments (30), and this targeting is likely mediated by distal determinants within the CD. However, the relationship between the potential signals

within the CD and the protein targeting and interactions necessary for HIV-1 replication and pathogenesis remains to be fully elucidated.

Lentiviral TM CD lengths, which are ~150 amino acids (aa) long, contrast with other retroviral TM lengths, which are 20-40 aa long (183); but why nature has conserved these long cytoplasmic tails is unclear. Do the motifs conserved within the gp41 CD mediate internalization to evade detection by the host immune system, or do they exist to optimize Env intracellular localization and incorporation into budding virions? There are many gaps in the intracellular trafficking map that exists for HIV-1 Env.

Two major types of trafficking and endocytosis motifs have been identified within the HIV-1 Env CD: tyrosine-based and dileucine-based motifs (78, 79). Di-leucine motifs typically contain a LL sequence, where L= Leucine; however, it has been found that one of the leucines may be replaced with an Ile, Val, Ala, or Met (328). The tyrosine-based motif has the general form YXXϕ, where Y is tyrosine, X is any residue, and ϕ is a residue with a bulky hydrophobic side chain (78, 368). Tyrosine-based and dileucine-based motifs have been demonstrated to mediate endocytosis, basolateral targeting in polarized epithelial cells, and targeting to specialized compartments (32).

Tyrosine and di-leucine motifs were each shown to be independently sufficient to induce internalization using TAC (T cell antigen receptor lacking zeta chains)-cluster of differentitation 3 (CD3) chimeras (219). Dileucine-like motifs in the glucose transporter GLUT4, yeast alkaline phosphatase (ALP), truncated growth hormone receptor (GHR), and interleukin (IL)-13 receptor all direct endocytosis (372, 378) (153, 198), whereas a dileucine motif in E-cadherin targets the protein to the basolateral surface of polarized epithelial cells (258). In Immunoglobulin G (IgG) Fc receptors, a di-Leu motif exhibited overlapping signals for both endocytosis and basolateral targeting (185). Furthermore, the cytoplasmic tail residues Y8 and I11of rat lgp120 (lamp-I) are equally important for endocytosis and basolateral targeting (178). Internalization studies of the interleukin 13 receptor α2 chain (IL-13Rα2) reveal that mutation of the LLL internalization motif to III resulted in only a 20% reduction in the endocytosis rate (198). Additionally, deletion of either the first or third Leu in the LLL motif also resulted in 20% reduction of the endocytosis rate (198, 303).

Internalization signals directed by dileucine motifs can also be strengthened by the presence of acidic residues or acidic cluster (AC) motifs in the cytoplasmic tail, as in GLUT4 and invariant chain (Iip31) (106, 303). In reference to Iip31, there are two

dileucine-like motifs in the cytoplasmic tail, each of which is preceded by critical acidic residues; these motifs together mediate internalization by providing a favorable structure for endocytosis (303). The data from the Iip31 studies imply that the role of the dileucine motifs in endocytosis may have different requirements from its role in endosomal targeting, which does not require the acidic residues (303). Proximal acidic residues can also regulate dileucine-based internalization signals. For example, a phosphorylated Ser facilitates the recognition of CD3γ by molecules involved in receptor-mediated endocytosis (105). The internalization of E-selectin is also regulated by the phosphorylation of a serine-type dileucine-like motif (208). In the cytoplasmic tail of furin, a novel, autonomous determinant, an AC motif, was shown to direct internalization (377). In conjunction with an FI motif, this AC motif was also observed to direct furin to the basolateral surface in Madin-Darby Canine Kidney epithelial (MDCK) cells (347).

Examination of the HIV-1 gp41 CD reveals four tyrosine motifs at 712, 768, 795, and 802, and six dileucine-based motifs at 774, 776, 784, 799, 814, and 855 (see Fig. 13). Different groups have made attempts to define the biological role of the motifs conserved within gp41 CD. Progressive truncations of the CD of the Env TM revealed that the CD

Figure 13: Classic Signaling Motifs within the Cytoplasmic Domain of gp41.

---TM—	712	768	774	776	784	795	799	802	814	855
	YSPL	YHRL	LL	LI	LL	YW	LL	YW	LL	LL

is dispensable for HIV-1 and SIV Env-mediated cell-cell fusion (112, 270, 387). SIV replication does not apparently require the gp41 CD (192, 409). SIV growth in human cells selects for a spontaneously truncated Env, which broadens the host range of the virus (192, 409). However, upon inoculation into Macaques, the virus encoding the truncated Env rapidly reverts back to wildtype (WT) (209). This reversion back to WT suggests that while this region is dispensable *in vitro*, it plays an important role *in vivo*; and a number of structural elements within the CD may contribute to this *in vivo* function.

The membrane-proximal tyrosine motif, at residue 712 in HIV-1 and 723 in SIV, has been shown to direct Env expression to the basolateral surface of polarized epithelial cells (201, 229, 230, 292). The basolateral surface of epithelial cells is similar to cell-cell contact points in non-polarized cells, such as lymphocytes (102). In human lymphocytes chronically infected by HIV-1 revealed that cell-cell transmission of virus is favored, and

this process is dependent on targeting of the virus to the region of cell-cell contact by the Y_{712} motif (102).

Thus, the Y_{712} motif exhibits overlapping functions since it also serves as the major endocytosis signal in HIV-1 Env (320). The role of the membrane-proximal Yxxϕ motif in internalization is suppressed in the presence of the Pr55gag precursor protein (114). This suggests that Env interaction with Gag may preclude its interaction with host adaptin molecules that recruit PM molecules into clathrin-coated pits and thereby effect incorporation of Env into budding virions (114). Moreover, it has been shown that Env cell surface expression is modulated by interactions between the gp41 CD and cell factors. The membrane proximal Yxxϕ motif of HIV-1 binds to the μ subunits of AP-1 and AP-2 complexes and C-terminal regions of these TM proteins interact with the β subunit of AP-2, thereby modulating intracellular and cell surface expression of Env glycoprotein (27).

Studies investigating the role of the tyrosine-motif at 802 of gp41 revealed that abolishing that signal abrogated both Env targeting to the TGN and Env interaction with TIP47, a protein required for the transport of mannose-6-phosphate receptors from

endosomes to the TGN (30). Mutation of $Y_{802}W_{803}$ resulted in a virus that was both poorly infectious and defective in Env incorporation, showing that $Y_{802}W_{803}$ directed gp41 TM targeting to the TGN after internalization. $Y_{802}W_{803}$ was also shown to be critical for retrograde transport from the endosome to the TGN, where in this study Env was shown to localize at steady state (30). It is not clear at the present time whether the lack of Env incorporation is the result of abrogation of the TIP47 interaction or the result of abolishing the Gag-Env binding site for assembly. The MA domain of Gag has previously been shown in the context of infected T-cell lines to interact with the gp41 CD at the α helix 2 region, which includes the conserved motifs Y_{795}, LL_{799}, and $Y_{802}W_{803}$ (269).

Studies using C-terminal truncations of the gp41 CD revealed that mutation of the LL_{855} motif disrupted AP-1 binding to Env and altered the subcellular localization of Env, suggesting that proper post-Golgi routing of Env is dependent upon its recruitment of AP-1 (397). Furthermore, LL_{855} was shown to assist the $Y_{712}xx\phi$ in restricting the cell surface expression of Env (397). In this study, however, the investigators did not define whether the LL_{855} motif directed Env intracellular trafficking from the TGN to the PM or from the PM to the TGN.

The role of additional tyrosine-based and dileucine-based motifs within the HIV-1 CD has not been well defined, although there are a number of studies that suggest they may play a functional role in Env trafficking. Boge and colleagues determined that the Yxxϕ motif at 712 was important for AP-2 μ chain binding and endocytosis, but they did not demonstrate a role for Y768 in the internalization of Env (31). However, these results may be due to an oversight of the potential importance of a Leu_{765} in the Y-3 position of the Yxxϕ motif at 768. The highly conserved nature of this LxxYxxϕ sequence in gp41 resembles the "three pin plug" interaction required for P selectin protein binding of AP-2 μ chain (291). In the P selectin protein, a Leu in the Y-3 position of a Yxxϕ-like motif, as well as the Y+0 and the Y+3 residues, were shown to be important for μ2 binding. Owen *et al.* showed that in this structure internalization was not critically dependent on any one of the three interacting side chains (291). In the Boge studies, the role of the Y_{768} motif in endocytosis was determined based on the abrogation of only one residue within that conserved sequence, which would be sufficient to inactivate a "two pin plug" motif.

Bultmann *et al*. (46) attempted to identify sequence elements that influence the steady-state surface expression of Env, and they identified two sequences that inhibited cell

surface expression. These inhibitory sequences (is1 and is2) were shown to block cell surface expression of Env and to localize Env in the Golgi (46). The is2 segment includes the conserved motifs Y_{768}, LL_{774}, LI_{776}, and LL_{784}; but it was determined that LI_{776} and LL_{784} did not affect the surface expression of Env (46). The problem with these results is that mutation of a LL or LI to a LA may not necessarily abrogate the function of the motif because one Leu in a dileucine-based motif can be substituted with an alanine without losing function. Furthermore, the mutation of LI_{776} within the sequence LLLI (residues 774-777), leaves a LLLA, which may still be functional. This highlights the difficulty of dissecting out the role of these motifs in the presence of other potentially more dominant signals.

Conclusion

The major objective of this dissertation is to highlight the contributions of the highly conserved Y- and LL-motifs in the long cytoplasmic domain of gp41 on the HIV-1 life cycle. The difficulty in analyzing these motifs exists in a subtle contribution of a particular motif being masked by a potentially more dominant signal. We therefore employed a progressive mutagenesis strategy where all of the conserved Y- and LL-motifs were sequentially mutated in the Env CD. This approach allows us to directly

compare results from progressive mutagensis within the CD and to evaluate whether the sequential changes between the Env CD mutants enhibits a significant phenotype. It also affords us the opportunity to detect the first major threshold of defect in each step of the viral life cycle. The Env CD has been shown to mediate the interaction between Env and Gag, facilitate the polarized budding of HIV-1, modulate Env cell surface expression, and interact with cellular proteins. Only a few of these motifs have been characterized in contributing to the viral life cycle. Our strategy aims to highlight the critical motif(s) in each step of the HIV-1 life cycle.

Progressive Mutagenesis of Tyrosine and Di-leucine motifs in the HIV-1 Envelope Cytoplasmic Domain Results in a Loss of Env-mediated Fusion and Infectivity

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ABSTRACT

The gp41 component of the Human Immunodeficiency Virus (HIV) envelope glycoprotein (Env) contains a long cytoplasmic domain (CD) with multiple highly conserved tyrosine (Y) and dileucine (LL) putative trafficking motifs. Studies suggest that the trafficking motifs distal to major endocytosis motif (Y_{712}HRL), located at residues 712-715 of Env, may contribute to Env functionality in the viral life cycle. In order to examine the biological contribution of these motifs in the biosynthesis, transport, and function of Env, we constructed two panels of mutants in which the conserved Y- and LL-motifs were sequentially substituted by alternative residues, either in the presence or absence of Y_{712}. All of the mutant Envs, when expressed in the absence of other viral proteins, maintained at least WT levels of Env surface staining by multiple antibodies. The Y_{712} mutation (Y712C) contributed to at least a 4-fold increase in surface expression for all mutants containing this change. Sequential mutagenesis of the Y- and LL-motifs resulted in a generally progressive decrease in Env fusogenicity. However, mutation of dileucine motifs beyond the tyrosine at residue 768 resulted in the most dramatic effects on Env incorporation into virions, viral infectivity, and virus fusion with target cells. These mutations effectively abrogate the amphipathic nature of the lytic

peptide 2 (LLP2) domain and show that this can dramatically interfere with biological functions of Env that are distinct from intracellular transport to the plasma membrane.

INTRODUCTION

The envelope glycoprotein (Env) cytoplasmic domain (CD) is a key determinant in the replication of Human Immunodeficiency Virus type I (HIV-1) at two pivotal steps: (*i*) at the point of viral assembly, where Env must be incorporated into budding virions, and (*ii*) at the stage of viral entry into host target cells. The Env CD has been shown to interact with domains of Gag during assembly (30, 57, 84), interact with cellular components during intracellular transport (4, 5, 25, 78), modulate the fusogenicity of the Env complex both in the cell and within the virion (4, 66, 79), and regulate the cell surface expression of Env (8, 65, 68, 84). However, exactly which Env CD sequences mediate these phenotypically important roles remains to be elucidated.

Env, a type I trans-membrane protein, is synthesized as the precursor protein, gp160, on ribosomes associated with the endoplasmic reticulum (ER) (36). Upon oligomerization and correct folding of gp160 (36), the stable complex is then transported from the ER to the Golgi complex, where Env is terminally glycosylated and then processed into gp120, the receptor-binding surface (SU) protein, and gp41, the trans-membrane (TM) component, by a furin-like protease (36). In the mature form of Env, gp120 and gp41 are non-covalently linked. The mature Env complex, which facilitates viral entry into host

cells (67, 81), is then transported to and expressed on the cell surface, where either of two events may occur: Env is either incorporated into budding virions or it is rapidly internalized.

In the context of the mature virion, Env mediates virion attachment to the HIV-1 receptor, the CD4 molecule, and its chemokine co-receptor, CXCR4 or CCR5, and mediates fusion of the viral and cellular membranes (3, 30, 57, 79, 84), thereby facilitating entry of the virus into the host target cell. Viral infectivity depends on Env incorporation into budding virions and the subsequent entry into and infection of target cells.

Lentiviruses, such as HIV-1 and SIV, contain TM proteins with unusually long CD of ~150 amino acids (aa), in contrast to other retroviral TM CD, which are 20-40 aa long (36), but why nature has conserved these long cytoplasmic tails remains unclear. Truncation and elongation of the TM CD have been shown to alter the functionality of Env in the viral life cycle. Truncation studies reveal that the CD is dispensable for Env-mediated cell-cell fusion (26, 57, 80) and for SIV replication (39, 87). SIV growth in human cells selects for a spontaneously truncated Env, which broadens the host range of

the virus (39, 87). However, the virus encoding the truncated Env reverts back to wild type (WT) upon inoculation into macaques (43). This reversion back to WT suggests that while this region is dispensable *in vitro*, it plays an important role *in vivo*; and a number of structural elements within the CD may contribute to this *in vivo* function (31).

In HIV-1, truncation of the CD by as few as 20 aa significantly reduced viral replication in most cell types (26, 30, 32, 80). The Env CD is required in a cell-type dependent manner for incorporation into virions and for generating a productive, transmissible infection in most of the T cell lines tested (57). Cell-type dependence may be due to differences in expression and localization of host factors (58), suggesting that gp41 CD interactions with cellular proteins are important for efficient assembly. Similarly, it appears necessary for this region of Env to interact with the matrix (MA) domain of the Gag polyprotein precursor for incorporation of full-length proteins (20), and mutations in the CD, which block Env incorporation, can be rescued by amino acid changes in MA (57).

The HIV-1 gp41 CD contains several potential internalization and trafficking motifs, including four tyrosine motifs at $712_{Yxx\phi}$, $768_{Yxx\phi}$, 795_{YW}, and 802_{YW}, and six dileucine

motifs at 774_{LL}, 776_{LI}, 784_{LL}, 799_{LL}, 814_{LL}, and 855_{LL}, that have been conserved in the majority of HIV-1 patient isolates (81). Both tyrosine-based (Yxxϕ) and dileucine-based (LL) motifs can play individual or overlapping roles (35, 37, 47). These overlapping roles are modulated by different requirements for proximity to trans-membrane domains and to the carboxy or amino terminus (7). Residues near the motif itself can strengthen or specialize the signal or the mediating interaction (7). These motifs have been shown to facilitate endocytosis, basolateral targeting in polarized cells, and targeting to specialized compartments within the cells (7).

The membrane proximal Yxxϕ motif has been established as the major endocytosis signal for gp41 (6, 8, 45, 59, 65), which is suppressed in the presence of Pr55gag (28). The Y712 motif has been shown to direct the basolateral targeting of Env and the polarized budding of HIV-1 (24, 51) and to interact with the μ1 and μ2 chains of adaptin (AP) complexes (4, 6, 59). Mutagenesis of this motif in both the HIV and SIV Env CD has consistently resulted in increased levels of cell surface expression (8, 24, 45, 68, 78), although in one study this resulted in decreased infectivity, entry, and poorly replicating virus, independent of Env incorporation into virions (22). Further, a study in SIV demonstrated that abrogation of the membrane proximal Yxxϕ motif through deletion of

a highly conserved GY amino acid pair yielded replication competent virus that was highly attenuated in vivo (31).

The YW_{802} motif has been well studied and reported to interact with TIP47, implicated in linking the Env-Gag interaction (52), resulting in the retrograde transport of Env from the endosome to the Golgi (5). Abrogation or deletion of YW_{802} also resulted in decreased Env incorporation, infectivity, and replication (46, 57). The C-terminal LL_{855} has also been shown to interact with AP-1 and to regulate the subcellular localization of Env (84), with varying reports regarding its role in the endocytosis of Env (13, 84). The $Y_{768}xx\phi$ motif, in addition to LL_{774}, LI_{776}, and LL_{784}, overlaps with the inhibitory sequence 2, *is2*, described as inhibiting the surface expression of Env (10), although mutagenesis of Y_{768} alone did not result in a distinct phenotype or loss of AP-2 μ chain binding by Env (6). Interestingly, this tyrosine motif resembles the "three-pin plug" motif previously described for μ2 binding to the P-selectin protein (60), with an upstream leucine residue ($LxxY_{768}xxL$) that could also contribute to adaptin binding.

A number of the conserved motifs also overlap with the amphipathic α-helical lentiviral lytic peptides LLP1 (aa 828-856) (33, 55), LLP2 (aa 770-795) (70), and LLP3 (aa 789-

815) (42). This complicates mutational analyses since LLP1 and LLP2 have been reported to play a role in the fusion process (53). Further complicating the biological role of the Env CD, is a novel coupling of the fusion process with virion maturation (83) and its role in impeding the entry of immature virions into target cells through its interaction with the immature Gag core (38, 44, 53, 56). The complexity of these trafficking motifs, located within close proximity to each other and physically overlapping with other functional domains, exemplifies the difficulty in dissecting out the roles of the trafficking motifs conserved along the Env CD.

In order to better understand why HIV-1 has conserved tyrosine and di-leucine motifs within the unusually long CD of Env, we have employed a progressive mutagenesis strategy to sequentially mutate out all of the conserved Y- and LL-based motifs in the gp41 CD. For each of these mutants, we have determined surface expression, fusogenicity, incorporation, and the ability to facilitate entry and infection in target cells. Sequential mutagenesis generally resulted in progressive impairment of Env fusogenicity, Env incorporation, viral infectivity and entry, despite efficient transport and expression of Env on the cell surface. The most dramatic phenotype was observed

following mutation of Y_{768}, and adjacent dileucine motifs within LLP2, which points to a critical role for the amphipathic nature of this region in modulating Env function.

METHODS AND MATERIALS

Cell lines and culture. COS-1 and 293T cells were obtained from the American Type Culture Collection (Manassas, Va.), and TZM-bl were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: TZM-bl from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc. (23, 63, 71, 76). Cells were maintained in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), and 100 U/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate (Gibco BRL, Rockville, MD), at 37°C and 5% CO_2. All transfections were performed using the Fugene 6 (Roche, Indianapolis, IN) protocol at ~70% confluency of cells. All infections were conducted in DMEM containing 1% FBS and 80 μg/ml DEAE-dextran.

Antibodies. The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 gp120 Monoclonal Antibody (IgG1 b12) from Dr. Dennis Burton and Carlos Barbas (2, 11, 12, 64), Hybridoma 902 (anti-gp120) from Dr. Bruce Chesebro (17, 62), HIV-1 gp120 Monoclonal Antibody (2G12) from Dr. Herman Katinger (9, 21, 29, 54, 73), HIV-1 p24 Monoclonal Antibody (183-H12-5C) from Dr. Bruce Chesebro and Kathy Wehrly (18,

72, 75), and HIV-IG from NABI and NHLBI. The HIV-1 patient sera were obtained through the Emory CFAR Clinical Core. The horseradish peroxidase conjugated goat anti-human (heavy and light chains) monoclonal antibody (mAb) and the sheep anti-HIV-1 gp120 Polyclonal Antibody were purchased from Pierce (Rockford, IL) and Cliniqa Corp (San Marcos, CA), respectively. The Anti-HIV-1 gp120 D7324 mAb was purchased from Aalto Bio Reagents Ltd (Dublin, IE). AlexaFluor®647 Goat anti-human IgG (Heavy and Long Chain) was purchased from Invitrogen (Carlsbad, CA).

Glycoprotein and proviral expression constructs. The HIV-1 Env CD trafficking motif mutants were generated by employing either a Quickchange polymerase chain reaction (PCR) mutagenesis strategy (Stratagene, La Jolla, CA) or a multi-step overlapping PCR mutgenesis strategy using Expand High Fidelity PCR System (Roche, Indianapolis, IN). The resulting Env CD clones are referred to as follows: WT, Y, A, B, C, D, E, YA, YB, YC, YD, and YE. The second open reading frame (ORF) of *tat*, which overlaps with the gp41 CD between the motifs at 712 and 768, is unaffected by the substitutions made in these Env constructs. Because *rev* contains a second ORF that overlaps with seven of the ten trafficking motifs within the Env CD, the mutagenesis

strategy employed focused on maintaining the integrity of *rev* while mutating out the Y and LL motifs within Env (shown in Fig. 1).

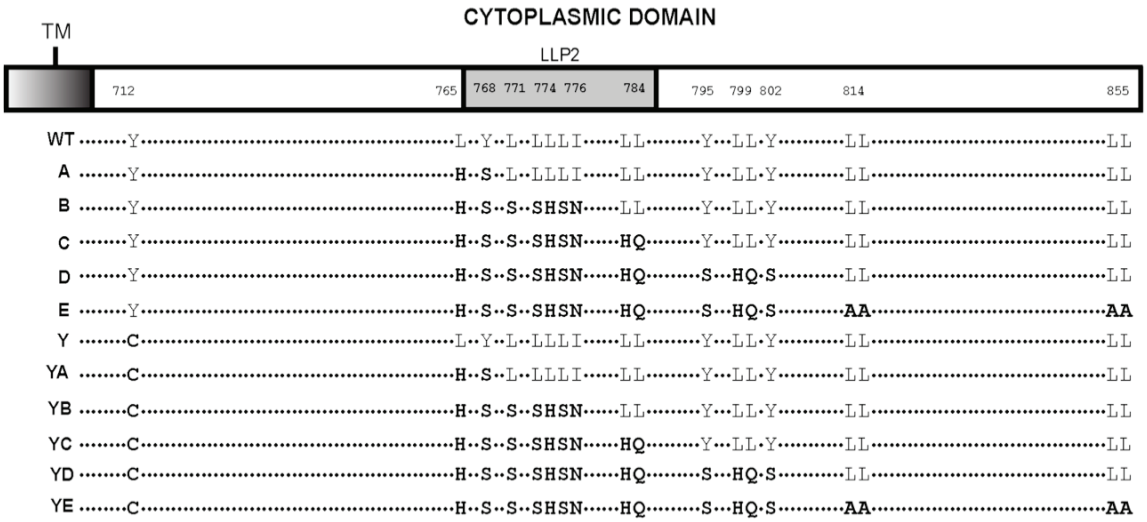

Fig. 1. gp41 cytoplasmic domain trafficking motif mutagenesis strategy. The key amino acids of the Y- and LL-motifs targeted for mutagenesis are listed under the corresponding location in the NL4-3 envelope cytoplasmic domain. WT residues are indicated by regular-faced type, and the residues in bold-faced type represent the progressive mutations for each mutant. The glycoproteins are separated by those containing the WT Y_{712} motif (top half) and by those containing the Y712C mutation (bottom half).

The following primers were used for mutagenesis:

Y712CFP 5'GGCAGGGATGTTCACCATTATCG3',

Y712CRP 5'CGATAATGGTGAACATCCCTGCCTAACTC3',

LY768HSFP 5'GCCTGTGCCACTTCAGCTCCCACCGC3',

LY768HSRP 5'GCGGTGGGAGCTGAAGTGGCACAGGC3',

L771/LLLI774SHSSFP

5’GCTCCCACCGCTCGAAAGACTCACACTCGAATGTAACGAGG3’,

L771/LLLI774SHSSRP

5’CCTCGTTACATTCGAGTGTGAGTCTTTCGAGCGGTGGGAGC3’,

LL784HQFP 5’CGAGGATTGTGGAACTTCTGGGACGCAGGGGG3’,

LL784HQRP 5’CCCCCTGCGTCC CAGAAGTTCCACAATCCTCG3’,

Y795S/LL799HQ/Y802SFP 5’GGAAGCCCTCAAACTTGGTGGAATCACCAACAGT

CTTGGAGTCAGG3’,

Y795S/LL799HQ/Y802SRP 5’CCTGACTCCAAGACTGTTGGTGATTCCACCAAGA

TTTGAGGGCTTCC3’,

LL814AAFP 5’GC TGTTAACGCGGCCAATGCCAATGCCACAGC3’,

LL814AARP 5’GGCATTGGCCGCGT TAACAGCACTATTC3’,

LL855AAFP5’GGGCTTGGAAAGGATTGCGGCATAAGATGG G3’,

LL855ARP5’CCCATCTTATGCCGCAATCCTTTCCAAGCCC3’,

All Env CD mutants were created in or from pSPEX-NL, a pSP-based vector (Promega, Madison, WI) containing the *Eco*RI-*Xho*I sequences of HIV-1 NL4-3, including the full-length cytoplasmic tail. Subsequent to verification in pSPEX, the mutant PCR fragments

were subcloned at the unique sites *Nhe*I to *Xho*I from the pSPEX shuttle vector to the mammalian expression vector pSRH, a simian virus 40 late-promoter-based expression vector containing the Mason-Pfizer Monkey Virus constitutive transport element, to create the pSRHS construct, which expresses a full-length Env from NL4-3. The HIV-1 Env expression vector also encodes the *tat* and *rev* genes from NL4.3 (66).

To measure the surface expression of the mutant Env glycoproteins, an enhanced blue florescent protein (EBFP) expression cassette was cloned into the pSRHS vectors at the unique restriction sites *NheI* and *BlpI* to create the pSRHS-EB vectors. The EBFP cassette was excised from the previously described vector (48). For use in single-round infectivity and Env incorporation assays, the mutant Envs were also cloned into the proviral vector pNL4-3 via the unique sites *Nhe*I and *Blp*I. All mutations were confirmed by DNA sequencing and by using primers that flank the Env CD.

Glycoprotein expression and immunoprecipitation. Env trafficking motif mutants in pSRHS expression vectors were transfected into COS-1 cells (2.5×10^5) seeded in 6-well plates. To verify protein expression, processing, and stability, the transfected cells were metabolically labeled 36-48 hours posttransfection. The transfected cells were starved

for 15 min in methionine-free and cysteine-free DMEM (Gibco-BRL, Rockville, MD) and pulse-labeled for 30 min in the same medium supplemented with [^{35}S]Methionine and [^{35}S]Cysteine (125μCi/well) protein-labeling mix (Perkin-Elmer NEN, Boston, MA). The labeled cells were then chased for 2 h and 4 h in unlabeled complete DMEM. The chase supernatants were removed and filtered through a 0.45μm membrane to remove cellular debris. Cell lysates were prepared on ice by addition of 0.5ml ice-cold lysis buffer (1% Triton X-100, 50mM NaCl in 25 mM Tris-HCl [pH 8.0]), and nuclei were removed from lysates by centrifugation at 13,200 revolutions per minute, rpm, for 10 min at 4°C in a microcentrifuge (Beckman, Palo Alto, CA). HIV-1 Env proteins were immunoprecipitated from cell lysates and supernatants by incubating at 4°C with HIV-1 patient sera. Immunoprecipitated proteins were then precipitated with formalin-fixed *Staphylococcus aureus* (protein A) and washed three times in lysis buffer containing 0.1% sodium dodecyl sulfate (SDS). The labeled proteins were resolved by 10% SDS-PAGE, visualized by autoradiography, and quantified using a Cyclone phosphorimaging system (Packard, Meridian, CT) as previously described (77).

Cell-cell fusion assay. COS-1 cells (2.5 x 10^5) were seeded in 6-well plates, transfected with the pSRHS-EB Env expression vectors at ~70% confluency, resuspended by

trypsinization, and co-cultured with TZM-bl cells at a ratio of 1:5. The co-cultures of cells were incubated for 24 h and then lysed in the luciferase reporter buffer (Promega, Madison, WI). The cells were twice subjected to freezing for 1 hour and then thawing for 20 min, followed by centrifugation (Beckman, Palo Alto, CA) of the lysates at 13,200 rpm for 10 min to remove any cellular debris. Each cell lysate (40 μl) was added to a well in a 96-well plate, and then combined with 100 μl of the luciferase substrate (Promega, Madison, WI). Light emission was then measured using a Synergy multi-detector microplate reader (Biotek, Vinooski, VT) as previously described (69).

Cell surface expression of Env glycoprotein. Surface expression of WT and mutant Env glycoproteins was measured using Flow cytometry in both a primary and secondary antibody (Ab) detection system. Env surface expression was measured by the human anti-gp120 mAb b12 and the mouse anti-gp120 mAb 902 each conjugated to AlexaFluor®647 (Invitrogen, Carlsbad, CA) in a primary detection system. The human 2G12 Ab was used in conjunction with the AlexaFluor®647 Goat anti-human IgG (H+L) to measure Env surface expression in a secondary Ab detection system. The Env proteins were expressed from the pSRHS-EB vector. EBFP expression served as a positive transfection control for these experiments. COS-1 cells were transiently

transfected with pSRHS-EB and cultured for 36-48 h. Cells were then resuspended by trypsinization**,** washed three times, and stained for 1 h at RT with 5μg/ml of the primary Ab. Cells stained with b12-Alexa®647 or 902-AlexaFluor®647 were washed three times prior to flow cytometry analysis. Cells stained with 2G12 were washed three times and then stained with the secondary Ab, AlexaFluor®647 Goat anti-human IgG (H+L), at 2μg/ml for 1 h at RT. Double-stained cells were washed three times. Env surface expression was measured by flow cytometry analysis utilizing the LSRII system and the FACSDiva software (version 6.1), and analyzed using FlowJo software (version 8.8.4). Samples for each mutant were stained in triplicate, and a total of 50,000 events were accumulated for each sample. For each of these experiments, the mean fluorescence intensity (MFI) was calculated and multiplied by the percent of the cell population positive for both EBFP and R (660/20), to produce the MFI Index. (69)

Single-round infection. Single round infectivity was measured in a luciferase-based virus-cell fusion assay. COS-1 cells were seeded at a density of 2.5×10^5 in 6-well plates and co-transfected with the pSRHS expression vector and the pSG3Δ*env* proviral vector. The pSG3Δ*env* proviral vector was used as a negative control. At 72 h posttransfection, viral supernatants were clarified by centrifugation at 3,000 rpm for 20 min at 4°C to

remove cellular debris. TZM-bl indicator cells (1 x 10^5) seeded in 12-well plates were then infected with equivalent amounts of virus (5ng), which were normalized by p24 enzyme-linked immunosorbent assay (ELISA) (34, 69). Complete DMEM was added after a 2 h incubation at 37°C, and luciferase activity was measured 48 h post infection as described above.

Virus-cell fusion assay. A virion-based fusion assay was performed as previously described by Cavrois (14, 49, 69). BlaM-Vpr incorporated NL4.3 viruses were produced by transient co-transfection of the proviral plasmid pNL4.3, the pCMV-BlaM-Vpr vector (kindly provided by W. Greene, UCSF), and the pAdvantage vector (Promega, Madison, WI) by employing calcium phosphate precipitation of the DNA. BlaM-Vpr incorporated viruses containing WT and mutant Env glycoproteins were collected 48 h post-transfection and filtered through a 0.45-μm membrane. Viral supernatants were then loaded onto a 25% sucrose cushion (in PBS [pH 7.2]) and centrifuged at 100,000 x g for 2.5 h at 4°C as described above. The supernatant and sucrose layers were then removed and the resulting viral pellets were resuspended in serum-free DMEM. The virus titers were normalized by p24 ELISAs, and equivalent amounts of virus (200 ng p24) were then added to TZM-bl cells (3 x 10^5), which were cultured in CO_2-independent medium

(Gibco-BRL, Rockville, MD) supplemented with 1% fetal bovine serum. The samples were incubated at 37 °C for 6 h, followed by removal of free viruses with a wash in serum-free CO_2-independent medium. Because of a difference in temperature requirement, the fluorescent dye, CCF2-AM, was then loaded into these cells by passive diffusion for 2 h at room temperature, following the β-lactamase loading kit protocol (Invitrogen, Carlsbad, CA). Following washing with serum-free CO_2-independent medium to remove any residual extracellular dye, the cells were resuspended in CO_2-independent medium supplemented with 10% fetal bovine serum and 2.5mM probenecid. Subsequent to incubation at room temperature in the dark for 16 h, the cells were fixed with 4% paraformaldehyde at 4°C for 20 min. The cells were then subjected to flow cytometry analysis in a Beckman Dickinson LSRII cytometer.

Env incorporation into virions. 293T cells (1 X 10^6) were transfected with proviral vectors. Viral supernatants were harvested and clarified 72 h post transfection and were pelleted through a 25% sucrose cushion by ultracentrifugation at 100, 000 x g for 2.5 h. The layers of supernatant and sucrose were carefully removed, and the resulting viral pellets were resuspended in 200 μl PBS (pH 7.2). The viral pellets were subjected to p24

and gp120 ELISAs to determine the amount of p24 and gp120 (34, 69). Incorporation was determined by calculating the ratio of ng/ml of gp120 to p24.

RESULTS

Generation of Env mutants. The unusually long CD of gp41 contains multiple Y- and LL-motifs. In order to define the functional role played by these motifs in the HIV-1 life cycle, a progressive mutagenesis strategy was employed in which the Y- and LL-based motifs were sequentially mutated along the Env CD. Several of these motifs overlap the Rev open reading frame, necessitating substitutions that maintain Rev function. The mutants were classified based on their location in the NL4-3 Env and are shown in Fig. 1. Site-directed mutagenesis was employed to introduce the trafficking motif mutations into the *env* gene. A complex overlapping PCR strategy was then utilized to create progressive mutants in the CD. Introduction of the L765H/Y768S mutations into the *env* sequence generated mutant A. The subsequent addition of L771S/LLLI774SHSN to mutant A results in mutant B, the addition of LL784HQ to mutant B results in mutant C, the additional changes of Y795S/LL799HQ/Y802S to mutant C produce mutant D, and LL814AA/LL855AA was combined with mutant D to create mutant E. Introduction of the Y712C mutation to WT and the Env mutants A, B, C, D, and E resulted in the generation of the Y, YA, YB, YC, YD, and YE mutants. All Env CD mutants were cloned into the Env expression vectors pSRHS and pSRHS-EB, as well as the proviral vector pNL4.3.

Envelope biosynthesis, processing, and stability. In order to investigate the effects of this mutagenesis on the biosynthesis, processing, and stability of the glycoproteins, WT and mutant Envelopes were expressed from the SV40-based pSRHS vector, which also expresses Rev and Tat. Env expression was under the control of the SV40 late promoter and polyadenylation signals provided by the long terminal repeat (LTR) of the Mason-Pfizer monkey virus (26, 39). The WT and mutant glycoproteins were expressed in COS-1 cells, which have been shown to facilitate high expression of Env from pSRHS (26). Two days after transfection, the Env proteins were metabolically labeled for 30 min with [^{35}S] and further chased for 2 h and 4 h in complete unlabeled media. Following lysis of the cells, the glycoproteins within the cell lysates and supernatants were immunoprecipitated with HIV-1 patient sera, resolved by SDS-PAGE, and visualized by autoradiography (26, 39). Sequential mutagenesis of the Y- and LL-based motifs in the CD mutants did not decrease the level of expression of gp160, or the processing of precursor to gp120 and gp41, indicating normal intracellular transport to the trans-Golgi complex, as seen in Fig. 2A. Examination of the amount of gp120 shed into the supernatant also revealed that the mutagenesis of these motifs did not alter the stability of gp120, represented in Fig. 2B. Similar results were seen in pulse-chase experiments conducted on the pSRHS-EB Env expression constructs (data not shown).

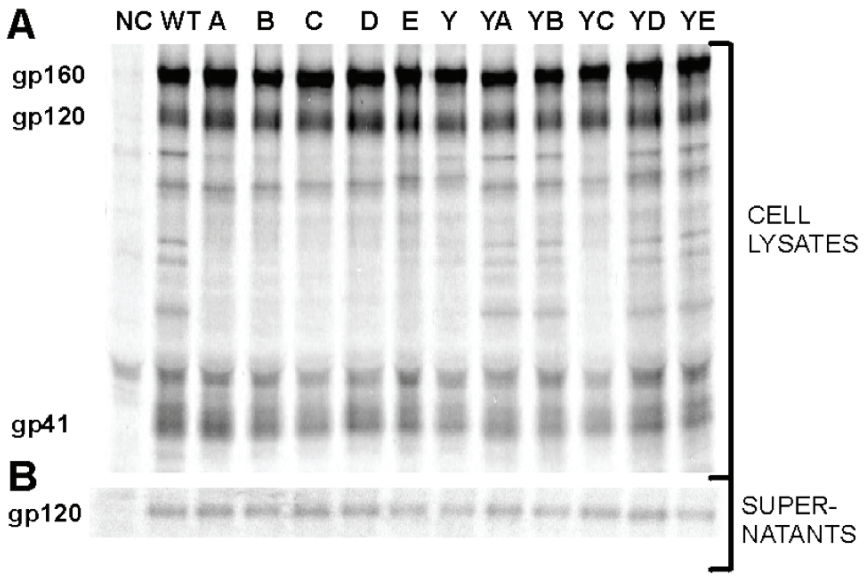

Fig. 2. Biosynthesis and processing of mutant glycoproteins. COS-1 cells transiently transfected with the Env expression vector pSRHS were metabolically labeled and immunoprecipitated with anti-HIV-1 patient sera. The locations of the Env precursor and the components of the mature Env complex are indicated at the left of the gel. The pulse cell lysates of glycoproteins expressed from the pSRHS vector (A) are shown in the gel at the top, and the corresponding gel for the amount of gp120 shed into the supernatant (B), is shown in the gel at the bottom.

Effects of sequential mutagenesis in the cytoplasmic domain of Env on cell-cell fusion. Because the Env trafficking motif mutants maintained WT levels of biosynthesis, processing, and stability, we wanted to screen the glycoproteins for functionality. In order to measure Env-mediated cell-cell fusion, a luciferase-based fusion assay was utilized. The Env expression vector containing WT and mutant *env* genes, including both the *rev* and *tat* genes, were expressed in COS-1 cells. Two days after transfection, the transiently transfected COS-1 cells were co-cultured and mixed

with TZM-bl indicator cells, which contain an HIV-2 LTR driven luciferase gene and express the HIV-1 receptor, CD4, and coreceptors CCR5 and CXCR4. Upon fusion of the cellular membranes of the Env expressing COS-1 cells and the target TZM-bl cells, Tat, which is also expressed from pSRHS-EB, activates the HIV-2 LTR and drives luciferase production (76). A quantitative analysis of Envelope mediated cell-cell fusion was measured for each of the mutants by calculating their relative luciferase enzyme activity compared to WT. The relative luciferase activity for each of the mutants was averaged from three independent experiments performed in triplicate; these results are shown in Fig. 3. The low background resulting from the EBFP control, expressed from the pEBFP-N1 construct lacking the *env*, *rev*, and *tat* genes, was subtracted from the experimental values to give a baseline for fusion activity.

In Fig. 3, the Env mutants have been separated into two series, those containing the WT Y_{712} motif and those containing the Y712C mutation. Direct comparison of the two panels indicates that the Y712C mutation did not affect the fusogenicity of the Env mutants in the context of cell-cell fusion, with the Y mutant maintaining 96% fusion activity compared to WT. Mutagenesis of the first two pins of the Y_{768} motif resulted in 62% and 63% the fusogenicity of WT for mutants A and YA, respectively. Subsequent

mutagenesis of the third pin in the Y_{768} motif and the $LL_{774}LI_{776}$ motifs resulted in a significant decrease of fusion compared to WT with B inducing 41% and YB inducing 35% fusogenicity to WT. Fusion activity decreased in the remaining mutants to approximately 30% that of WT, while mutant YE had a greater decrease at 17% of WT.

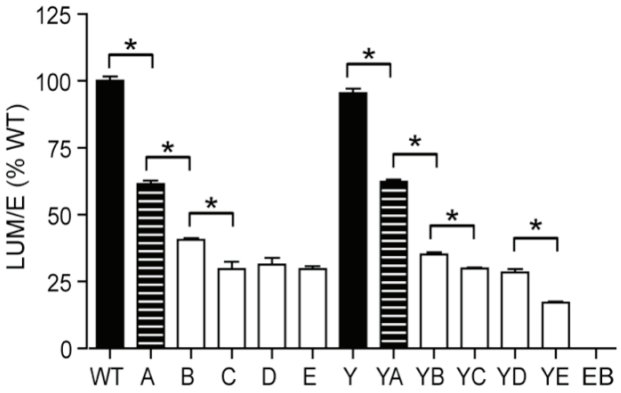

Fig. 3. Envelope-mediated cell-cell fusion. COS-1 cells transiently transfected with the Env expression vector were cultured for 24h. The COS-1 cells transiently expressing WT and mutant glycoproteins were co-cultured with TZM-bl indicator cells. Following a 24 h incubation, the co-culture of cells was lysed and measured for luciferase activity. P values were calculated by using Tukey's T-test and a value <0.001 are shown with an asterisk. The data represents results from three independent experiments conducted in triplicate. The error bars represent the standard deviation of the means.

Thus, sequential mutagenesis of the Y- and LL- based motifs within the long CD of HIV-1 Env resulted in a progressive decrease of Env mediated cell-cell fusion activity. These results show that mutation of the Y- and LL-based motifs contained within the Env CD can modulate fusion activity of the Env glycoprotein.

Effects of mutagenesis in the cytoplasmic domain on Env cell surface expression.

Because sequential mutagenesis of the trafficking motifs within the CD resulted in a progressive decrease in Env fusion activity, we wanted to establish whether this resulted from an altered transport to and expression on the cell surface. COS-1 cells expressing the WT and mutant envelopes were stained with each of three monoclonal antibodies (mAb): 902, which recognizes a linear epitope on the gp120 V3 loop (62, 75), b12, which recognizes an epitope that overlaps the CD4 binding site (61, 64), and 2G12, which recognizes a complex of carbohydrates on the surface of gp120 (73). The first two were directly conjugated to AlexaFluor®647, while 2G12 was detected using Alexa647 labeled Goat anti-human IgG (H+L). Following immunostaining, the cells were subjected to flow cytometry analysis. EBFP expression from the Env expression vector served as the experimental transfection control. The results from the flow cytometry analysis are shown in Fig. 4. Once again, the samples have been separated into two series: those containing the WT Y_{712} motif and those containing the Y712C mutation. The MFI Index value was calculated for each of the samples. The results indicate that all of the Env CD mutants maintained at least WT levels of surface expression, while introduction of the Y712C mutation into the CD resulted in an increase in glycoprotein cell surface expression, following immunostaining with all three antibodies. In Fig. 4A,

the flow cytometry dot plots of mAb 902 stained cells reveal an distinct shift in the staining pattern between the WT Y_{712} panels and the Y712C panels, with a greater proportion of the cells staining and with higher intensity in the latter, consistent with increased levels of surface expression; the corresponding MFI Index values are shown in Fig. 4B. The MFI Index values for the WT Y_{712} panel of mutants were similar to WT Env levels with A at 101%, B at 195%, C at 125%, D at 120%, and E at 136% that of WT. By inserting the Y712C mutation into WT Env, the MFI Index value of the Y mutant increased to 447% of WT. This increase was reflected in the MFI Index values of the other mutants containing the Y712C, including YA at 563%, YB at 396%, YC at 563%, YD at 409%, and YE at 194%.

We confirmed the increased surface expression with the 2 additional monoclonal antibodies. The results for immunostaining with mAb b12 are shown in Fig. 4C and those for mAb 2G12 in Fig. 4D. The patterns of staining for both antibodies are similar to that observed with mAb 902, with a majority of the Y_{712}WT mutant-expressing cells

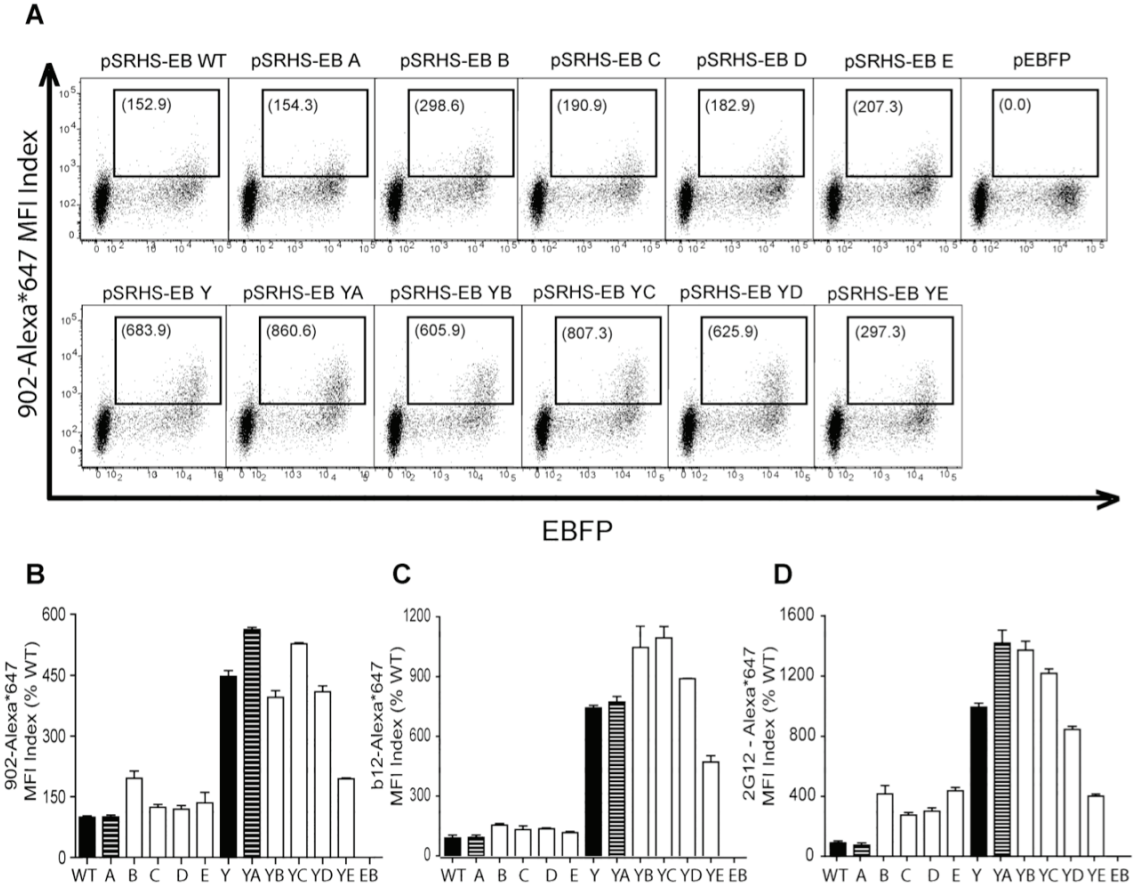

Fig. 4. Cell surface expression of envelope glycoproteins. (A) COS-1 cells transiently transfected with each of the pSRHS-EB Env expression vectors were immunostained with Alexa*647-conjugated anti-g120 mAb 902. The dot plot panels are separated into two series for analysis: (1) those containing the WT Y_{712} motif in the top row, and (2) those containing the Y712C motif in the bottom row. (B) The quantified surface expression levels of the Env glycoproteins are shown as the relative mean fluorescence intensity (MFI) Index (MFI x % of cells double positive for EBFP and Alexa*647). Additional cells were stained with Alexa*647-conjugated anti-gp120 mAb b12 (C) and anti-gp120 mAb 2G12 + Alexa*647-Goat anti-hu IgG (H+L) (D). The error bars represent the standard deviation of the means.

exhibiting MFI indices similar to WT Env, although for 2G12 a 3-4-fold increase in surface staining was observed for mutants B-E. As with 902, a majority of the cells

expressing the Y712C-containing mutants exhibited much higher levels of surface staining with b12 and 2G12, although the absolute increase differed (approximately 8 and 10-fold for Y respectively). In each case cells expressing the YE mutant showed the smallest increase in Env surface expression of the Y712C-containing mutants relative to WT.

Env CD mutants exhibit a defect in virus entry and virus-cell fusion. Because the levels of surface expression of the Env CD mutants did not correspond to the observed defects in cell-cell fusion, we examined the Env mutants, in the context of pSG3ΔEnv pseudotyped virus, for their capacity to mediate virus entry and virus-cell fusion.

A luciferase-based single round virus entry assay was conducted, utilizing the same target cell fusion system as described above. Equivalent amounts of pseudotyped virus (normalized for p24), produced in COS-1 cells, were used to infect TZM-bl indicator cells. The cells were measured for luciferase activity at 48 h post-infection. The SG3ΔEnv virus was used as the background control. The results indicate that the sequential mutagenesis of the Env CD trafficking motifs resulted in much more pronounced defective phenotypes in the context of pseudotyped virus as shown in Fig.

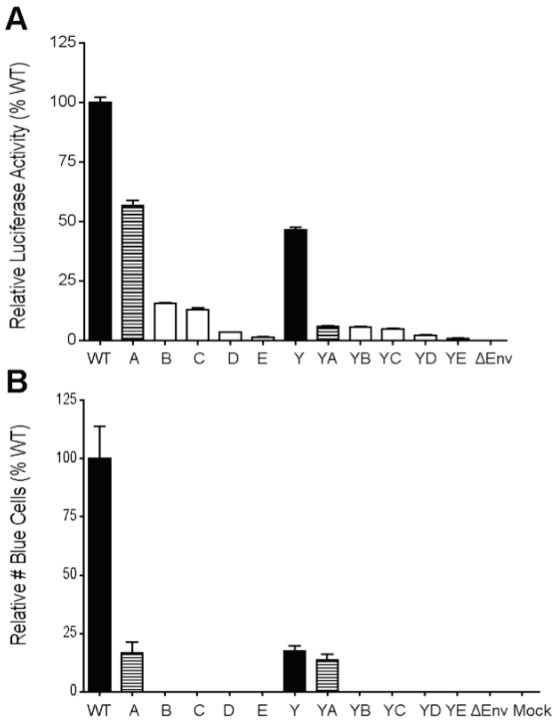

Fig. 5. Infectivity and entry of envelope cytoplasmic domain mutants. (A) Single round infectivity. Env-pseudotyped SG3ΔEnv viruses produced in 293T cells and p24-normalized, were used to infect TZM-bl indicator cells. After a 48 h incubation, the cell mixtures were lysed and luciferase activity was assayed. (B) Virus-cell fusion assay. Env CD mutants in NL4-3, pseudotyped with pCMV-BlaM-Vpr, were produced in 293T cells and subjected to gradient ultracentrifugation. Resuspended viral pellets were then normalized using p24 ELISA assays and used to infect TZM-bl indicator cells. The CCF2-AM fluorescent dye was loaded into the cells and incubated for 16 h at room temperature. The data represents results from three independent experiments conducted in triplicate. The error bars represent the standard deviation of the means.

5A. In contrast to the cell-cell fusion results, where the maximum decrease observed for mutant E was 70%, infectivity of virus pseudotyped by this Env was reduced 99%. Even mutant B, in which just the Y_{768} motif and two adjacent dileucine motifs are mutated,

exhibited only 16% the virus entry activity of WT Env. While the Y712C substitution in mutant Y had little effect on cell-cell fusion, the infectivity of viruses pseudotyped with this Env was 47% that of WT, and the remaining Y712C-containing mutants were reduced in virus entry by more than 94% compared to WT.

In order to define further the defect in entry, we utilized the ß-lactamase virus-cell fusion assay described previously (14, 15, 49, 69). For this assay, pNL4-3 proviral clones were co-transfected with a β-lactamase-Vpr fusion protein (BlaM-Vpr) expression vector, and the released virus was used to infect TZM-bl cells as described in Materials and Methods. The extent of virus-cell fusion, as assessed by intracellular ß-lactamase activity is shown in Fig. 5B. The results of this assay were similar to those observed in the virus entry assay (Fig. 5A), with only mutants A, Y and YA exhibiting low levels of ß-lactamase activity, 14-17% that of WT.

Glycoprotein incorporation into virions.

To establish whether a defect in Env incorporation into virions contributed to the infectivity impairment of the Env CD mutants, we employed an ELISA-based approach to determine the efficiency of Env incorporation. The CD mutant viruses were recovered

from provirus transfected 293T cells. Viral supernatants were pelleted through a 25% sucrose cushion and subjected to p24 and gp120 ELISAs. To rule out that residual gp120 may contaminate the viral pellets following resuspension in PBS, gp120-containing supernatant from pSRHS-transfected 293T cells was combined with SG3ΔEnv virus produced from 293T cells, and subjected to the same protocol as the NL4.3 viruses to determine the amount of background gp120 in viral pellets. The ratios of gp120/p24 were calculated for each virus to measure Env incorporation into virions, and are shown in Fig. 6.

Mutant A incorporated near WT levels of Env, but the level of incorporation rapidly decreased, with mutants B through E incorporating between 24 and 38% the amount of gp120 compared to WT. The Y712C mutation reduced the level of incorporation to 49% that of WT, and mutants YA-YE, showed a similar pattern of incorporation to their non-Y712C counterparts, except for YE, in which the levels were somewhat lower.

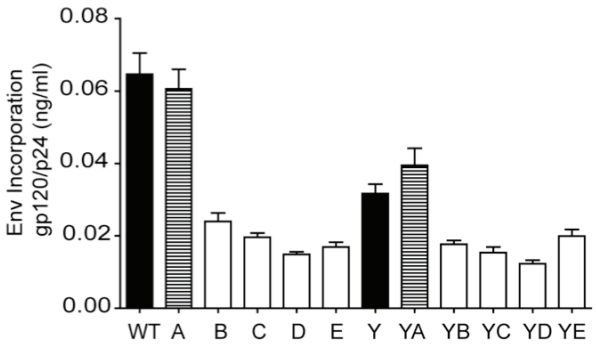

Fig. 6. Incorporation of envelope glycoproteins into virions. NL4-3 viruses containing the WT and mutant Env proteins were produced in 293T cells and pelleted through a 25% sucrose cushion. Resuspended viral pellets were then subjected to gp120 ELISAs and p24 ELISAs. Incorporation of Env into virions is shown as the ratio of gp120/p24. The data represents results from three independent experiments conducted in triplicate. The error bars represent the standard deviation of the means.

DISCUSSION

The objective of this study was to investigate the role of the highly conserved Y- and LL-based motifs conserved within the gp41 CD in the HIV-1 life cycle. To this end, we have employed a progressive mutagenesis strategy, in which all of these motifs were sequentially mutated throughout the CD. Previous studies have attempted to study the role of the CD in the context of chimeric proteins (4, 8, 10, 13, 84), while others have truncated the CD in order to determine the affects on Env functionality (1, 16, 26, 85, 86). However, while such an approach allows removal of all trafficking motifs in the CD, there appears to be a functional dependence between the gp41 CD and its ectodomain, as well as a conformational dependence of gp120 on the Env CD (27). This makes studying Env in the context of the full-length CD even more crucial. Truncation of the CD results in an increased susceptibility to neutralization by antibodies, likely due to a more open trimer conformation (64, 82), and an increase in viral entry by non-replicating immature virions (44, 83). Similar studies also demonstrated that production of fully infectious virus requires the long CD (58).

Env glycoprotein biosynthesis, processing, stability, and transport to the Golgi (based on cleavage of gp160 to gp120 and gp41) were unaffected by the mutation of trafficking

motifs. These motifs also appear, for the most part, to be dispensable for transport of Env to the cell surface. The Y_{712} motif, however, appears to be important for regulating the cell surface expression of the HIV-1 Env, as evidenced by a minimum 4-fold increase in surface expression of the Y (Y712C) mutant. Because the b12 mAb binds to an epitope that overlaps with the CD4-binding site on gp120 and because we were concerned with the structural dependence of gp120 on the gp41 CD, we performed surface immunostaining with three monoclonal antibodies, including mAb 902 and mAb 2G12, which bind a linear protein epitope and a complex carbohydrate epitope, respectively. All three mAb showed an increase in surface expression of the Y-mutants compared to the WT Y_{712} mutant panel, and a slight decrease in YE compared to the rest of the Y-mutants. All of the mutants maintained at least WT levels of surface expression in COS-1 cells, while all of the Y-mutants exhibited an increase in surface expression. This is consistent with previous studies of the membrane proximal Yxxϕ motif in Env of both HIV and SIV (8, 24, 45, 68, 78, 84).

A consistently lower level of surface staining relative to the other Y-mutants was observed for the YE mutant, even though this still exceeded that of WT Env for each mAb. In contrast this was not observed for the E mutant, which exhibited surface staining

levels equivalent to the B, C and D mutants. Because YE lacks any of the conserved tyrosine- and dileucine-based trafficking motifs, and so is unlikely to be more efficiently endocytosed, the reduced surface staining is most easily explained by less efficient transport of this mutant to the PM, perhaps because in the absence of Y_{712} necessary adaptin interactions are impaired.

Env Mutants	Env-mediated Cell-Cell Fusion[a]	Cell Surface Expression (MAb 902)[a]	Pseudotype Virus Infectivity[a]	Virus-Cell Fusion[a]	Incorporation[a]
WT	100	100	100	100	100
A	62	101	57	17	94
B	41	195	16	0	38
C	30	125	13	0	31
D	31	120	4	0	24
E	30	136	1	0	28
Y	96	447	47	17	49
YA	62	563	6	14	53
YB	35	396	6	0	29
YC	30	563	5	0	24
YD	29	495	2	0	20
YE	17	194	1	0	32

Table 1. Table of results for Env cytoplasmic domain mutants. [a] Values are expressed as percentages of WT.

Despite an increase in surface expression in the Y712C-containing mutants, there was a progressive decrease in Env fusogenicity from WT through C, after which Env fusogenicity stabilized (summarized in Table 1). Similar results were observed with the Y-mutants, although YE again was the most defective. Thus changes in these tyrosine and dileucine motifs within the cytoplasmic domain are capable of inducing phenotypic effects on an event that is commonly associated with the ectodomain of Env (receptor and co-receptor binding, 6-helix bundle formation). The motifs mutated in A, B, and C are also of interest because they overlap with the LLP2 motif (aa 765-788; see Fig. 1) in the NL4-3 gp41 CD, which has been proposed to play a role in fusion (41, 53). Indeed, Lu et al., (53) showed that at sub-optimal temperatures (31.5°C), antibodies to this region could bind to the interface of fusing cells and inhibit fusion. They proposed that, following formation of the gp41 HR1/HR2 6-helix bundle, the LLP2 peptide region is transiently exposed and modulates fusion by interacting with this helical complex. Consistent with this model, it is of interest that the reduction in fusion we observed for the CD mutants described here is maximal at mutant C (or YC), in which 7/9 hydrophobic residues within LLP2 are mutated and where the amphipathic nature of this region has been completely abrogated.

The effect of the CD mutations on viral infectivity in TZM-bl cells was much more pronounced than on cell-cell fusion (summarized in Table 1). In assays of Env pseudotyped virus significantly reduced levels of infectivity were observed for all of the mutants. The A and Y mutants retained approximately 50% infectivity in pseudotyped virus assays, but the remaining mutants exhibited less than 20% (16-1%) that of WT. The defective stage in virus entry appeared to be at the level of virus-cell fusion, since the results of BLAM assays closely paralleled the infectivity results observed, in that only A, Y and YA exhibited any virus-cell fusion and at a level of approximately 20% that of WT.

It seems likely that the defects in virus infectivity represent the sum of defects in Env fusion and reduced levels of Env incorporation into virions (Table 1). Env incorporation decreased as more motifs were mutated, with the greatest drop being observed between mutants A and B (and in parallel between YA and YB); mutant A virions incorporating WT levels of Env and mutant B only 1/3 that of WT. This is again consistent with a role for the hydrophobic residues within LLP2 region of the CD, since in mutants B and YB all of the hydrophobic residues in the N-terminal half of this region have been mutated to polar residues.

The Y mutant virions also showed reduced levels of incorporation (49%), similar to that described in previous studies (22, 46, 78). This result seems paradoxical to our observation of increased levels of Env at the cell surface, which is where virus buds (40). The basis for reduced levels of Env incorporation are at present unclear, although it may reflect altered intracellular trafficking of Env and an inability of Env and Gag to be directed to a common site for assembly. Env clearly has the capacity to redirect where in the cell virus assembly occurs. In polarized epithelial cells Env directs budding to the basolateral membrane and in CD4 T cells to a single pole of the cell (19, 24, 50, 51). Mutation of the major endocytosis motif at Y_{712} has been shown to disrupt polarized budding in both systems (24, 50, 51). The loss of additional tyrosine and di-leucine motifs in mutants B-E (and YB-YE) could alter potential interactions of LLP2 with LLP1 and the membrane (74), which might further reduce the potential for co-localization of Env and Gag, and explain the observed reduction in incorporation. Previous studies on the role of tyrosine-based motifs directing incorporation of HIV Env into virions have focused on the YW_{802} motif, which has been postulated to interact with the cellular trafficking protein TIP47 in retrograde transport of Env from the endosome to the Golgi (5). Mutation of the motif in Env or silencing of TIP47 expression resulted in reduced Env incorporation and virus infectivity (5, 52). In the studies presented here, we did not

observe any additional reduction in Env incorporation following mutagenesis of Y_{802} (mutant D vs. C and YD vs YC). Thus it appears that the defects imposed by the changes in mutants B and C were upstream of any role that TIP47 might play in this process.

From the studies reported here, we show that sequential mutagenesis of the Y- and LL-based motifs located within the CD of HIV-1 gp41 had a profound effect on Env function and demonstrates a critical role for hydrophobic residues in this region of the CD. This was evident in decreased Env-mediated cell-cell fusion, Env incorporation into virions, and viral entry into target cells. Env transport to the plasma membrane occurred in the absence of all of the conserved Y and LL motifs in the CD, arguing against a critical role for them in outward transport of the protein. Plasma membrane location alone was clearly not sufficient for efficient assembly of Env into virions, since a majority of the mutants exhibited reduced levels of Env incorporation and this, coupled with decreased fusogenicity of Env, resulted in them being non-infectious. The greatest phenotypic effects were linked to multiple changes in the LLP2 region of the CD, but additional experiments will be required to determine whether this reflects a distinct role for this region in late stages of Env-induced cell fusion, an alteration in CD-membrane

interactions, or changes in protein-protein interactions within or between gp41 monomers necessary for the fusion process.

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REFERENCES

1. **Affranchino, J. L., and S. A. Gonzalez.** 2006. Mutations at the C-terminus of the simian immunodeficiency virus envelope glycoprotein affect gp120-gp41 stability on virions. Virology **347:**217-25.

2. **Barbas, C. F., 3rd, E. Bjorling, F. Chiodi, N. Dunlop, D. Cababa, T. M. Jones, S. L. Zebedee, M. A. Persson, P. L. Nara, E. Norrby, and et al.** 1992. Recombinant human Fab fragments neutralize human type 1 immunodeficiency virus in vitro. Proc Natl Acad Sci U S A **89:**9339-43.

3. **Berger, E. A., P. M. Murphy, and J. M. Farber.** 1999. Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. Annu Rev Immunol **17:**657-700.

4. **Berlioz-Torrent, C., B. L. Shacklett, L. Erdtmann, L. Delamarre, I. Bouchaert, P. Sonigo, M. C. Dokhelar, and R. Benarous.** 1999. Interactions of the cytoplasmic domains of human and simian retroviral transmembrane proteins with components of the clathrin adaptor complexes modulate intracellular and cell surface expression of envelope glycoproteins. J Virol **73:**1350-61.

5. **Blot, G., K. Janvier, S. Le Panse, R. Benarous, and C. Berlioz-Torrent.** 2003. Targeting of the human immunodeficiency virus type 1 envelope to the trans-Golgi network through binding to TIP47 is required for env incorporation into virions and infectivity. J Virol **77:**6931-45.

6. **Boge, M., S. Wyss, J. S. Bonifacino, and M. Thali.** 1998. A membrane-proximal tyrosine-based signal mediates internalization of the HIV-1 envelope glycoprotein via interaction with the AP-2 clathrin adaptor. J Biol Chem **273:**15773-8.

7. **Bonifacino, J. S., and L. M. Traub.** 2003. Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annu Rev Biochem **72:**395-447.

8. **Bowers, K., A. Pelchen-Matthews, S. Honing, P. J. Vance, L. Creary, B. S. Haggarty, J. Romano, W. Ballensiefen, J. A. Hoxie, and M. Marsh.** 2000. The simian immunodeficiency virus envelope glycoprotein contains multiple signals that regulate its cell surface expression and endocytosis. Traffic **1:**661-74.

9. **Buchacher, A., R. Predl, K. Strutzenberger, W. Steinfellner, A. Trkola, M. Purtscher, G. Gruber, C. Tauer, F. Steindl, A. Jungbauer, and et al.** 1994. Generation of human monoclonal antibodies against HIV-1 proteins; electrofusion and Epstein-Barr virus transformation for peripheral blood lymphocyte immortalization. AIDS Res Hum Retroviruses **10:**359-69.

10. **Bultmann, A., W. Muranyi, B. Seed, and J. Haas.** 2001. Identification of two sequences in the cytoplasmic tail of the human immunodeficiency virus type 1 envelope glycoprotein that inhibit cell surface expression. J Virol **75:**5263-76.

11. **Burton, D. R., C. F. Barbas, 3rd, M. A. Persson, S. Koenig, R. M. Chanock, and R. A. Lerner.** 1991. A large array of human monoclonal antibodies to type 1 human immunodeficiency virus from combinatorial libraries of asymptomatic seropositive individuals. Proc Natl Acad Sci U S A **88:**10134-7.

12. **Burton, D. R., J. Pyati, R. Koduri, S. J. Sharp, G. B. Thornton, P. W. Parren, L. S. Sawyer, R. M. Hendry, N. Dunlop, P. L. Nara, and et al.** 1994. Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. Science **266:**1024-7.

13. **Byland, R., P. J. Vance, J. A. Hoxie, and M. Marsh.** 2007. A conserved dileucine motif mediates clathrin and AP-2-dependent endocytosis of the HIV-1 envelope protein. Mol Biol Cell **18:**414-25.

14. **Cavrois, M., C. De Noronha, and W. C. Greene.** 2002. A sensitive and specific enzyme-based assay detecting HIV-1 virion fusion in primary T lymphocytes. Nat Biotechnol **20:**1151-4.

15. **Cavrois, M., J. Neidleman, M. Bigos, and W. C. Greene.** 2004. Fluorescence resonance energy transfer-based HIV-1 virion fusion assay. Methods Mol Biol **263:**333-44.

16. **Celma, C. C., J. M. Manrique, J. L. Affranchino, E. Hunter, and S. A. Gonzalez.** 2001. Domains in the simian immunodeficiency virus gp41 cytoplasmic tail required for envelope incorporation into particles. Virology **283:**253-61.

17. **Chesebro, B., and K. Wehrly.** 1988. Development of a sensitive quantitative focal assay for human immunodeficiency virus infectivity. J Virol **62:**3779-88.

18. **Chesebro, B., K. Wehrly, J. Nishio, and S. Perryman.** 1992. Macrophage-tropic human immunodeficiency virus isolates from different patients exhibit unusual V3 envelope sequence homogeneity in comparison with T-cell-tropic isolates: definition of critical amino acids involved in cell tropism. J Virol **66:**6547-54.

19. **Compans, R. W.** 1995. Virus entry and release in polarized epithelial cells. Curr Top Microbiol Immunol **202:**209-19.

20. **Cosson, P.** 1996. Direct interaction between the envelope and matrix proteins of HIV-1. Embo J **15:**5783-8.

21. **Crawford, J. M., P. L. Earl, B. Moss, K. A. Reimann, M. S. Wyand, K. H. Manson, M. Bilska, J. T. Zhou, C. D. Pauza, P. W. Parren, D. R. Burton, J. G. Sodroski, N. L. Letvin, and D. C. Montefiori.** 1999. Characterization of primary isolate-like variants of simian-human immunodeficiency virus. J Virol **73:**10199-207.

22. **Day, J. R., C. Munk, and J. C. Guatelli.** 2004. The membrane-proximal tyrosine-based sorting signal of human immunodeficiency virus type 1 gp41 is required for optimal viral infectivity. J Virol **78:**1069-79.

23. **Derdeyn, C. A., J. M. Decker, J. N. Sfakianos, X. Wu, W. A. O'Brien, L. Ratner, J. C. Kappes, G. M. Shaw, and E. Hunter.** 2000. Sensitivity of human immunodeficiency virus type 1 to the fusion inhibitor T-20 is modulated by coreceptor specificity defined by the V3 loop of gp120. J Virol **74:**8358-67.

24. **Deschambeault, J., J. P. Lalonde, G. Cervantes-Acosta, R. Lodge, E. A. Cohen, and G. Lemay.** 1999. Polarized human immunodeficiency virus budding in lymphocytes involves a tyrosine-based signal and favors cell-to-cell viral transmission. J Virol **73:**5010-7.

25. **Dong, X., H. Li, A. Derdowski, L. Ding, A. Burnett, X. Chen, T. R. Peters, T. S. Dermody, E. Woodruff, J. J. Wang, and P. Spearman.** 2005. AP-3 directs the intracellular trafficking of HIV-1 Gag and plays a key role in particle assembly. Cell **120:**663-74.

26. **Dubay, J. W., S. J. Roberts, B. H. Hahn, and E. Hunter.** 1992. Truncation of the human immunodeficiency virus type 1 transmembrane glycoprotein cytoplasmic domain blocks virus infectivity. J Virol **66:**6616-25.

27. **Edwards, T. G., S. Wyss, J. D. Reeves, S. Zolla-Pazner, J. A. Hoxie, R. W. Doms, and F. Baribaud.** 2002. Truncation of the cytoplasmic domain induces exposure of conserved regions in the ectodomain of human immunodeficiency virus type 1 envelope protein. J Virol **76:**2683-91.

28. **Egan, M. A., L. M. Carruth, J. F. Rowell, X. Yu, and R. F. Siliciano.** 1996. Human immunodeficiency virus type 1 envelope protein endocytosis mediated by a highly conserved intrinsic internalization signal in the cytoplasmic domain of gp41 is suppressed in the presence of the Pr55gag precursor protein. J Virol **70:**6547-56.

29. **Etemad-Moghadam, B., Y. Sun, E. K. Nicholson, G. B. Karlsson, D. Schenten, and J. Sodroski.** 1999. Determinants of neutralization resistance in the envelope glycoproteins of a simian-human immunodeficiency virus passaged in vivo. J Virol **73:**8873-9.

30. **Freed, E. O., and M. A. Martin.** 1996. Domains of the human immunodeficiency virus type 1 matrix and gp41 cytoplasmic tail required for envelope incorporation into virions. J Virol **70:**341-51.

31. **Fultz, P. N., P. J. Vance, M. J. Endres, B. Tao, J. D. Dvorin, I. C. Davis, J. D. Lifson, D. C. Montefiori, M. Marsh, M. H. Malim, and J. A. Hoxie.** 2001. In vivo attenuation of simian immunodeficiency virus by disruption of a tyrosine-dependent sorting signal in the envelope glycoprotein cytoplasmic tail. J Virol **75:**278-91.

32. **Gabuzda, D., U. Olshevsky, P. Bertani, W. A. Haseltine, and J. Sodroski.** 1991. Identification of membrane anchorage domains of the HIV-1 gp160 envelope glycoprotein precursor. J Acquir Immune Defic Syndr **4:**34-40.

33. **Gawrisch, K., K. H. Han, J. S. Yang, L. D. Bergelson, and J. A. Ferretti.** 1993. Interaction of peptide fragment 828-848 of the envelope glycoprotein of human immunodeficiency virus type I with lipid bilayers. Biochemistry **32:**3112-8.

34. **Hammonds, J., X. Chen, L. Ding, T. Fouts, A. De Vico, J. zur Megede, S. Barnett, and P. Spearman.** 2003. Gp120 stability on HIV-1 virions and Gag-Env pseudovirions is enhanced by an uncleaved Gag core. Virology **314:**636-49.

35. **Honing, S., and W. Hunziker.** 1995. Cytoplasmic determinants involved in direct lysosomal sorting, endocytosis, and basolateral targeting of rat lgp120 (lamp-I) in MDCK cells. J Cell Biol **128:**321-32.

36. **Hunter, E., and R. Swanstrom.** 1990. Retrovirus envelope glycoproteins. Curr Top Microbiol Immunol **157:**187-253.

37. **Hunziker, W., and C. Fumey.** 1994. A di-leucine motif mediates endocytosis and basolateral sorting of macrophage IgG Fc receptors in MDCK cells. Embo J **13:**2963-9.

38. **Jiang, J., and C. Aiken.** 2006. Maturation of the viral core enhances the fusion of HIV-1 particles with primary human T cells and monocyte-derived macrophages. Virology **346:**460-8.

39. **Johnston, P. B., J. W. Dubay, and E. Hunter.** 1993. Truncations of the simian immunodeficiency virus transmembrane protein confer expanded virus host range by removing a block to virus entry into cells. J Virol **67:**3077-86.

40. **Jouvenet, N., P. D. Bieniasz, and S. M. Simon.** 2008. Imaging the biogenesis of individual HIV-1 virions in live cells. Nature **454:**236-40.

41. **Kalia, V., S. Sarkar, P. Gupta, and R. C. Montelaro.** 2003. Rational site-directed mutations of the LLP-1 and LLP-2 lentivirus lytic peptide domains in the intracytoplasmic tail of human immunodeficiency virus type 1 gp41 indicate common functions in cell-cell fusion but distinct roles in virion envelope incorporation. J Virol **77:**3634-46.

42. **Kliger, Y., A. Aharoni, D. Rapaport, P. Jones, R. Blumenthal, and Y. Shai.** 1997. Fusion peptides derived from the HIV type 1 glycoprotein 41 associate within phospholipid membranes and inhibit cell-cell Fusion. Structure-function study. J Biol Chem **272:**13496-505.

43. **Kodama, T., D. P. Wooley, Y. M. Naidu, H. W. Kestler, 3rd, M. D. Daniel, Y. Li, and R. C. Desrosiers.** 1989. Significance of premature stop codons in env of simian immunodeficiency virus. J Virol **63:**4709-14.

44. **Kol, N., Y. Shi, M. Tsvitov, D. Barlam, R. Z. Shneck, M. S. Kay, and I. Rousso.** 2007. A stiffness switch in human immunodeficiency virus. Biophys J **92:**1777-83.

45. **LaBranche, C. C., M. M. Sauter, B. S. Haggarty, P. J. Vance, J. Romano, T. K. Hart, P. J. Bugelski, M. Marsh, and J. A. Hoxie.** 1995. A single amino acid change in the cytoplasmic domain of the simian immunodeficiency virus transmembrane molecule increases envelope glycoprotein expression on infected cells. J Virol **69:**5217-27.

46. **Lambele, M., B. Labrosse, E. Roch, A. Moreau, B. Verrier, F. Barin, P. Roingeard, F. Mammano, and D. Brand.** 2007. Impact of natural polymorphism within the gp41 cytoplasmic tail of human immunodeficiency virus type 1 on the intracellular distribution of envelope glycoproteins and viral assembly. J Virol **81:**125-40.

47. **Letourneur, F., and R. D. Klausner.** 1992. A novel di-leucine motif and a tyrosine-based motif independently mediate lysosomal targeting and endocytosis of CD3 chains. Cell **69:**1143-57.

48. **Lin, X., C. A. Derdeyn, R. Blumenthal, J. West, and E. Hunter.** 2003. Progressive truncations C terminal to the membrane-spanning domain of simian immunodeficiency virus Env reduce fusogenicity and increase concentration dependence of Env for fusion. J Virol **77:**7067-77.

49. **Lineberger, J. E., R. Danzeisen, D. J. Hazuda, A. J. Simon, and M. D. Miller.** 2002. Altering expression levels of human immunodeficiency virus type 1 gp120-gp41 affects efficiency but not kinetics of cell-cell fusion. J Virol **76:**3522-33.

50. **Lodge, R., H. Gottlinger, D. Gabuzda, E. A. Cohen, and G. Lemay.** 1994. The intracytoplasmic domain of gp41 mediates polarized budding of human immunodeficiency virus type 1 in MDCK cells. J Virol **68:**4857-61.

51. **Lodge, R., J. P. Lalonde, G. Lemay, and E. A. Cohen.** 1997. The membrane-proximal intracytoplasmic tyrosine residue of HIV-1 envelope glycoprotein is critical for basolateral targeting of viral budding in MDCK cells. Embo J **16:**695-705.

52. **Lopez-Verges, S., G. Camus, G. Blot, R. Beauvoir, R. Benarous, and C. Berlioz-Torrent.** 2006. Tail-interacting protein TIP47 is a connector between Gag and Env and is required for Env incorporation into HIV-1 virions. Proc Natl Acad Sci U S A **103:**14947-52.

53. **Lu, L., Y. Zhu, J. Huang, X. Chen, H. Yang, S. Jiang, and Y. H. Chen.** 2008. Surface exposure of the HIV-1 env cytoplasmic tail LLP2 domain during the membrane fusion process: interaction with gp41 fusion core. J Biol Chem **283:**16723-31.

54. **Mascola, J. R., M. G. Lewis, G. Stiegler, D. Harris, T. C. VanCott, D. Hayes, M. K. Louder, C. R. Brown, C. V. Sapan, S. S. Frankel, Y. Lu, M. L. Robb, H. Katinger, and D. L. Birx.** 1999. Protection of Macaques against pathogenic simian/human immunodeficiency virus 89.6PD by passive transfer of neutralizing antibodies. J Virol **73:**4009-18.

55. **Miller, M. A., M. W. Cloyd, J. Liebmann, C. R. Rinaldo, Jr., K. R. Islam, S. Z. Wang, T. A. Mietzner, and R. C. Montelaro.** 1993. Alterations in cell membrane permeability by the lentivirus lytic peptide (LLP-1) of HIV-1 transmembrane protein. Virology **196:**89-100.

56. **Murakami, T., S. Ablan, E. O. Freed, and Y. Tanaka.** 2004. Regulation of human immunodeficiency virus type 1 Env-mediated membrane fusion by viral protease activity. J Virol **78:**1026-31.

57. **Murakami, T., and E. O. Freed.** 2000. Genetic evidence for an interaction between human immunodeficiency virus type 1 matrix and alpha-helix 2 of the gp41 cytoplasmic tail. J Virol **74:**3548-54.

58. **Murakami, T., and E. O. Freed.** 2000. The long cytoplasmic tail of gp41 is required in a cell type-dependent manner for HIV-1 envelope glycoprotein incorporation into virions. Proc Natl Acad Sci U S A **97:**343-8.

59. **Ohno, H., R. C. Aguilar, M. C. Fournier, S. Hennecke, P. Cosson, and J. S. Bonifacino.** 1997. Interaction of endocytic signals from the HIV-1 envelope glycoprotein complex with members of the adaptor medium chain family. Virology **238:**305-15.

60. **Owen, D. J., H. Setiadi, P. R. Evans, R. P. McEver, and S. A. Green.** 2001. A third specificity-determining site in mu 2 adaptin for sequences upstream of Yxx phi sorting motifs. Traffic **2:**105-10.

61. **Parren, P. W., P. Fisicaro, A. F. Labrijn, J. M. Binley, W. P. Yang, H. J. Ditzel, C. F. Barbas, 3rd, and D. R. Burton.** 1996. In vitro antigen challenge of human antibody libraries for vaccine evaluation: the human immunodeficiency virus type 1 envelope. J Virol **70:**9046-50.

62. **Pincus, S. H., K. Wehrly, and B. Chesebro.** 1989. Treatment of HIV tissue culture infection with monoclonal antibody-ricin A chain conjugates. J Immunol **142:**3070-5.

63. **Platt, E. J., K. Wehrly, S. E. Kuhmann, B. Chesebro, and D. Kabat.** 1998. Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1. J Virol **72:**2855-64.

64. **Roben, P., J. P. Moore, M. Thali, J. Sodroski, C. F. Barbas, 3rd, and D. R. Burton.** 1994. Recognition properties of a panel of human recombinant Fab fragments to the CD4 binding site of gp120 that show differing abilities to neutralize human immunodeficiency virus type 1. J Virol **68:**4821-8.

65. **Rowell, J. F., P. E. Stanhope, and R. F. Siliciano.** 1995. Endocytosis of endogenously synthesized HIV-1 envelope protein. Mechanism and role in processing for association with class II MHC. J Immunol **155:**473-88.

66. **Salzwedel, K., J. T. West, and E. Hunter.** 1999. A conserved tryptophan-rich motif in the membrane-proximal region of the human immunodeficiency virus type 1 gp41 ectodomain is important for Env-mediated fusion and virus infectivity. J Virol **73:**2469-80.

67. **Sattentau, Q. J., S. Zolla-Pazner, and P. Poignard.** 1995. Epitope exposure on functional, oligomeric HIV-1 gp41 molecules. Virology **206:**713-7.

68. **Sauter, M. M., A. Pelchen-Matthews, R. Bron, M. Marsh, C. C. LaBranche, P. J. Vance, J. Romano, B. S. Haggarty, T. K. Hart, W. M. Lee, and J. A. Hoxie.** 1996. An internalization signal in the simian immunodeficiency virus transmembrane protein cytoplasmic domain modulates expression of envelope glycoproteins on the cell surface. J Cell Biol **132:**795-811.

69. **Shang, L., L. Yue, and E. Hunter.** 2008. Role of the membrane-spanning domain of human immunodeficiency virus type 1 envelope glycoprotein in cell-cell fusion and virus infection. J Virol **82:**5417-28.

70. **Srinivas, S. K., R. V. Srinivas, G. M. Anantharamaiah, J. P. Segrest, and R. W. Compans.** 1992. Membrane interactions of synthetic peptides corresponding to amphipathic helical segments of the human immunodeficiency virus type-1 envelope glycoprotein. J Biol Chem **267:**7121-7.

71. **Takeuchi, Y., M. O. McClure, and M. Pizzato.** 2008. Identification of gammaretroviruses constitutively released from cell lines used for human immunodeficiency virus research. J Virol **82:**12585-8.

72. **Toohey, K., K. Wehrly, J. Nishio, S. Perryman, and B. Chesebro.** 1995. Human immunodeficiency virus envelope V1 and V2 regions influence replication efficiency in macrophages by affecting virus spread. Virology **213:**70-9.

73. **Trkola, A., M. Purtscher, T. Muster, C. Ballaun, A. Buchacher, N. Sullivan, K. Srinivasan, J. Sodroski, J. P. Moore, and H. Katinger.** 1996. Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. J Virol **70:**1100-8.

74. **Venable, R. M., R. W. Pastor, B. R. Brooks, and F. W. Carson.** 1989. Theoretically determined three-dimensional structures for amphipathic segments of the HIV-1 gp41 envelope protein. AIDS Res Hum Retroviruses **5:**7-22.

75. **Wehrly, K., and B. Chesebro.** 1997. p24 antigen capture assay for quantification of human immunodeficiency virus using readily available inexpensive reagents. Methods **12:**288-93.

76. **Wei, X., J. M. Decker, H. Liu, Z. Zhang, R. B. Arani, J. M. Kilby, M. S. Saag, X. Wu, G. M. Shaw, and J. C. Kappes.** 2002. Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. Antimicrob Agents Chemother **46:**1896-905.

77. **West, J. T., P. B. Johnston, S. R. Dubay, and E. Hunter.** 2001. Mutations within the putative membrane-spanning domain of the simian immunodeficiency virus transmembrane glycoprotein define the minimal requirements for fusion, incorporation, and infectivity. J Virol **75:**9601-12.

78. **West, J. T., S. K. Weldon, S. Wyss, X. Lin, Q. Yu, M. Thali, and E. Hunter.** 2002. Mutation of the dominant endocytosis motif in human immunodeficiency virus type 1 gp41 can complement matrix mutations without increasing Env incorporation. J Virol **76:**3338-49.

79. **White, J. M.** 1992. Membrane fusion. Science **258:**917-24.

80. **Wilk, T., T. Pfeiffer, and V. Bosch.** 1992. Retained in vitro infectivity and cytopathogenicity of HIV-1 despite truncation of the C-terminal tail of the env gene product. Virology **189:**167-77.

81. **Willey, R. L., and M. A. Martin.** 1993. Association of human immunodeficiency virus type 1 envelope glycoprotein with particles depends on interactions between the third variable and conserved regions of gp120. J Virol **67:**3639-43.

82. **Wu, L., T. Zhou, Z. Y. Yang, K. Svehla, S. O'Dell, M. K. Louder, L. Xu, J. R. Mascola, D. R. Burton, J. A. Hoxie, R. W. Doms, P. D. Kwong, and G. J. Nabel.** 2009. Enhanced exposure of the CD4-binding site to neutralizing antibodies by structural design of a membrane-anchored human immunodeficiency virus type 1 gp120 domain. J Virol **83:**5077-86.

83. **Wyma, D. J., J. Jiang, J. Shi, J. Zhou, J. E. Lineberger, M. D. Miller, and C. Aiken.** 2004. Coupling of human immunodeficiency virus type 1 fusion to virion maturation: a novel role of the gp41 cytoplasmic tail. J Virol **78:**3429-35.

84. **Wyss, S., C. Berlioz-Torrent, M. Boge, G. Blot, S. Honing, R. Benarous, and M. Thali.** 2001. The highly conserved C-terminal dileucine motif in the cytosolic domain of the human immunodeficiency virus type 1 envelope glycoprotein is critical for its association with the AP-1 clathrin adaptor [correction of adapter]. J Virol **75:**2982-92.

85. **Wyss, S., A. S. Dimitrov, F. Baribaud, T. G. Edwards, R. Blumenthal, and J. A. Hoxie.** 2005. Regulation of human immunodeficiency virus type 1 envelope glycoprotein fusion by a membrane-interactive domain in the gp41 cytoplasmic tail. J Virol **79:**12231-41.

86. **Yuste, E., J. D. Reeves, R. W. Doms, and R. C. Desrosiers.** 2004. Modulation of Env content in virions of simian immunodeficiency virus: correlation with cell surface expression and virion infectivity. J Virol **78:**6775-85.

87. **Zingler, K., and D. R. Littman.** 1993. Truncation of the cytoplasmic domain of the simian immunodeficiency virus envelope glycoprotein increases env incorporation into particles and fusogenicity and infectivity. J Virol **67:**2824-31.

DISCUSSION

The work illustrated within this dissertation highlights the contributions of a key component of HIV-1 infectivity, the envelope glycoprotein. In the scope of the viral life cycle, Env determines host and cell tropism and facilitates the entry of HIV-1 virions into target host cells. Production of infection-competent virions requires the interaction of the Env and Gag proteins and the subsequent incorporation of Env into budding virions, which is mediated by the gp41 cytoplasmic domain. Although the interaction between Env and Gag resulting in Env incorporation has been considered to be the major contribution of the gp41 CD (84, 374), its role in regulating the cell surface expression of Env, directing the intracellular trafficking of the Env glycoprotein, and in mediating the fusogenicity of mature virions have become of particular interest.

HIV-1 follows a C-type assembly, in which the viral components traffic to the location of particle budding. The Env-Gag interaction, which is necessary for Env incorporation into budding virions, requires that both components traffic to a central location within the cell,

which remains to be determined. In the context of our study, progressive mutation of the conserved Y- and LL-motifs in the CD did not affect the transport of Env from the ER to the Golgi, as evident from WT levels of Env precursor and cleavage products produced during Pulse-chase experiments. The putative trafficking motifs are also dispensable for the subsequent transport of Env from the TGN to the PM, as indicated by at least WT levels of glycoprotein surface expression by immunostaining Env with three different Abs.

Env is endocytosed into intracellular compartments following expression at the cell surface, and sequences in the gp41 CD have been shown to mediate the internalization of Env from the plasma membrane, with the membrane-proximal Yxxϕ motif being established as the major endocytosis signal (31, 283, 320). Within the scope of our progressive mutagenic study of Env, abrogation of the Y_{712} motif had the most dramatic effect on Env expression at the PM of COS-1 cells. Interestingly, in the WT Y_{712} panel of Env glycoproteins, progressive mutagenesis of the CD resulted in WT levels of Env surface expression, arguing that these motifs are not required for transport of Env to the cell surface. Because these types of motifs have been shown to play functional roles in addition to endocytosis, including polarized targeting of proteins and polarized budding

of virions, interaction with cellular proteins, and intracellular trafficking, further characterization of the putative trafficking motifs was warranted.

A number of studies suggest that there may be additional determinants distal to the membrane-proximal tyrosine motif (35). One study proposed an endocytic role for the LL_{855} motif, but suggested that it was less active than Y_{712} (51, 397). The caveat of this report, as well as others regarding the endocytosis of the Env glycoprotein, is that they examine the contribution of the Env CD towards internalization in the context of CD4- or cluster of differnentiation 8 (CD8)-gp41CD chimeras, not the native Env protein. While such an approach may provide more quantifiable data, it does not take into account the potential for trimerization of Env altering access of cellular proteins to the trafficking motifs. The results of our surface expression studies of the progressively mutated envelopes, however, did not reveal an increase in Env surface expression of mutants E and YE compared to the corresponding WT and Y712C constructs, respectively, which would be expected if mutation of an endocytic motif at 855 reduced endocytosis. Comparison of Env mutants within the Y panel revealed that abrogation of the LL_{855} motif in YE actually resulted in a decrease in Env surface expression compared to the Y mutant itself, although the surface levels remained higher than that of WT. Additional

studies are necessary to characterize the contributions of these motifs on Env internalization.

Following internalization, Env has been shown to localize in discrete intracellular compartments (30), and this targeting is likely mediated by distal determinants within the CD. Env endocytosis may facilitate the co-localization of Env and Gag at an intracellular site to facilitate Env incorporation into assembling particles, or it could provide unincorporated Env glycoproteins a second opportunity to be incorporated into budding virions. The internalization of Env, which is highly immunogenic, may also serve to minimize the exposure of unincorporated Env to immune detection.

Studies indicate interactions between the conserved Y- and LL-motifs in the Env CD and key cellular proteins, known to mediate intracellular trafficking, internalization, and polarized protein targeting and viral budding. These interactions include AP-1 to Y_{712}, Y_{768}, and LL_{855} motifs (27, 283, 397); AP-2 with Y_{712} and Y_{768} (27, 31, 51, 283), and AP-3 with Y_{712} and Y_{768} (283). AP-3, which transports proteins in the normal direction from the Golgi to vacuoles or lysosomes, has been determined to be essential for Env incorporation into budding virions (108), but in what capacity, remains to be shown.

Despite the contribution of the AP complexes to Env trafficking, none have been shown to directly mediate the interaction between Env and Gag.

The cellular tail interacting protein of 47 kDa, TIP47, which mediates the retrograde transport of proteins from the late endosome to the TGN, has been shown to link the Env and Gag interaction and is critical for incorporation of Env into HIV-1 virions (30, 232). A deletion in the Env CD called del8 (amino acids 802-806 of Env), which includes the YW_{802} motif critical to TIP47 interaction and Env incorporation, resulted in a decrease in incorporation and a decrease in infectivity to 25% that of WT (269). The major defects of Env incorporation in our Env CD study occur upstream of the YW_{802} motif, which interacts with TIP47. Substitution of YW_{802}, essential to the Env-Gag interaction, did not result in a significant change in Env incorporation from the progressive mutagenesis of mutants C and YC to mutants D and YD, respectively. This result suggests that the Y- and LL-motifs upstream of YW_{802} may also contribute to Env incorporation.

Interestingly, mutation of the first two pins of the Y_{768} motif in mutants A and YA resulted in a 94% and 63% incorporation of Env compared to WT; and the Y mutant resulted in 50% Env incorporation that of WT. Mutagenesis beyond this point in the CD

resulted in a dramatic decrease in Env incorporation to ~20-38% that of WT (refer to Table 1). Although the conserved Y- and LL-motifs located upstream of YW_{802} have not been directly described in the Env-Gag interaction, they may contribute to Env CD binding to host cellular proteins and Env trafficking that occurs prior to its co-localization and interaction with Gag, thereby affecting the relative incorporation of mutant Env into assembling virions compared to WT or they may facilitate the direct interaction between Env and Gag.

Env, an N-terminal type I fusion protein, exists in a metastable state in the virion. The conversion of the Env precursor, gp160, into the metastable form of Env occurs during proteolytic processing into gp120 and gp41 during transport of the trimer through the Golgi. The gp120 SU component of the Env complex facilitates attachment of the virion to the CD4 receptor, upon which a conformational change occurs in gp120, thus allowing co-receptor binding. Following gp120 binding of the co-receptor, gp41 undergoes a conformational change, which thereby activates the fusion peptide and facilitates viral entry into the host target cell. A number of studies highlight that efficient entry resulting in productive infection of the target cells requires the interaction between the full-length Env CD and the MA domain of Gag within the mature virion.

The results from our investigation of the progressively mutated Env CD reflected a decrease in viral infectivity and virus-cell fusion (viral entry), exhibiting the most significant defects upon complete abrogation of all three pins of Y_{768} and the following dileucine-motifs at 774 and 776 in mutants B and YB and resulting in virus incapable of mediating virus-cell fusion with and infection of target TZM-bl cells (refer to Table 1). Although the gp41 CD has been characterized to function in both a structural and immunogenic capacity in the HIV-1 life cycle (113, 398), our results suggest that it may also contribute to virion fusogenicity and viral entry.

The Env CD-Gag interactions in immature particles have been shown to suppress fusion until the virus is mature and capable of initiating RT. Previous studies of the interaction of the Env CD and unprocessed Gag, describe an Env-mediated suppression of fusion of the viral and cellular membranes following CD4 and co-receptor binding activation of the Env conformational change (268, 396), where the fusion block was not a function of virus-associated Env levels (268). A novel role for Env CD coupling fusion to virion maturation was thus established (396). More recent, Kol and colleagues reported that an Env-mediated "stiffness switch", from rigid immature particles to flexible mature virions, correlates with efficient viral entry, suggesting the "first mechanistic role" of Env in

Env Mutants	Env-mediated Cell-Cell Fusion[a]	Cell Surface Expression (MAb 902) [a]	Pseudotype Virus Infectivity[a]	Virus-Cell Fusion[a]	Incorporation[a]
WT	100	100	100	100	100
A	62	101	57	17	94
B	41	195	16	0	38
C	30	125	13	0	31
D	31	120	4	0	24
E	30	136	1	0	28
Y	96	447	47	17	49
YA	62	563	6	14	53
YB	35	396	6	0	29
YC	30	563	5	0	24
YD	29	495	2	0	20
YE	17	194	1	0	32

Table 1. Table of results for Env cytoplasmic domain mutants. [a] Values are expressed as percentages of WT.

HIV-1 entry and infection (210). They also reported that the entry defect of immature virions was rescued by pesudotyping virus with CD-deleted Env (210), proposing that the CD plays a role in particle fusogenicity. The study further suggested that the "stiffness switch" may serve as a mechanism of screening the entry of non-replicating immature virions into host cells, thereby improving the replication fitness of the virus (210). Although this study compared virus containing the full-length Env CD versus those lacking the gp41 cytoplasmic tail, it establishes a role for the CD in virion fusogenicity

and entry. In the context of our study, it is possible that the Env CD mutations could modify the "lateral diffusion" of the Env trimers in the viral membrane, thereby affecting the fusion process. It is also plausible that the substitutions made in the CD may have altered the conformation of the mature Env complex, which could affect the interaction between Env and Gag and alter the rigidity or flexibility of the virion as to either inhibit entry of the virion or allow non-replicating virions to enter target cells.

Interestingly, the Y_{768} and $LL_{774}LI_{776}$ motifs in mutants B and YB, which exhibited the greatest phenotypic effects from mutagenesis, are located within the region defined for the LLP (lentiviral lytic peptide) 2 region of the Env CD. A number of reports indicate a functional role for the LLP2 region of the Env CD in the fusion process (196, 197, 235, 276, 398). Truncation of Env just upstream of the $LxxY_{768}xxL$ motif, which includes the entire LLP2 region, resulted in an increase in fusion efficiency and exposure of CD4-induced neutralization sensitive epitopes in the gp41 ectodomain, suggesting that the conserved length of the CD facilitates optimal fusion activity (398). Another group investigated R (Arginine) to E (Glutamic acid) changes in two critical residues (770 and 788 in reference to NL4-3 sequence) located within the hydrophilic interface of the LLP2 domain of the dual-tropic B clade HIV-1 strains ME46 (196, 197, 276) and 89.6 (276).

The premise of this mutagenic strategy (R to E) was to reduce the net positive charge of the amphipathic helices, which this group previously showed to abrogate the membrane disrupting activities of the LLP regions (257), while preserving the hydrophobic moment and structural integrity of the protein (196, 197, 257, 276). Mutagenesis of the conserved R_{770} and R_{788} residues resulted in a 90% reduction in Env-mediated cell-cell fusion compared to WT Env, which did not correlate to the WT levels of Env surface expression (197), but supports a role for the defined LLP2 region in the fusion process.

The LLP2 region has been directly shown to associate with the process of membrane fusion (197, 398). Lu and colleagues further explored the LLP2 region, located inside the viral membrane, and suggested a transient exposure of the region on the membrane surface during the fusion process (235). This group took advantage of a suboptimal temperature (31.5°C), which has previously been shown to slow down the fusion process and prolong the fusion intermediate stage (150). The report indicates that at the suboptimal temperature (31.5°C), anti-LLP2 antibodies were capable of inhibiting HIV-1 Env-mediated cell-cell fusion of effecter (transiently expressing Env) and target (expressing the receptor and co-receptors) cells and binding to the interface between those cells (235). The results from this study suggest that the LLP2 region, located inside

the viral membrane, is transiently exposed on the surface of the membrane during the fusion process (235). Further, they showed a high affinity binding between a synthetic LLP2 peptide and the gp41 six-helix bundle core structure (235). The caveat of this study, however, is that they examined fusion in the context of HIV-1 Env expression in cells, which may not exhibit an identical phenotype as cell-free virions in the fusion process. This study illustrates the potential role of the LLP2 domain as a therapeutic target in the development of fusion and entry inhibitors.

The transition from mutants A to B indicates a role for the LLP2 region of the HIV-1 Env CD. The LLP2 region consists of an amphipathic α-helix, which includes charged residues on one side of the helix and hydrophobic residues on the other side (Fig. 1). Our mutagenesis strategy resulted in the substitution of the conserved non-polar LL-motifs within the LLP2 region with either basic or polar residues. These mutagenic changes may affect the fusion process by disrupting the hydrogen bonding of the existing polar groups within the α-helix, abrogating the amphipathic nature of the α-helix in turn disabling its ability to insert into or interact at the interface of the lipid membrane, or reducing the hydrophobic moment of LLP2 thereby decreasing the helicity of the

structure. Interestingly, the most dramatic phenotypes resulted from complete abrogation of the three pins of Y_{768} and $LL_{774}LI_{776}$ motifs, which overlap with LLP2.

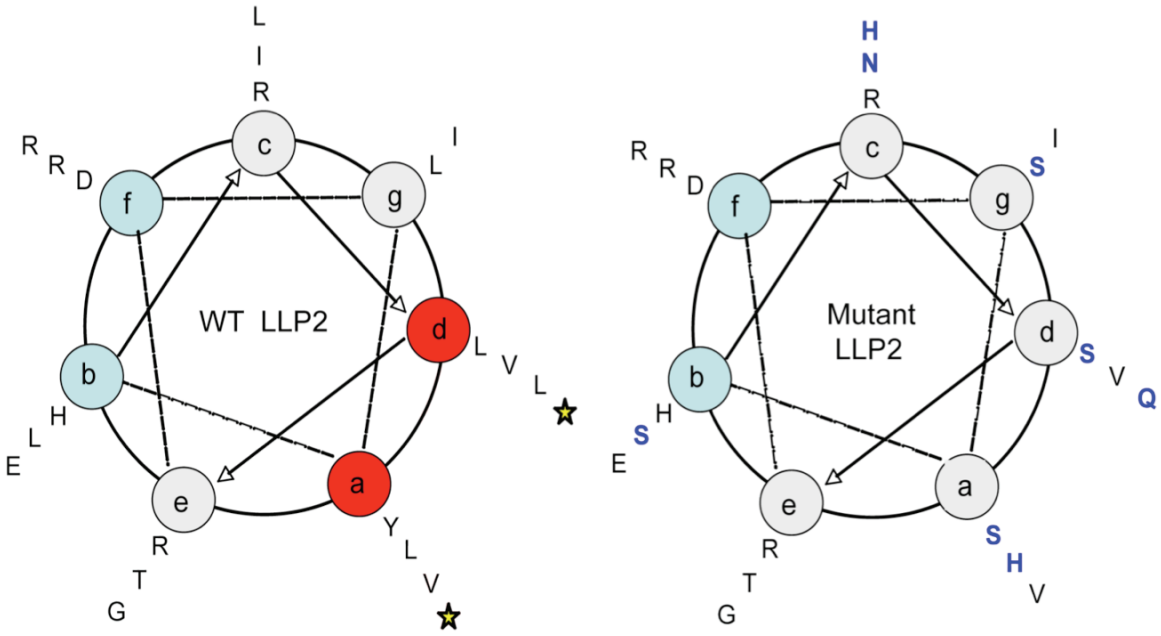

Fig. 1. A bird's eye view of the LLP2 amphipathic alpha helix. The WT LLP2 sequence is depicted by the helix on the left (A), whereas the mutagenic substitutions introduced into this region are represented in the helix on the right (B). Each turn of the helix begins at the N-terminal residue of the region, represented by ***a,*** and proceeds through ***b***, ***c***, ***d***, ***e***, ***f***, and ***g***, and then back to ***a***, with the corresponding amino acids listed by their location within the helix. Polar residues (light blue circles) on one side of the helix and non-polar residues (red circles) on the opposite side of the helix characterize the amphipathic nature of LLP2 region. In the helix on the right, grey circles represent the disruption of the non-polar amino acids by both charged and polar residues.

The LLP2 region has been proposed to play a role in the fusion process (257). Transient exposure of the LLP2 region on the extracellular surface during the fusion process has also been suggested more recently (235). Venable and colleagues have postulated an

interaction between the LLP2 and LLP1 regions of the Env CD within the lipid membrane (370), while another group suggested that the LLP regions might form viroporins that affect the membrane ionic permeability through the formation of ion channels and by creating disturbances in the lipid bilayer (85).

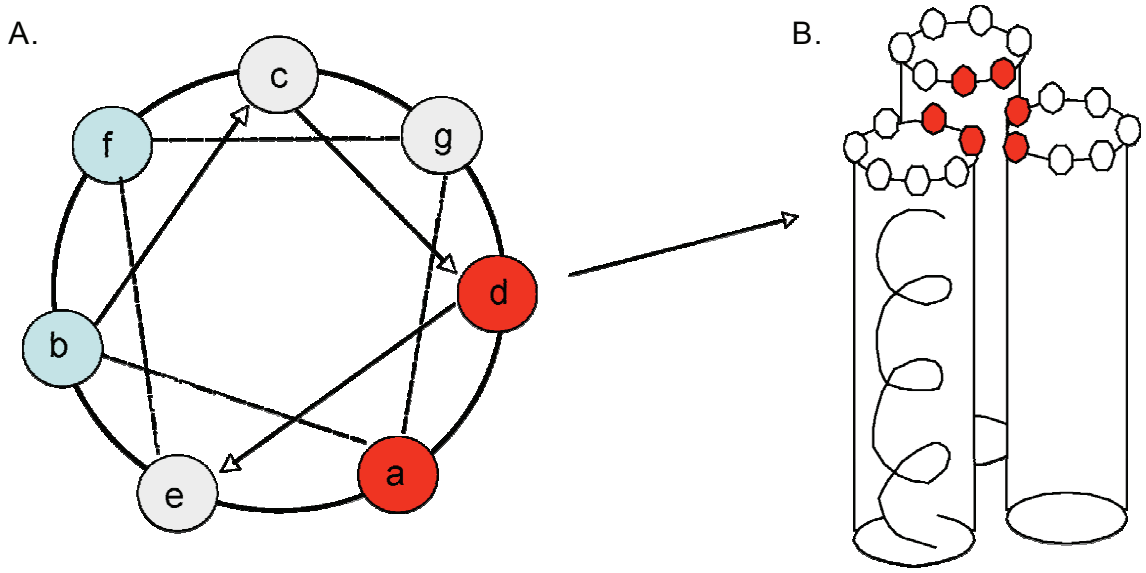

Fig. 2. Amphipathic alpha helix. A bird's eye view of the amphipathic LLP2 region is shown on the left (A) , which includes distinct polar (light blue) and non-polar (red circles) sides. The timeric nture of the mature envelope glycoprotein is represented by the figure on the right (B), in which the non-polar residues of the LLP2 region form contact points within the trimer f the gp41 cytoplasmic domain.

A bird's eye view of the LLP2 amphipathic helix from its amino-terminus is shown in Fig. 2, comparing the WT sequence to the substitutions made in the LLP2 region as a result of our mutagenesis strategy. This particular view of the amphipathic helices

clearly depicts the disruption of the hydrophobicity of the helix and, in turn, the amphipathic nature of the helix.

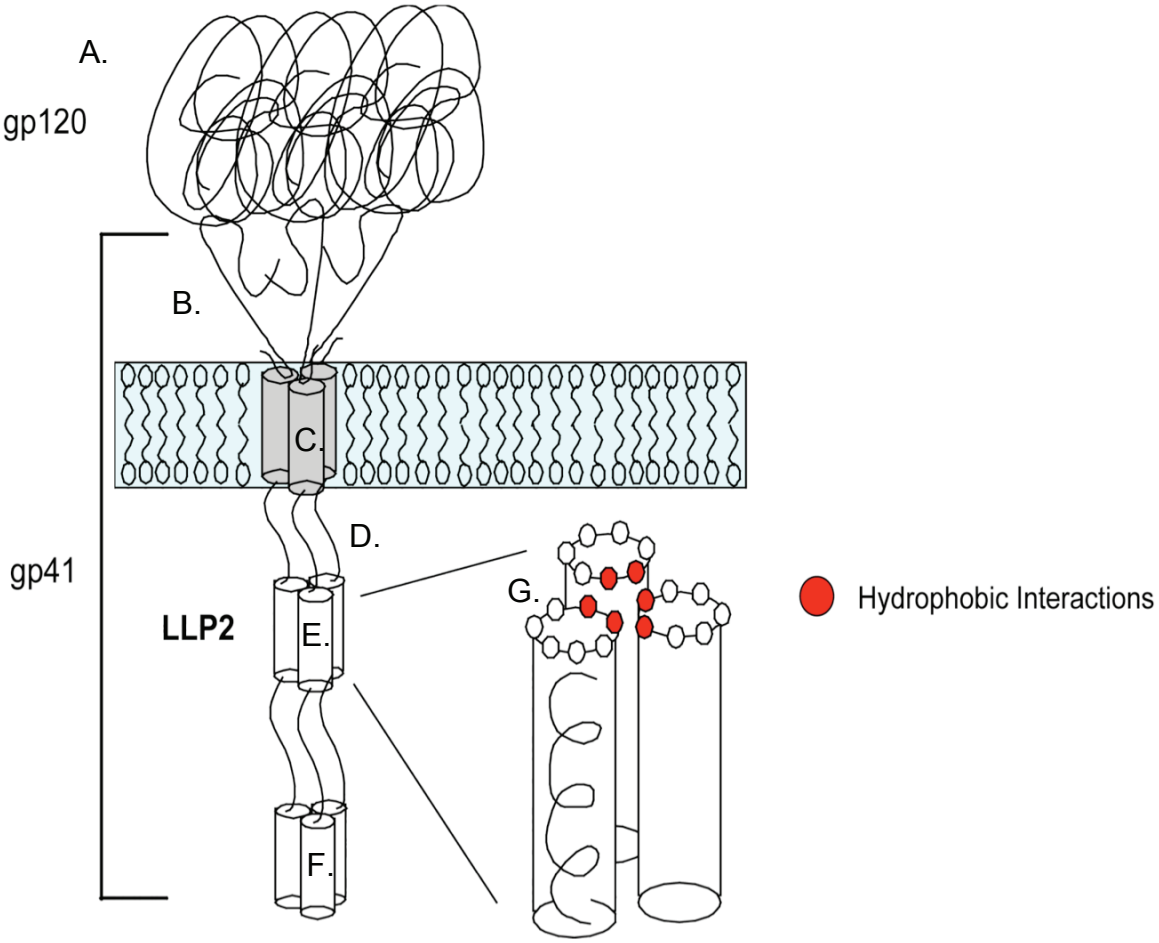

Fig. 3. Proposed model for the Env cytoplasmic domain. (A) gp120, (B) gp41 ectodomain, (C) gp41 transmembrane domain, (D) gp41 cytoplasmic domain, (E) LLP2, (F) LLP1, and (G) hydrophobic interactions within trimer of amphipathic LLP2.

In the context of the mature Env complex trimer, one could invisage how the hydrophobic side of the LLP2 amphipathic helix could form contact points within the

trimer of three amphipathic LLP2 regions of the Env complex (Fig. 3). These hydrophobic residues could function to stabilize the Env protein (Fig. 3), both in the context of the mature Env complex located in the cellular lipid membrane and in its interaction with the structural components of the particle while embedded in the virion lipid membrane.

Amphipathic helices have been described to function as cytolytic compounds that disrupt bacterial lipid membranes through insertion into the membrane (5, 28). They have also been shown to interact with the cellular lipid membrane at the interface between the membrane and the aqueous surface of the cytosol, with helical amphiphilicity being more important than hydrophobicity with regard to interfacial binding (122). Further, interfacially adsorbed amphipathic helices have also been shown to increase membrane curvature, which may facilitate the formation of a fusion intermediate during the fusion process with regard to the function of LLP2 (405). Whether the LLP2 region functions to stabilize the Env trimer, to form a viroporin by insertion into the cell membrane or to bring the membranes closer by increasing curvature through its interaction with the lipid membrane, it is evident that reduction of the hydrophobic moment and abrogation of the

amphipathic nature and helicity of the LLP2 region could disrupt the function of the Env CD.

The future directions of this study include investigation of Y (Tyrosine) to S (Serine) and LL (Leucine) to AA (Alanine) mutagenesis of the conserved Y- and LL-motifs in the HIV-1 Env CD, which would maintain the hydrophobicity, amphipathic nature, and helicity of the LLP2 region. By maintaining the structural integrity of the LLP2 region, the contribution of the conserved Y- and LL-motifs in the fusion process could be further investigated. Studying these mutants would require the insertion of an upstream stop codon in *rev* that would allow the MPMV Constituitive Transport Element (CTE) engineered into our Env expression vector to function in the nuclear export of viral transcripts, particularly for Env, in order to analyze the contribution of the mutant Env proteins in the fusion process. It would also be of interest to explore the role of the conserved Y- and LL-motifs in virion fusogenicity, as described by Kol and colleagues (210), and during the fusion process at the suboptimal temperature of 31.5°C, as reported by Lu and colleagues (235).

Future investigation of the HIV-1 Env CD mutants could also move towards the investigation of the leucine-rich activation domain of Rev. Because the second ORF of Rev overlaps with the Env CD (Fig. 4), employment of our mutagenesis strategies of the gp41 cytoplasmic tail have been complicated. Previously contructed Env CD mutants,

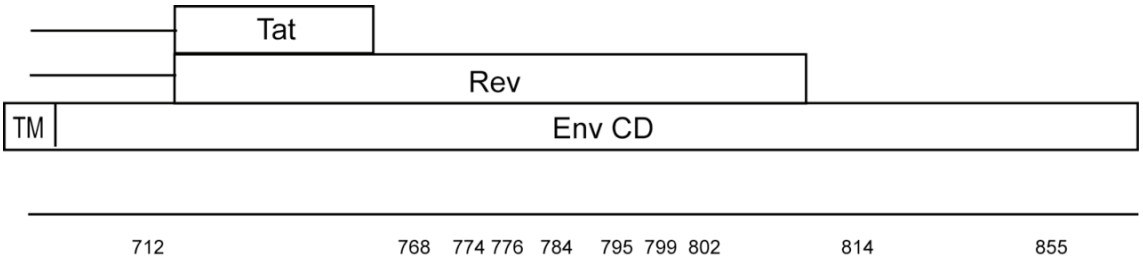

Fig. 4. Overlapping reading frames of Env, Rev, and Tat. The second open reading frames (ORF) of *rev* and *tat* overlap with the *env* sequence through the cytoplasmic domain. The *rev* ORF, unlike the *tat* ORF, overlaps with *env* motifs 768 through 802, indicated by the motif locations within Env a the bottom of the figure.

that implement a Y to S and a LL to AA progressive mutagenic strategy (Fig. 5), resulted in decreased levels of Env biosynthesis in pulse-chase experiments (Fig. 6), even in the presence of the MPMV CTE. These Env substitutions produced dominant negative mutations of the Rev activation domain, containing the Rev nuclear export signal, resulted in a decrease in the nuclear export of viral transcripts and, therefore, Env biosynthesis. Additionally, we created a deletion mutant, Δ771-775, in the Env glycoprotein CD, which happened to delete critical residues in the Rev activation domain.

This deletion completely abrogated Env biosynthesis, consequently resulting in a dominant negative Rev phenotype in the presence of the MPMV CTE.

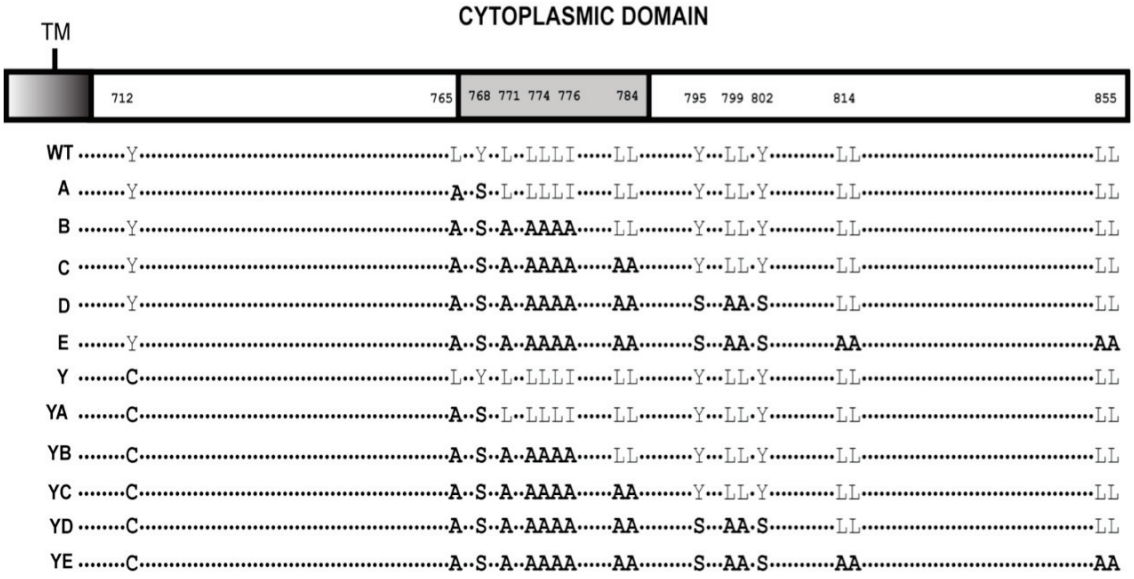

Fig. 5. Traditional mutagenesis strategy of the gp41 cytoplasmic domain Y- and LL-motifs. The key amino acids of the Y- and LL-motifs targeted for mutagenesis are listed under the corresponding location in the NL4-3 envelope cytoplasmic domain. WT residues are indicated by regular-faced type, and the residues in bold-faced type represent the progressive mutations for each mutant. The glycoproteins containing the WT Y_{712} motif (top half) are separated from those containing the Y712C mutation (bottom half).

Studies of transdominant negative Rev mutants report successful inhibition of HIV-1 infection in human T cell lines that either transiently express or that have been transduced with the dominant negative M10 *rev* gene (12, 240). Although lentiviral vectors have been used to transduce human cluster of differentiation 34 (CD34)+ hematopoietic

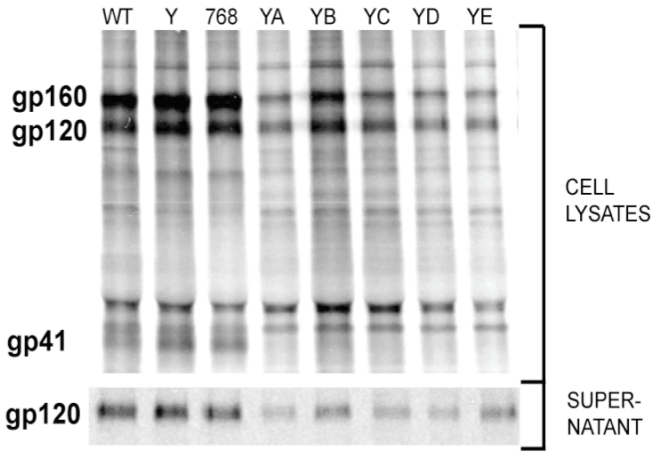

Fig. 6. Biosynthesis and processing of traditionally mutated Env glycoproteins. COS-1 cells transiently transfected with the Env expression vector pSRHS were metabolically labeled and immunoprecipitated with anti-HIV-1 patient sera. The locations of the Env precursor and the components of the mature Env complex are indicated at the left of the gel. The pulse cell lysates of glycoproteins expressed from the pSRHS vector (A) are shown in the gel at the top, and the corresponding gel for the amount of gp120 shed into the supernatant (B), is shown in the gel at the bottom.

progenitor cells (12, 240), nanomicelle technology could also be engineered to specifically target particular cell populations for delivery of the (Δ771-775) mutant *rev* gene, which can then be studied for inhibition of HIV-1 infection. The pragmatic application of nanomicelle targeting of cells that can be infected by HIV-1 for delivery of the transdominant negative *rev* gene is the gene therapy administration of a transdominant block of HIV-1 infection in humans without consequence to the function of the targeted cells.

The work highlighted in this dissertation sheds light on the contribution of the Y- and LL-motifs conserved with the long CD of gp41. In a progressive comparison of mutations made along the length of the CD, we were able to detect critical thresholds, in different stages of the HIV-1 life cycle, beyond which the virus could not function. The motifs were not necessary for transport of Env from the ER through the TGN to the PM. The membrane-proximal $Y_{712}xx\phi$ motif, however, appears to regulates the surface expression of Env. Our results also suggest that motifs upstream of the critical TIP47-binding YW_{802} motif contribute to the incorporation of Env into budding virions. Whether this contribution includes an interaction with Gag or with cellular proteins resulting in upstream trafficking to Envs interaction with Gag remains to be determined. Independent of virus, our study shows a role for these motifs in Env-mediated cell-cell fusion. The most dramatic defects in viral entry and infectivity from our progressive mutagenesis comparison resulted upon complete abrogation of the Y_{768} and and LL_{774} LI_{776} motifs, which overlap with the defined region for LLP2. Our study has brought attention to the contributions of motifs that have not previously been characterized. Moreover, the potential transient exposure of the LLP2 region on the surface of the viral membrane (235) combined with our results, defining the conserved motifs located within that region, highlight a potential target for the development of fusion or entry inhibitors.

GENERAL LIST OF REFERENCES

1. **Affranchino, J. L., and S. A. Gonzalez.** 2006. Mutations at the C-terminus of the simian immunodeficiency virus envelope glycoprotein affect gp120-gp41 stability on virions. Virology **347:**217-25.

2. **Aiyar, A., D. Cobrinik, Z. Ge, H. J. Kung, and J. Leis.** 1992. Interaction between retroviral U5 RNA and the T psi C loop of the tRNA(Trp) primer is required for efficient initiation of reverse transcription. J Virol **66:**2464-72.

3. **Aiyar, A., Z. Ge, and J. Leis.** 1994. A specific orientation of RNA secondary structures is required for initiation of reverse transcription. J Virol **68:**611-8.

4. **Alkhatib, G., C. Combadiere, C. C. Broder, Y. Feng, P. E. Kennedy, P. M. Murphy, and E. A. Berger.** 1996. CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. Science **272:**1955-8.

5. **Almeida, P. F., and A. Pokorny.** 2009. Mechanisms of antimicrobial, cytolytic, and cell-penetrating peptides: from kinetics to thermodynamics. Biochemistry **48:**8083-93.

6. **Aloia, R. C., H. Tian, and F. C. Jensen.** 1993. Lipid composition and fluidity of the human immunodeficiency virus envelope and host cell plasma membranes. Proc Natl Acad Sci U S A **90:**5181-5.

7. **Amarasinghe, G. K., R. N. De Guzman, R. B. Turner, and M. F. Summers.** 2000. NMR structure of stem-loop SL2 of the HIV-1 psi RNA packaging signal reveals a novel A-U-A base-triple platform. J Mol Biol **299:**145-56.

8. **Amarasinghe, G. K., J. Zhou, M. Miskimon, K. J. Chancellor, J. A. McDonald, A. G. Matthews, R. R. Miller, M. D. Rouse, and M. F. Summers.** 2001. Stem-loop SL4 of the HIV-1 psi RNA packaging signal exhibits weak affinity for the nucleocapsid protein. structural studies and implications for genome recognition. J Mol Biol **314:**961-70.

9. **Ames, J. B., R. Ishima, T. Tanaka, J. I. Gordon, L. Stryer, and M. Ikura.** 1997. Molecular mechanics of calcium-myristoyl switches. Nature **389:**198-202.

10. **Ames, J. B., T. Tanaka, L. Stryer, and M. Ikura.** 1996. Portrait of a myristoyl switch protein. Curr Opin Struct Biol **6:**432-8.

11. **Arrigo, S. J., and I. S. Chen.** 1991. Rev is necessary for translation but not cytoplasmic accumulation of HIV-1 vif, vpr, and env/vpu 2 RNAs. Genes Dev **5:**808-19.

12. **Bahner, I., T. Sumiyoshi, M. Kagoda, R. Swartout, D. Peterson, K. Pepper, F. Dorey, J. Reiser, and D. B. Kohn.** 2007. Lentiviral vector transduction of a dominant-negative Rev gene into human CD34+ hematopoietic progenitor cells potently inhibits human immunodeficiency virus-1 replication. Mol Ther **15:**76-85.

13. **Banapour, B., M. L. Marthas, R. J. Munn, and P. A. Luciw.** 1991. In vitro macrophage tropism of pathogenic and nonpathogenic molecular clones of simian immunodeficiency virus (SIVmac). Virology **183:**12-9.

14. **Bannwarth, S., and A. Gatignol.** 2005. HIV-1 TAR RNA: the target of molecular interactions between the virus and its host. Curr HIV Res **3:**61-71.

15. **Barbas, C. F., 3rd, E. Bjorling, F. Chiodi, N. Dunlop, D. Cababa, T. M. Jones, S. L. Zebedee, M. A. Persson, P. L. Nara, E. Norrby, and et al.** 1992. Recombinant human Fab fragments neutralize human type 1 immunodeficiency virus in vitro. Proc Natl Acad Sci U S A **89:**9339-43.

16. **Barboric, M., and B. M. Peterlin.** 2005. A new paradigm in eukaryotic biology: HIV Tat and the control of transcriptional elongation. PLoS Biol **3:**e76.

17. **Barre-Sinoussi, F., J. C. Chermann, F. Rey, M. T. Nugeyre, S. Chamaret, J. Gruest, C. Dauguet, C. Axler-Blin, F. Vezinet-Brun, C. Rouzioux, W. Rozenbaum, and L. Montagnier.** 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science **220:**868-71.

18. **Bartel, D. P., M. L. Zapp, M. R. Green, and J. W. Szostak.** 1991. HIV-1 Rev regulation involves recognition of non-Watson-Crick base pairs in viral RNA. Cell **67:**529-36.

19. **Baudin, F., R. Marquet, C. Isel, J. L. Darlix, B. Ehresmann, and C. Ehresmann.** 1993. Functional sites in the 5' region of human immunodeficiency virus type 1 RNA form defined structural domains. J Mol Biol **229:**382-97.

20. **Beerens, N., and B. Berkhout.** 2002. The tRNA primer activation signal in the human immunodeficiency virus type 1 genome is important for initiation and processive elongation of reverse transcription. J Virol **76:**2329-39.

21. **Beerens, N., F. Groot, and B. Berkhout.** 2001. Initiation of HIV-1 reverse transcription is regulated by a primer activation signal. J Biol Chem **276:**31247-56.

22. **Beerens, N., F. Groot, and B. Berkhout.** 2000. Stabilization of the U5-leader stem in the HIV-1 RNA genome affects initiation and elongation of reverse transcription. Nucleic Acids Res **28:**4130-7.

23. **Behnia, R., and S. Munro.** 2005. Organelle identity and the signposts for membrane traffic. Nature **438:**597-604.

24. **Benn, S., R. Rutledge, T. Folks, J. Gold, L. Baker, J. McCormick, P. Feorino, P. Piot, T. Quinn, and M. Martin.** 1985. Genomic heterogeneity of AIDS retroviral isolates from North America and Zaire. Science **230:**949-51.

25. **Berger, E. A., R. W. Doms, E. M. Fenyo, B. T. Korber, D. R. Littman, J. P. Moore, Q. J. Sattentau, H. Schuitemaker, J. Sodroski, and R. A. Weiss.** 1998. A new classification for HIV-1. Nature **391:**240.

26. **Berger, E. A., P. M. Murphy, and J. M. Farber.** 1999. Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. Annu Rev Immunol **17:**657-700.

27. **Berlioz-Torrent, C., B. L. Shacklett, L. Erdtmann, L. Delamarre, I. Bouchaert, P. Sonigo, M. C. Dokhelar, and R. Benarous.** 1999. Interactions of the cytoplasmic domains of human and simian retroviral transmembrane proteins with components of the clathrin adaptor complexes modulate intracellular and cell surface expression of envelope glycoproteins. J Virol **73:**1350-61.

28. **Bernheimer, A. W., and L. S. Avigad.** 1970. Nature and properties of a cytolytic agent produced by Bacillus subtilis. J Gen Microbiol **61:**361-9.

29. **Berthet-Colominas, C., S. Monaco, A. Novelli, G. Sibai, F. Mallet, and S. Cusack.** 1999. Head-to-tail dimers and interdomain flexibility revealed by the crystal structure of HIV-1 capsid protein (p24) complexed with a monoclonal antibody Fab. Embo J **18:**1124-36.

30. **Blot, G., K. Janvier, S. Le Panse, R. Benarous, and C. Berlioz-Torrent.** 2003. Targeting of the human immunodeficiency virus type 1 envelope to the trans-Golgi network through binding to TIP47 is required for env incorporation into virions and infectivity. J Virol **77:**6931-45.

31. **Boge, M., S. Wyss, J. S. Bonifacino, and M. Thali.** 1998. A membrane-proximal tyrosine-based signal mediates internalization of the HIV-1 envelope glycoprotein via interaction with the AP-2 clathrin adaptor. J Biol Chem **273:**15773-8.

32. **Bonifacino, J. S., and L. M. Traub.** 2003. Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annu Rev Biochem **72:**395-447.

33. **Boulanger, M. C., C. Liang, R. S. Russell, R. Lin, M. T. Bedford, M. A. Wainberg, and S. Richard.** 2005. Methylation of Tat by PRMT6 regulates human immunodeficiency virus type 1 gene expression. J Virol **79:**124-31.

34. **Bour, S., R. Geleziunas, and M. A. Wainberg.** 1995. The human immunodeficiency virus type 1 (HIV-1) CD4 receptor and its central role in promotion of HIV-1 infection. Microbiol Rev **59:**63-93.

35. **Bowers, K., A. Pelchen-Matthews, S. Honing, P. J. Vance, L. Creary, B. S. Haggarty, J. Romano, W. Ballensiefen, J. A. Hoxie, and M. Marsh.** 2000. The simian immunodeficiency virus envelope glycoprotein contains multiple signals that regulate its cell surface expression and endocytosis. Traffic **1:**661-74.

36. **Bowman, E. H., V. K. Pathak, and W. S. Hu.** 1996. Efficient initiation and strand transfer of polypurine tract-primed plus-strand DNA prevent strand transfer of internally initiated plus-strand DNA. J Virol **70:**1687-94.

37. **Brady, J., and F. Kashanchi.** 2005. Tat gets the "green" light on transcription initiation. Retrovirology **2:**69.

38. **Brasey, A., M. Lopez-Lastra, T. Ohlmann, N. Beerens, B. Berkhout, J. L. Darlix, and N. Sonenberg.** 2003. The leader of human immunodeficiency virus type 1 genomic RNA harbors an internal ribosome entry segment that is active during the G2/M phase of the cell cycle. J Virol **77:**3939-49.

39. **Bres, V., R. Kiernan, S. Emiliani, and M. Benkirane.** 2002. Tat acetyl-acceptor lysines are important for human immunodeficiency virus type-1 replication. J Biol Chem **277:**22215-21.

40. **Brigati, C., M. Giacca, D. M. Noonan, and A. Albini.** 2003. HIV Tat, its TARgets and the control of viral gene expression. FEMS Microbiol Lett **220:**57-65.

41. **Brugger, B., B. Glass, P. Haberkant, I. Leibrecht, F. T. Wieland, and H. G. Krausslich.** 2006. The HIV lipidome: a raft with an unusual composition. Proc Natl Acad Sci U S A **103:**2641-6.

42. **Buchacher, A., R. Predl, K. Strutzenberger, W. Steinfellner, A. Trkola, M. Purtscher, G. Gruber, C. Tauer, F. Steindl, A. Jungbauer, and et al.** 1994. Generation of human monoclonal antibodies against HIV-1 proteins; electrofusion and Epstein-Barr virus transformation for peripheral blood lymphocyte immortalization. AIDS Res Hum Retroviruses **10:**359-69.

43. **Buck, C. B., X. Shen, M. A. Egan, T. C. Pierson, C. M. Walker, and R. F. Siliciano.** 2001. The human immunodeficiency virus type 1 gag gene encodes an internal ribosome entry site. J Virol **75:**181-91.

44. **Bukrinsky, M. I., and O. K. Haffar.** 1999. HIV-1 nuclear import: in search of a leader. Front Biosci **4:**D772-81.

45. **Bullough, P. A., F. M. Hughson, J. J. Skehel, and D. C. Wiley.** 1994. Structure of influenza haemagglutinin at the pH of membrane fusion. Nature **371:**37-43.

46. **Bultmann, A., W. Muranyi, B. Seed, and J. Haas.** 2001. Identification of two sequences in the cytoplasmic tail of the human immunodeficiency virus type 1 envelope glycoprotein that inhibit cell surface expression. J Virol **75:**5263-76.

47. **Burke, C. J., G. Sanyal, M. W. Bruner, J. A. Ryan, R. L. LaFemina, H. L. Robbins, A. S. Zeft, C. R. Middaugh, and M. G. Cordingley.** 1992. Structural implications of spectroscopic characterization of a putative zinc finger peptide from HIV-1 integrase. J Biol Chem **267:**9639-44.

48. **Burton, D. R., C. F. Barbas, 3rd, M. A. Persson, S. Koenig, R. M. Chanock, and R. A. Lerner.** 1991. A large array of human monoclonal antibodies to type 1 human immunodeficiency virus from combinatorial libraries of asymptomatic seropositive individuals. Proc Natl Acad Sci U S A **88:**10134-7.

49. **Burton, D. R., J. Pyati, R. Koduri, S. J. Sharp, G. B. Thornton, P. W. Parren, L. S. Sawyer, R. M. Hendry, N. Dunlop, P. L. Nara, and et al.** 1994. Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. Science **266:**1024-7.

50. **Bushman, F. D., A. Engelman, I. Palmer, P. Wingfield, and R. Craigie.** 1993. Domains of the integrase protein of human immunodeficiency virus type 1 responsible for polynucleotidyl transfer and zinc binding. Proc Natl Acad Sci U S A **90:**3428-32.

51. **Byland, R., P. J. Vance, J. A. Hoxie, and M. Marsh.** 2007. A conserved dileucine motif mediates clathrin and AP-2-dependent endocytosis of the HIV-1 envelope protein. Mol Biol Cell **18:**414-25.

52. **Caffrey, M., M. Cai, J. Kaufman, S. J. Stahl, P. T. Wingfield, D. G. Covell, A. M. Gronenborn, and G. M. Clore.** 1998. Three-dimensional solution structure of the 44 kDa ectodomain of SIV gp41. Embo J **17:**4572-84.

53. **Cai, R., B. Carpick, R. F. Chun, K. T. Jeang, and B. R. Williams.** 2000. HIV-I TAT inhibits PKR activity by both RNA-dependent and RNA-independent mechanisms. Arch Biochem Biophys **373:**361-7.

54. **Cambi, A., F. de Lange, N. M. van Maarseveen, M. Nijhuis, B. Joosten, E. M. van Dijk, B. I. de Bakker, J. A. Fransen, P. H. Bovee-Geurts, F. N. van Leeuwen, N. F. Van Hulst, and C. G. Figdor.** 2004. Microdomains of the C-type lectin DC-SIGN are portals for virus entry into dendritic cells. J Cell Biol **164:**145-55.

55. **Campbell, S., and A. Rein.** 1999. In vitro assembly properties of human immunodeficiency virus type 1 Gag protein lacking the p6 domain. J Virol **73:**2270-9.

56. **Campbell, S., and V. M. Vogt.** 1995. Self-assembly in vitro of purified CA-NC proteins from Rous sarcoma virus and human immunodeficiency virus type 1. J Virol **69:**6487-97.

57. **Cannon, P. M., S. Matthews, N. Clark, E. D. Byles, O. Iourin, D. J. Hockley, S. M. Kingsman, and A. J. Kingsman.** 1997. Structure-function studies of the human immunodeficiency virus type 1 matrix protein, p17. J Virol **71:**3474-83.

58. **Cao, J., L. Bergeron, E. Helseth, M. Thali, H. Repke, and J. Sodroski.** 1993. Effects of amino acid changes in the extracellular domain of the human immunodeficiency virus type 1 gp41 envelope glycoprotein. J Virol **67:**2747-55.

59. **Carr, C. M., and P. S. Kim.** 1993. A spring-loaded mechanism for the conformational change of influenza hemagglutinin. Cell **73:**823-32.

60. **Carter, C., and G. Zybarth.** 1994. Processing of retroviral Gag polyproteins: an in vitro approach. Methods Enzymol **241:**227-53.

61. **Cavrois, M., C. De Noronha, and W. C. Greene.** 2002. A sensitive and specific enzyme-based assay detecting HIV-1 virion fusion in primary T lymphocytes. Nat Biotechnol **20:**1151-4.

62. **Cavrois, M., J. Neidleman, M. Bigos, and W. C. Greene.** 2004. Fluorescence resonance energy transfer-based HIV-1 virion fusion assay. Methods Mol Biol **263:**333-44.

63. **Celma, C. C., J. M. Manrique, J. L. Affranchino, E. Hunter, and S. A. Gonzalez.** 2001. Domains in the simian immunodeficiency virus gp41 cytoplasmic tail required for envelope incorporation into particles. Virology **283:**253-61.

64. **Chan, D. C., D. Fass, J. M. Berger, and P. S. Kim.** 1997. Core structure of gp41 from the HIV envelope glycoprotein. Cell **89:**263-73.

65. **Chazal, N., B. Gay, C. Carriere, J. Tournier, and P. Boulanger.** 1995. Human immunodeficiency virus type 1 MA deletion mutants expressed in baculovirus-infected cells: cis and trans effects on the Gag precursor assembly pathway. J Virol **69:**365-75.

66. **Cherepanov, P., G. Maertens, P. Proost, B. Devreese, J. Van Beeumen, Y. Engelborghs, E. De Clercq, and Z. Debyser.** 2003. HIV-1 integrase forms stable tetramers and associates with LEDGF/p75 protein in human cells. J Biol Chem **278:**372-81.

67. **Chesebro, B., and K. Wehrly.** 1988. Development of a sensitive quantitative focal assay for human immunodeficiency virus infectivity. J Virol **62:**3779-88.

68. **Chesebro, B., K. Wehrly, J. Nishio, and S. Perryman.** 1992. Macrophage-tropic human immunodeficiency virus isolates from different patients exhibit unusual V3 envelope sequence homogeneity in comparison with T-cell-tropic isolates: definition of critical amino acids involved in cell tropism. J Virol **66:**6547-54.

69. **Choe, H., M. Farzan, Y. Sun, N. Sullivan, B. Rollins, P. D. Ponath, L. Wu, C. R. Mackay, G. LaRosa, W. Newman, N. Gerard, C. Gerard, and J. Sodroski.** 1996. The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. Cell **85:**1135-48.

70. **Cimarelli, A., S. Sandin, S. Hoglund, and J. Luban.** 2000. Basic residues in human immunodeficiency virus type 1 nucleocapsid promote virion assembly via interaction with RNA. J Virol **74:**3046-57.

71. **Clever, J., C. Sassetti, and T. G. Parslow.** 1995. RNA secondary structure and binding sites for gag gene products in the 5' packaging signal of human immunodeficiency virus type 1. J Virol **69:**2101-9.

72. **Cochrane, A. W., C. H. Chen, and C. A. Rosen.** 1990. Specific interaction of the human immunodeficiency virus Rev protein with a structured region in the env mRNA. Proc Natl Acad Sci U S A **87:**1198-202.

73. **Coffin, J. M.** 1992. Genetic diversity and evolution of retroviruses. Curr Top Microbiol Immunol **176:**143-64.

74. **Coffin, J. M.** 1995. HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy. Science **267:**483-9.

75. **Coffin, J. M.** 1996. HIV viral dynamics. Aids **10 Suppl 3:**S75-84.

76. **Cohen, E. A., G. Dehni, J. G. Sodroski, and W. A. Haseltine.** 1990. Human immunodeficiency virus vpr product is a virion-associated regulatory protein. J Virol **64:**3097-9.

77. **Cohen, E. A., E. F. Terwilliger, Y. Jalinoos, J. Proulx, J. G. Sodroski, and W. A. Haseltine.** 1990. Identification of HIV-1 vpr product and function. J Acquir Immune Defic Syndr **3:**11-8.

78. **Collawn, J. F., A. Lai, D. Domingo, M. Fitch, S. Hatton, and I. S. Trowbridge.** 1993. YTRF is the conserved internalization signal of the transferrin receptor, and a second YTRF signal at position 31-34 enhances endocytosis. J Biol Chem **268:**21686-92.

79. **Collawn, J. F., M. Stangel, L. A. Kuhn, V. Esekogwu, S. Q. Jing, I. S. Trowbridge, and J. A. Tainer.** 1990. Transferrin receptor internalization sequence YXRF implicates a tight turn as the structural recognition motif for endocytosis. Cell **63:**1061-72.

80. **Colombini, S., S. K. Arya, M. S. Reitz, L. Jagodzinski, B. Beaver, and F. Wong-Staal.** 1989. Structure of simian immunodeficiency virus regulatory genes. Proc Natl Acad Sci U S A **86:**4813-7.

81. **Compans, R. W.** 1995. Virus entry and release in polarized epithelial cells. Curr Top Microbiol Immunol **202:**209-19.

82. **Conticello, S. G., R. S. Harris, and M. S. Neuberger.** 2003. The Vif protein of HIV triggers degradation of the human antiretroviral DNA deaminase APOBEC3G. Curr Biol **13:**2009-13.

83. **Copeland, K. F.** 2005. Modulation of HIV-1 transcription by cytokines and chemokines. Mini Rev Med Chem **5:**1093-101.

84. **Cosson, P.** 1996. Direct interaction between the envelope and matrix proteins of HIV-1. Embo J **15:**5783-8.

85. **Costin, J. M., J. M. Rausch, R. F. Garry, and W. C. Wimley.** 2007. Viroporin potential of the lentivirus lytic peptide (LLP) domains of the HIV-1 gp41 protein. Virol J **4:**123.

86. **Crawford, J. M., P. L. Earl, B. Moss, K. A. Reimann, M. S. Wyand, K. H. Manson, M. Bilska, J. T. Zhou, C. D. Pauza, P. W. Parren, D. R. Burton, J. G. Sodroski, N. L. Letvin, and D. C. Montefiori.** 1999. Characterization of primary isolate-like variants of simian-human immunodeficiency virus. J Virol **73:**10199-207.

87. **D'Agostino, D. M., B. K. Felber, J. E. Harrison, and G. N. Pavlakis.** 1992. The Rev protein of human immunodeficiency virus type 1 promotes polysomal association and translation of gag/pol and vpu/env mRNAs. Mol Cell Biol **12:**1375-86.

88. **D'Souza, V., and M. F. Summers.** 2005. How retroviruses select their genomes. Nat Rev Microbiol **3:**643-55.

89. **Daniel, M. D., N. L. Letvin, N. W. King, M. Kannagi, P. K. Sehgal, R. D. Hunt, P. J. Kanki, M. Essex, and R. C. Desrosiers.** 1985. Isolation of T-cell tropic HTLV-III-like retrovirus from macaques. Science **228:**1201-4.

90. **Dannull, J., A. Surovoy, G. Jung, and K. Moelling.** 1994. Specific binding of HIV-1 nucleocapsid protein to PSI RNA in vitro requires N-terminal zinc finger and flanking basic amino acid residues. Embo J **13:**1525-33.

91. **Day, J. R., C. Munk, and J. C. Guatelli.** 2004. The membrane-proximal tyrosine-based sorting signal of human immunodeficiency virus type 1 gp41 is required for optimal viral infectivity. J Virol **78:**1069-79.

92. **Dayton, A. I., J. G. Sodroski, C. A. Rosen, W. C. Goh, and W. A. Haseltine.** 1986. The trans-activator gene of the human T cell lymphotropic virus type III is required for replication. Cell **44:**941-7.

93. **Dayton, E. T., D. M. Powell, and A. I. Dayton.** 1989. Functional analysis of CAR, the target sequence for the Rev protein of HIV-1. Science **246:**1625-9.

94. **De Guzman, R. N., Z. R. Wu, C. C. Stalling, L. Pappalardo, P. N. Borer, and M. F. Summers.** 1998. Structure of the HIV-1 nucleocapsid protein bound to the SL3 psi-RNA recognition element. Science **279:**384-8.

95. **Dedera, D., W. Hu, N. Vander Heyden, and L. Ratner.** 1989. Viral protein R of human immunodeficiency virus types 1 and 2 is dispensable for replication and cytopathogenicity in lymphoid cells. J Virol **63:**3205-8.

96. **Demirov, D. G., and E. O. Freed.** 2004. Retrovirus budding. Virus Res **106:**87-102.

97. **Demirov, D. G., A. Ono, J. M. Orenstein, and E. O. Freed.** 2002. Overexpression of the N-terminal domain of TSG101 inhibits HIV-1 budding by blocking late domain function. Proc Natl Acad Sci U S A **99:**955-60.

98. **Demirov, D. G., J. M. Orenstein, and E. O. Freed.** 2002. The late domain of human immunodeficiency virus type 1 p6 promotes virus release in a cell type-dependent manner. J Virol **76:**105-17.

99. **Deng, H., R. Liu, W. Ellmeier, S. Choe, D. Unutmaz, M. Burkhart, P. Di Marzio, S. Marmon, R. E. Sutton, C. M. Hill, C. B. Davis, S. C. Peiper, T. J. Schall, D. R. Littman, and N. R. Landau.** 1996. Identification of a major co-receptor for primary isolates of HIV-1. Nature **381:**661-6.

100. **Deng, L., C. de la Fuente, P. Fu, L. Wang, R. Donnelly, J. D. Wade, P. Lambert, H. Li, C. G. Lee, and F. Kashanchi.** 2000. Acetylation of HIV-1 Tat by CBP/P300 increases transcription of integrated HIV-1 genome and enhances binding to core histones. Virology **277:**278-95.

101. **Derdeyn, C. A., J. M. Decker, J. N. Sfakianos, X. Wu, W. A. O'Brien, L. Ratner, J. C. Kappes, G. M. Shaw, and E. Hunter.** 2000. Sensitivity of human immunodeficiency virus type 1 to the fusion inhibitor T-20 is modulated by coreceptor specificity defined by the V3 loop of gp120. J Virol **74:**8358-67.

102. **Deschambeault, J., J. P. Lalonde, G. Cervantes-Acosta, R. Lodge, E. A. Cohen, and G. Lemay.** 1999. Polarized human immunodeficiency virus budding in lymphocytes involves a tyrosine-based signal and favors cell-to-cell viral transmission. J Virol **73:**5010-7.

103. **Desfarges, S., B. Salin, C. Calmels, M. L. Andreola, V. Parissi, and M. Fournier.** 2009. HIV-1 integrase trafficking in S. cerevisiae: a useful model to dissect the microtubule network involvement of viral protein nuclear import. Yeast **26:**39-54.

104. **Diaz, E., and S. R. Pfeffer.** 1998. TIP47: a cargo selection device for mannose 6-phosphate receptor trafficking. Cell **93:**433-43.

105. **Dietrich, J., X. Hou, A. M. Wegener, L. O. Pedersen, N. Odum, and C. Geisler.** 1996. Molecular characterization of the di-leucine-based internalization motif of the T cell receptor. J Biol Chem **271:**11441-8.

106. **Dietrich, J., J. Kastrup, B. L. Nielsen, N. Odum, and C. Geisler.** 1997. Regulation and function of the CD3gamma DxxxLL motif: a binding site for adaptor protein-1 and adaptor protein-2 in vitro. J Cell Biol **138:**271-81.

107. **Dillon, P. J., P. Nelbock, A. Perkins, and C. A. Rosen.** 1990. Function of the human immunodeficiency virus types 1 and 2 Rev proteins is dependent on their ability to interact with a structured region present in env gene mRNA. J Virol **64:**4428-37.

108. **Dong, X., H. Li, A. Derdowski, L. Ding, A. Burnett, X. Chen, T. R. Peters, T. S. Dermody, E. Woodruff, J. J. Wang, and P. Spearman.** 2005. AP-3 directs the intracellular trafficking of HIV-1 Gag and plays a key role in particle assembly. Cell **120:**663-74.

109. **Dorfman, T., J. Luban, S. P. Goff, W. A. Haseltine, and H. G. Gottlinger.** 1993. Mapping of functionally important residues of a cysteine-histidine box in the human immunodeficiency virus type 1 nucleocapsid protein. J Virol **67:**6159-69.

110. **Dorfman, T., F. Mammano, W. A. Haseltine, and H. G. Gottlinger.** 1994. Role of the matrix protein in the virion association of the human immunodeficiency virus type 1 envelope glycoprotein. J Virol **68:**1689-96.

111. **Du, Z., P. O. Ilyinskii, K. Lally, R. C. Desrosiers, and A. Engelman.** 1997. A mutation in integrase can compensate for mutations in the simian immunodeficiency virus att site. J Virol **71:**8124-32.

112. **Dubay, J. W., S. J. Roberts, B. H. Hahn, and E. Hunter.** 1992. Truncation of the human immunodeficiency virus type 1 transmembrane glycoprotein cytoplasmic domain blocks virus infectivity. J Virol **66:**6616-25.

113. **Edwards, T. G., S. Wyss, J. D. Reeves, S. Zolla-Pazner, J. A. Hoxie, R. W. Doms, and F. Baribaud.** 2002. Truncation of the cytoplasmic domain induces exposure of conserved regions in the ectodomain of human immunodeficiency virus type 1 envelope protein. J Virol **76:**2683-91.

114. **Egan, M. A., L. M. Carruth, J. F. Rowell, X. Yu, and R. F. Siliciano.** 1996. Human immunodeficiency virus type 1 envelope protein endocytosis mediated by a highly conserved intrinsic internalization signal in the cytoplasmic domain of gp41 is suppressed in the presence of the Pr55gag precursor protein. J Virol **70:**6547-56.

115. **Endres, M. J., P. R. Clapham, M. Marsh, M. Ahuja, J. D. Turner, A. McKnight, J. F. Thomas, B. Stoebenau-Haggarty, S. Choe, P. J. Vance, T. N. Wells, C. A. Power, S. S. Sutterwala, R. W. Doms, N. R. Landau, and J. A. Hoxie.** 1996. CD4-independent infection by HIV-2 is mediated by fusin/CXCR4. Cell **87:**745-56.

116. **Etemad-Moghadam, B., Y. Sun, E. K. Nicholson, G. B. Karlsson, D. Schenten, and J. Sodroski.** 1999. Determinants of neutralization resistance in the envelope glycoproteins of a simian-human immunodeficiency virus passaged in vivo. J Virol **73:**8873-9.

117. **Feinberg, M. B., D. Baltimore, and A. D. Frankel.** 1991. The role of Tat in the human immunodeficiency virus life cycle indicates a primary effect on transcriptional elongation. Proc Natl Acad Sci U S A **88:**4045-9.

118. **Feinberg, M. B., R. F. Jarrett, A. Aldovini, R. C. Gallo, and F. Wong-Staal.** 1986. HTLV-III expression and production involve complex regulation at the levels of splicing and translation of viral RNA. Cell **46:**807-17.

119. **Feinberg, M. B., and D. Trono.** 1992. Intracellular immunization: trans-dominant mutants of HIV gene products as tools for the study and interruption of viral replication. AIDS Res Hum Retroviruses **8:**1013-22.

120. **Felber, B. K., M. Hadzopoulou-Cladaras, C. Cladaras, T. Copeland, and G. N. Pavlakis.** 1989. rev protein of human immunodeficiency virus type 1 affects the stability and transport of the viral mRNA. Proc Natl Acad Sci U S A **86:**1495-9.

121. **Feng, Y., C. C. Broder, P. E. Kennedy, and E. A. Berger.** 1996. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science **272:**872-7.

122. **Fernandez-Vidal, M., S. Jayasinghe, A. S. Ladokhin, and S. H. White.** 2007. Folding amphipathic helices into membranes: amphiphilicity trumps hydrophobicity. J Mol Biol **370:**459-70.

123. **Fisher, A. G., M. B. Feinberg, S. F. Josephs, M. E. Harper, L. M. Marselle, G. Reyes, M. A. Gonda, A. Aldovini, C. Debouk, R. C. Gallo, and et al.** 1986. The trans-activator gene of HTLV-III is essential for virus replication. Nature **320:**367-71.

124. **Fisher, R. J., A. Rein, M. Fivash, M. A. Urbaneja, J. R. Casas-Finet, M. Medaglia, and L. E. Henderson.** 1998. Sequence-specific binding of human immunodeficiency virus type 1 nucleocapsid protein to short oligonucleotides. J Virol **72:**1902-9.

125. **Franke, E. K., H. E. Yuan, and J. Luban.** 1994. Specific incorporation of cyclophilin A into HIV-1 virions. Nature **372:**359-62.

126. **Freed, E. O.** 1998. HIV-1 gag proteins: diverse functions in the virus life cycle. Virology **251:**1-15.

127. **Freed, E. O.** 2006. HIV-1 Gag: flipped out for PI(4,5)P(2). Proc Natl Acad Sci U S A **103:**11101-2.

128. **Freed, E. O.** 2002. Viral late domains. J Virol **76:**4679-87.

129. **Freed, E. O., and M. A. Martin.** 1996. Domains of the human immunodeficiency virus type 1 matrix and gp41 cytoplasmic tail required for envelope incorporation into virions. J Virol **70:**341-51.

130. **Freed, E. O., D. J. Myers, and R. Risser.** 1990. Characterization of the fusion domain of the human immunodeficiency virus type 1 envelope glycoprotein gp41. Proc Natl Acad Sci U S A **87:**4650-4.

131. **Freed, E. O., D. J. Myers, and R. Risser.** 1991. Identification of the principal neutralizing determinant of human immunodeficiency virus type 1 as a fusion domain. J Virol **65:**190-4.

132. **Freed, E. O., J. M. Orenstein, A. J. Buckler-White, and M. A. Martin.** 1994. Single amino acid changes in the human immunodeficiency virus type 1 matrix protein block virus particle production. J Virol **68:**5311-20.

133. **Fu, W., R. J. Gorelick, and A. Rein.** 1994. Characterization of human immunodeficiency virus type 1 dimeric RNA from wild-type and protease-defective virions. J Virol **68:**5013-8.

134. **Fultz, P. N., P. J. Vance, M. J. Endres, B. Tao, J. D. Dvorin, I. C. Davis, J. D. Lifson, D. C. Montefiori, M. Marsh, M. H. Malim, and J. A. Hoxie.** 2001. In vivo attenuation of simian immunodeficiency virus by disruption of a tyrosine-dependent sorting signal in the envelope glycoprotein cytoplasmic tail. J Virol **75:**278-91.

135. **Furuta, R. A., C. T. Wild, Y. Weng, and C. D. Weiss.** 1998. Capture of an early fusion-active conformation of HIV-1 gp41. Nat Struct Biol **5:**276-9.

136. **Gabuzda, D., U. Olshevsky, P. Bertani, W. A. Haseltine, and J. Sodroski.** 1991. Identification of membrane anchorage domains of the HIV-1 gp160 envelope glycoprotein precursor. J Acquir Immune Defic Syndr **4:**34-40.

137. **Gallo, R. C., P. S. Sarin, E. P. Gelmann, M. Robert-Guroff, E. Richardson, V. S. Kalyanaraman, D. Mann, G. D. Sidhu, R. E. Stahl, S. Zolla-Pazner, J. Leibowitch, and M. Popovic.** 1983. Isolation of human T-cell leukemia virus in acquired immune deficiency syndrome (AIDS). Science **220:**865-7.

138. **Gamble, T. R., F. F. Vajdos, S. Yoo, D. K. Worthylake, M. Houseweart, W. I. Sundquist, and C. P. Hill.** 1996. Crystal structure of human cyclophilin A bound to the amino-terminal domain of HIV-1 capsid. Cell **87:**1285-94.

139. **Gao, F., E. Bailes, D. L. Robertson, Y. Chen, C. M. Rodenburg, S. F. Michael, L. B. Cummins, L. O. Arthur, M. Peeters, G. M. Shaw, P. M. Sharp, and B. H. Hahn.** 1999. Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. Nature **397:**436-41.

140. **Gao, F., L. Yue, A. T. White, P. G. Pappas, J. Barchue, A. P. Hanson, B. M. Greene, P. M. Sharp, G. M. Shaw, and B. H. Hahn.** 1992. Human infection by genetically diverse SIVSM-related HIV-2 in west Africa. Nature **358:**495-9.

141. **Gao, K., R. J. Gorelick, D. G. Johnson, and F. Bushman.** 2003. Cofactors for human immunodeficiency virus type 1 cDNA integration in vitro. J Virol **77:**1598-603.

142. **Garcia, E., M. Pion, A. Pelchen-Matthews, L. Collinson, J. F. Arrighi, G. Blot, F. Leuba, J. M. Escola, N. Demaurex, M. Marsh, and V. Piguet.** 2005. HIV-1 trafficking to the dendritic cell-T-cell infectious synapse uses a pathway of tetraspanin sorting to the immunological synapse. Traffic **6:**488-501.

143. **Garcia, J. A., D. Harrich, L. Pearson, R. Mitsuyasu, and R. B. Gaynor.** 1988. Functional domains required for tat-induced transcriptional activation of the HIV-1 long terminal repeat. Embo J **7:**3143-7.

144. **Garrus, J. E., U. K. von Schwedler, O. W. Pornillos, S. G. Morham, K. H. Zavitz, H. E. Wang, D. A. Wettstein, K. M. Stray, M. Cote, R. L. Rich, D. G. Myszka, and W. I. Sundquist.** 2001. Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. Cell **107:**55-65.

145. **Gatignol, A., A. Buckler-White, B. Berkhout, and K. T. Jeang.** 1991. Characterization of a human TAR RNA-binding protein that activates the HIV-1 LTR. Science **251:**1597-600.

146. **Gawrisch, K., K. H. Han, J. S. Yang, L. D. Bergelson, and J. A. Ferretti.** 1993. Interaction of peptide fragment 828-848 of the envelope glycoprotein of human immunodeficiency virus type I with lipid bilayers. Biochemistry **32:**3112-8.

147. **Gendelman, H. E., L. M. Baca, J. Turpin, D. C. Kalter, B. Hansen, J. M. Orenstein, C. W. Dieffenbach, R. M. Friedman, and M. S. Meltzer.** 1990. Regulation of HIV replication in infected monocytes by IFN-alpha. Mechanisms for viral restriction. J Immunol **145:**2669-76.

148. **Gendelman, H. E., J. M. Orenstein, M. A. Martin, C. Ferrua, R. Mitra, T. Phipps, L. A. Wahl, H. C. Lane, A. S. Fauci, D. S. Burke, and et al.** 1988. Efficient isolation and propagation of human immunodeficiency virus on recombinant colony-stimulating factor 1-treated monocytes. J Exp Med **167:**1428-41.

149. **Gilboa, E., S. W. Mitra, S. Goff, and D. Baltimore.** 1979. A detailed model of reverse transcription and tests of crucial aspects. Cell **18:**93-100.

150. **Golding, H., M. Zaitseva, E. de Rosny, L. R. King, J. Manischewitz, I. Sidorov, M. K. Gorny, S. Zolla-Pazner, D. S. Dimitrov, and C. D. Weiss.** 2002. Dissection of human immunodeficiency virus type 1 entry with neutralizing antibodies to gp41 fusion intermediates. J Virol **76:**6780-90.

151. **Gottlinger, H. G., T. Dorfman, J. G. Sodroski, and W. A. Haseltine.** 1991. Effect of mutations affecting the p6 gag protein on human immunodeficiency virus particle release. Proc Natl Acad Sci U S A **88:**3195-9.

152. **Gottlinger, H. G., J. G. Sodroski, and W. A. Haseltine.** 1989. Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus type 1. Proc Natl Acad Sci U S A **86:**5781-5.

153. **Govers, R., P. van Kerkhof, A. L. Schwartz, and G. J. Strous.** 1998. Di-leucine-mediated internalization of ligand by a truncated growth hormone receptor is independent of the ubiquitin conjugation system. J Biol Chem **273:**16426-33.

154. **Graves, M. C., J. J. Lim, E. P. Heimer, and R. A. Kramer.** 1988. An 11-kDa form of human immunodeficiency virus protease expressed in Escherichia coli is sufficient for enzymatic activity. Proc Natl Acad Sci U S A **85:**2449-53.

155. **Groom, H. C., E. C. Anderson, J. A. Dangerfield, and A. M. Lever.** 2009. Rev regulates translation of human immunodeficiency virus type 1 RNAs. J Gen Virol **90:**1141-7.

156. **Gross, I., H. Hohenberg, and H. G. Krausslich.** 1997. In vitro assembly properties of purified bacterially expressed capsid proteins of human immunodeficiency virus. Eur J Biochem **249:**592-600.

157. **Guo, X., A. Roldan, J. Hu, M. A. Wainberg, and C. Liang.** 2005. Mutation of the SP1 sequence impairs both multimerization and membrane-binding activities of human immunodeficiency virus type 1 Gag. J Virol **79:**1803-12.

158. **Guy, B., M. Geist, K. Dott, D. Spehner, M. P. Kieny, and J. P. Lecocq.** 1991. A specific inhibitor of cysteine proteases impairs a Vif-dependent modification of human immunodeficiency virus type 1 Env protein. J Virol **65:**1325-31.

159. **Hadzopoulou-Cladaras, M., B. K. Felber, C. Cladaras, A. Athanassopoulos, A. Tse, and G. N. Pavlakis.** 1989. The rev (trs/art) protein of human immunodeficiency virus type 1 affects viral mRNA and protein expression via a cis-acting sequence in the env region. J Virol **63:**1265-74.

160. **Hahn, B. H., M. A. Gonda, G. M. Shaw, M. Popovic, J. A. Hoxie, R. C. Gallo, and F. Wong-Staal.** 1985. Genomic diversity of the acquired immune deficiency syndrome virus HTLV-III: different viruses exhibit greatest divergence in their envelope genes. Proc Natl Acad Sci U S A **82:**4813-7.

161. **Hammonds, J., X. Chen, L. Ding, T. Fouts, A. De Vico, J. zur Megede, S. Barnett, and P. Spearman.** 2003. Gp120 stability on HIV-1 virions and Gag-Env pseudovirions is enhanced by an uncleaved Gag core. Virology **314:**636-49.

162. **Harris, M., S. Hislop, P. Patsilinacos, and J. C. Neil.** 1992. In vivo derived HIV-1 nef gene products are heterogeneous and lack detectable nucleotide binding activity. AIDS Res Hum Retroviruses **8:**537-43.

163. **Harrison, G. P., and A. M. Lever.** 1992. The human immunodeficiency virus type 1 packaging signal and major splice donor region have a conserved stable secondary structure. J Virol **66:**4144-53.

164. **Hatziioannou, T., D. Perez-Caballero, S. Cowan, and P. D. Bieniasz.** 2005. Cyclophilin interactions with incoming human immunodeficiency virus type 1 capsids with opposing effects on infectivity in human cells. J Virol **79:**176-83.

165. **Hauber, J., and B. R. Cullen.** 1988. Mutational analysis of the trans-activation-responsive region of the human immunodeficiency virus type I long terminal repeat. J Virol **62:**673-9.

166. **Hauber, J., A. Perkins, E. P. Heimer, and B. R. Cullen.** 1987. Trans-activation of human immunodeficiency virus gene expression is mediated by nuclear events. Proc Natl Acad Sci U S A **84:**6364-8.

167. **Hayashi, T., Y. Ueno, and T. Okamoto.** 1993. Elucidation of a conserved RNA stem-loop structure in the packaging signal of human immunodeficiency virus type 1. FEBS Lett **327:**213-8.

168. **Heaphy, S., C. Dingwall, I. Ernberg, M. J. Gait, S. M. Green, J. Karn, A. D. Lowe, M. Singh, and M. A. Skinner.** 1990. HIV-1 regulator of virion expression (Rev) protein binds to an RNA stem-loop structure located within the Rev response element region. Cell **60:**685-93.

169. **Heinzinger, N. K., M. I. Bukinsky, S. A. Haggerty, A. M. Ragland, V. Kewalramani, M. A. Lee, H. E. Gendelman, L. Ratner, M. Stevenson, and M. Emerman.** 1994. The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. Proc Natl Acad Sci U S A **91:**7311-5.

170. **Henderson, A., A. Holloway, R. Reeves, and D. J. Tremethick.** 2004. Recruitment of SWI/SNF to the human immunodeficiency virus type 1 promoter. Mol Cell Biol **24:**389-97.

171. **Henderson, B. R., and P. Percipalle.** 1997. Interactions between HIV Rev and nuclear import and export factors: the Rev nuclear localisation signal mediates specific binding to human importin-beta. J Mol Biol **274:**693-707.

172. **Herbreteau, C. H., L. Weill, D. Decimo, D. Prevot, J. L. Darlix, B. Sargueil, and T. Ohlmann.** 2005. HIV-2 genomic RNA contains a novel type of IRES located downstream of its initiation codon. Nat Struct Mol Biol **12:**1001-7.

173. **Hermida-Matsumoto, L., and M. D. Resh.** 1999. Human immunodeficiency virus type 1 protease triggers a myristoyl switch that modulates membrane binding of Pr55(gag) and p17MA. J Virol **73:**1902-8.

174. **Hetzer, C., W. Dormeyer, M. Schnolzer, and M. Ott.** 2005. Decoding Tat: the biology of HIV Tat posttranslational modifications. Microbes Infect **7:**1364-9.

175. **Hill, C. P., D. Worthylake, D. P. Bancroft, A. M. Christensen, and W. I. Sundquist.** 1996. Crystal structures of the trimeric human immunodeficiency virus type 1 matrix protein: implications for membrane association and assembly. Proc Natl Acad Sci U S A **93:**3099-104.

176. **Hoffman, T. L., and R. W. Doms.** 1998. Chemokines and coreceptors in HIV/SIV-host interactions. Aids **12 Suppl A:**S17-26.

177. **Hoffman, T. L., C. C. LaBranche, W. Zhang, G. Canziani, J. Robinson, I. Chaiken, J. A. Hoxie, and R. W. Doms.** 1999. Stable exposure of the coreceptor-binding site in a CD4-independent HIV-1 envelope protein. Proc Natl Acad Sci U S A **96:**6359-64.

178. **Honing, S., and W. Hunziker.** 1995. Cytoplasmic determinants involved in direct lysosomal sorting, endocytosis, and basolateral targeting of rat lgp120 (lamp-I) in MDCK cells. J Cell Biol **128:**321-32.

179. **Hope, T. J., X. J. Huang, D. McDonald, and T. G. Parslow.** 1990. Steroid-receptor fusion of the human immunodeficiency virus type 1 Rev transactivator: mapping cryptic functions of the arginine-rich motif. Proc Natl Acad Sci U S A **87:**7787-91.

180. **Huang, C. C., M. Tang, M. Y. Zhang, S. Majeed, E. Montabana, R. L. Stanfield, D. S. Dimitrov, B. Korber, J. Sodroski, I. A. Wilson, R. Wyatt, and P. D. Kwong.** 2005. Structure of a V3-containing HIV-1 gp120 core. Science **310:**1025-8.

181. **Huang, M., J. M. Orenstein, M. A. Martin, and E. O. Freed.** 1995. p6Gag is required for particle production from full-length human immunodeficiency virus type 1 molecular clones expressing protease. J Virol **69:**6810-8.

182. **Hug, P., H. M. Lin, T. Korte, X. Xiao, D. S. Dimitrov, J. M. Wang, A. Puri, and R. Blumenthal.** 2000. Glycosphingolipids promote entry of a broad range of human immunodeficiency virus type 1 isolates into cell lines expressing CD4, CXCR4, and/or CCR5. J Virol **74:**6377-85.

183. **Hunter, E., and R. Swanstrom.** 1990. Retrovirus envelope glycoproteins. Curr Top Microbiol Immunol **157:**187-253.

184. **Hunziker, W., and C. Fumey.** 1994. A di-leucine motif mediates endocytosis and basolateral sorting of macrophage IgG Fc receptors in MDCK cells. Embo J **13:**2963-7.

185. **Hunziker, W., and C. Fumey.** 1994. A di-leucine motif mediates endocytosis and basolateral sorting of macrophage IgG Fc receptors in MDCK cells. Embo J **13:**2963-9.

186. **Iwai, S., C. Pritchard, D. A. Mann, J. Karn, and M. J. Gait.** 1992. Recognition of the high affinity binding site in rev-response element RNA by the human immunodeficiency virus type-1 rev protein. Nucleic Acids Res **20:**6465-72.

187. **Jacks, T., H. D. Madhani, F. R. Masiarz, and H. E. Varmus.** 1988. Signals for ribosomal frameshifting in the Rous sarcoma virus gag-pol region. Cell **55:**447-58.

188. **Jackson, R. J.** 2005. Alternative mechanisms of initiating translation of mammalian mRNAs. Biochem Soc Trans **33:**1231-41.

189. **Jetzt, A. E., H. Yu, G. J. Klarmann, Y. Ron, B. D. Preston, and J. P. Dougherty.** 2000. High rate of recombination throughout the human immunodeficiency virus type 1 genome. J Virol **74:**1234-40.

190. **Jewell, N. A., and L. M. Mansky.** 2000. In the beginning: genome recognition, RNA encapsidation and the initiation of complex retrovirus assembly. J Gen Virol **81:**1889-99.

191. **Jiang, J., and C. Aiken.** 2006. Maturation of the viral core enhances the fusion of HIV-1 particles with primary human T cells and monocyte-derived macrophages. Virology **346:**460-8.

192. **Johnston, P. B., J. W. Dubay, and E. Hunter.** 1993. Truncations of the simian immunodeficiency virus transmembrane protein confer expanded virus host range by removing a block to virus entry into cells. J Virol **67:**3077-86.

193. **Jolly, C., and Q. J. Sattentau.** 2005. Human immunodeficiency virus type 1 virological synapse formation in T cells requires lipid raft integrity. J Virol **79:**12088-94.

194. **Jordan, A., D. Bisgrove, and E. Verdin.** 2003. HIV reproducibly establishes a latent infection after acute infection of T cells in vitro. Embo J **22:**1868-77.

195. **Jouvenet, N., P. D. Bieniasz, and S. M. Simon.** 2008. Imaging the biogenesis of individual HIV-1 virions in live cells. Nature **454:**236-40.

196. **Kalia, V., S. Sarkar, P. Gupta, and R. C. Montelaro.** 2005. Antibody neutralization escape mediated by point mutations in the intracytoplasmic tail of human immunodeficiency virus type 1 gp41. J Virol **79:**2097-107.

197. **Kalia, V., S. Sarkar, P. Gupta, and R. C. Montelaro.** 2003. Rational site-directed mutations of the LLP-1 and LLP-2 lentivirus lytic peptide domains in the intracytoplasmic tail of human immunodeficiency virus type 1 gp41 indicate common functions in cell-cell fusion but distinct roles in virion envelope incorporation. J Virol **77:**3634-46.

198. **Kawakami, K., F. Takeshita, and R. K. Puri.** 2001. Identification of distinct roles for a dileucine and a tyrosine internalization motif in the interleukin (IL)-13 binding component IL-13 receptor alpha 2 chain. J Biol Chem **276:**25114-20.

199. **Kaye, J. F., and A. M. Lever.** 1998. Nonreciprocal packaging of human immunodeficiency virus type 1 and type 2 RNA: a possible role for the p2 domain of Gag in RNA encapsidation. J Virol **72:**5877-85.

200. **Kempf, D. J.** 1994. Design of symmetry-based, peptidomimetic inhibitors of human immunodeficiency virus protease. Methods Enzymol **241:**334-54.

201. **Keren, T., M. G. Roth, and Y. I. Henis.** 2001. Internalization-competent influenza hemagglutinin mutants form complexes with clathrin-deficient multivalent AP-2 oligomers in live cells. J Biol Chem **276:**28356-63.

202. **Kido, H., A. Fukutomi, and N. Katunuma.** 1990. A novel membrane-bound serine esterase in human T4+ lymphocytes immunologically reactive with antibody inhibiting syncytia induced by HIV-1. Purification and characterization. J Biol Chem **265:**21979-85.

203. **Kiernan, R. E., C. Vanhulle, L. Schiltz, E. Adam, H. Xiao, F. Maudoux, C. Calomme, A. Burny, Y. Nakatani, K. T. Jeang, M. Benkirane, and C. Van Lint.** 1999. HIV-1 tat transcriptional activity is regulated by acetylation. Embo J **18:**6106-18.

204. **Kimura, T., I. Hashimoto, M. Nishikawa, and J. I. Fujisawa.** 1996. A role for Rev in the association of HIV-1 gag mRNA with cytoskeletal beta-actin and viral protein expression. Biochimie **78:**1075-80.

205. **Kjems, J., M. Brown, D. D. Chang, and P. A. Sharp.** 1991. Structural analysis of the interaction between the human immunodeficiency virus Rev protein and the Rev response element. Proc Natl Acad Sci U S A **88:**683-7.

206. **Kjems, J., B. J. Calnan, A. D. Frankel, and P. A. Sharp.** 1992. Specific binding of a basic peptide from HIV-1 Rev. Embo J **11:**1119-29.

207. **Kliger, Y., A. Aharoni, D. Rapaport, P. Jones, R. Blumenthal, and Y. Shai.** 1997. Fusion peptides derived from the HIV type 1 glycoprotein 41 associate within phospholipid membranes and inhibit cell-cell Fusion. Structure-function study. J Biol Chem **272:**13496-505.

208. **Kluger, M. S., S. L. Shiao, A. L. Bothwell, and J. S. Pober.** 2002. Cutting Edge: Internalization of transduced E-selectin by cultured human endothelial cells: comparison of dermal microvascular and umbilical vein cells and identification of a phosphoserine-type di-leucine motif. J Immunol **168:**2091-5.

209. **Kodama, T., D. P. Wooley, Y. M. Naidu, H. W. Kestler, 3rd, M. D. Daniel, Y. Li, and R. C. Desrosiers.** 1989. Significance of premature stop codons in env of simian immunodeficiency virus. J Virol **63:**4709-14.

210. **Kol, N., Y. Shi, M. Tsvitov, D. Barlam, R. Z. Shneck, M. S. Kay, and I. Rousso.** 2007. A stiffness switch in human immunodeficiency virus. Biophys J **92:**1777-83.

211. **Kolchinsky, P., T. Mirzabekov, M. Farzan, E. Kiprilov, M. Cayabyab, L. J. Mooney, H. Choe, and J. Sodroski.** 1999. Adaptation of a CCR5-using, primary human immunodeficiency virus type 1 isolate for CD4-independent replication. J Virol **73:**8120-6.

212. **Kuiken, C. L., V. V. Lukashov, E. Baan, J. Dekker, J. A. Leunissen, and J. Goudsmit.** 1996. Evidence for limited within-person evolution of the V3 domain of the HIV-1 envelope in the amsterdam population. Aids **10:**31-7.

213. **Kwong, P. D., R. Wyatt, J. Robinson, R. W. Sweet, J. Sodroski, and W. A. Hendrickson.** 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature **393:**648-59.

214. **LaBranche, C. C., M. M. Sauter, B. S. Haggarty, P. J. Vance, J. Romano, T. K. Hart, P. J. Bugelski, M. Marsh, and J. A. Hoxie.** 1995. A single amino acid change in the cytoplasmic domain of the simian immunodeficiency virus transmembrane molecule increases envelope glycoprotein expression on infected cells. J Virol **69:**5217-27.

215. **Lambele, M., B. Labrosse, E. Roch, A. Moreau, B. Verrier, F. Barin, P. Roingeard, F. Mammano, and D. Brand.** 2007. Impact of natural polymorphism within the gp41 cytoplasmic tail of human immunodeficiency virus type 1 on the intracellular distribution of envelope glycoproteins and viral assembly. J Virol **81:**125-40.

216. **Lawrence, D. C., C. C. Stover, J. Noznitsky, Z. Wu, and M. F. Summers.** 2003. Structure of the intact stem and bulge of HIV-1 Psi-RNA stem-loop SL1. J Mol Biol **326:**529-42.

217. **Lawrence, J. B., A. W. Cochrane, C. V. Johnson, A. Perkins, and C. A. Rosen.** 1991. The HIV-1 Rev protein: a model system for coupled RNA transport and translation. New Biol **3:**1220-32.

218. **Leis, J., D. Baltimore, J. M. Bishop, J. Coffin, E. Fleissner, S. P. Goff, S. Oroszlan, H. Robinson, A. M. Skalka, H. M. Temin, and et al.** 1988. Standardized and simplified nomenclature for proteins common to all retroviruses. J Virol **62:**1808-9.

219. **Letourneur, F., and R. D. Klausner.** 1992. A novel di-leucine motif and a tyrosine-based motif independently mediate lysosomal targeting and endocytosis of CD3 chains. Cell **69:**1143-57.

220. **Levin, A., Z. Hayouka, M. Helfer, R. Brack-Werner, A. Friedler, and A. Loyter.** 2009. Peptides derived from HIV-1 integrase that bind Rev stimulate viral genome integration. PLoS One **4:**e4155.

221. **Levy, J. A., A. D. Hoffman, S. M. Kramer, J. A. Landis, J. M. Shimabukuro, and L. S. Oshiro.** 1984. Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. Science **225:**840-2.

222. **Li, F., C. Chen, B. A. Puffer, and R. C. Montelaro.** 2002. Functional replacement and positional dependence of homologous and heterologous L domains in equine infectious anemia virus replication. J Virol **76:**1569-77.

223. **Liang, C., J. Hu, R. S. Russell, A. Roldan, L. Kleiman, and M. A. Wainberg.** 2002. Characterization of a putative alpha-helix across the capsid-SP1 boundary that is critical for the multimerization of human immunodeficiency virus type 1 gag. J Virol **76:**11729-37.

224. **Liang, C., J. Hu, J. B. Whitney, L. Kleiman, and M. A. Wainberg.** 2003. A structurally disordered region at the C terminus of capsid plays essential roles in multimerization and membrane binding of the gag protein of human immunodeficiency virus type 1. J Virol **77:**1772-83.

225. **Lin, X., C. A. Derdeyn, R. Blumenthal, J. West, and E. Hunter.** 2003. Progressive truncations C terminal to the membrane-spanning domain of simian immunodeficiency virus Env reduce fusogenicity and increase concentration dependence of Env for fusion. J Virol **77:**7067-77.

226. **Lineberger, J. E., R. Danzeisen, D. J. Hazuda, A. J. Simon, and M. D. Miller.** 2002. Altering expression levels of human immunodeficiency virus type 1 gp120-gp41 affects efficiency but not kinetics of cell-cell fusion. J Virol **76:**3522-33.

227. **Linial, M. L., and A. D. Miller.** 1990. Retroviral RNA packaging: sequence requirements and implications. Curr Top Microbiol Immunol **157:**125-52.

228. **Littman, D. R.** 1998. Chemokine receptors: keys to AIDS pathogenesis? Cell **93:**677-80.

229. **Lodge, R., H. Gottlinger, D. Gabuzda, E. A. Cohen, and G. Lemay.** 1994. The intracytoplasmic domain of gp41 mediates polarized budding of human immunodeficiency virus type 1 in MDCK cells. J Virol **68:**4857-61.

230. **Lodge, R., J. P. Lalonde, G. Lemay, and E. A. Cohen.** 1997. The membrane-proximal intracytoplasmic tyrosine residue of HIV-1 envelope glycoprotein is critical for basolateral targeting of viral budding in MDCK cells. Embo J **16:**695-705.

231. **Lopez-Lastra, M., A. Rivas, and M. I. Barria.** 2005. Protein synthesis in eukaryotes: the growing biological relevance of cap-independent translation initiation. Biol Res **38:**121-46.

232. **Lopez-Verges, S., G. Camus, G. Blot, R. Beauvoir, R. Benarous, and C. Berlioz-Torrent.** 2006. Tail-interacting protein TIP47 is a connector between Gag and Env and is required for Env incorporation into HIV-1 virions. Proc Natl Acad Sci U S A **103:**14947-52.

233. **Louis, J. M., G. M. Clore, and A. M. Gronenborn.** 1999. Autoprocessing of HIV-1 protease is tightly coupled to protein folding. Nat Struct Biol **6:**868-75.

234. **Louis, J. M., E. M. Wondrak, A. R. Kimmel, P. T. Wingfield, and N. T. Nashed.** 1999. Proteolytic processing of HIV-1 protease precursor, kinetics and mechanism. J Biol Chem **274:**23437-42.

235. **Lu, L., Y. Zhu, J. Huang, X. Chen, H. Yang, S. Jiang, and Y. H. Chen.** 2008. Surface exposure of the HIV-1 env cytoplasmic tail LLP2 domain during the membrane fusion process: interaction with gp41 fusion core. J Biol Chem **283:**16723-31.

236. **Luban, J., K. L. Bossolt, E. K. Franke, G. V. Kalpana, and S. P. Goff.** 1993. Human immunodeficiency virus type 1 Gag protein binds to cyclophilins A and B. Cell **73:**1067-78.

237. **Lusic, M., A. Marcello, A. Cereseto, and M. Giacca.** 2003. Regulation of HIV-1 gene expression by histone acetylation and factor recruitment at the LTR promoter. Embo J **22:**6550-61.

238. **Maddon, P. J., A. G. Dalgleish, J. S. McDougal, P. R. Clapham, R. A. Weiss, and R. Axel.** 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell **47:**333-48.

239. **Maitra, R. K., N. A. McMillan, S. Desai, J. McSwiggen, A. G. Hovanessian, G. Sen, B. R. Williams, and R. H. Silverman.** 1994. HIV-1 TAR RNA has an intrinsic ability to activate interferon-inducible enzymes. Virology **204:**823-7.

240. **Malim, M. H., W. W. Freimuth, J. Liu, T. J. Boyle, H. K. Lyerly, B. R. Cullen, and G. J. Nabel.** 1992. Stable expression of transdominant Rev protein in human T cells inhibits human immunodeficiency virus replication. J Exp Med **176:**1197-201.

241. **Mann, D. A., I. Mikaelian, R. W. Zemmel, S. M. Green, A. D. Lowe, T. Kimura, M. Singh, P. J. Butler, M. J. Gait, and J. Karn.** 1994. A molecular rheostat. Co-operative rev binding to stem I of the rev-response element modulates human immunodeficiency virus type-1 late gene expression. J Mol Biol **241:**193-207.

242. **Mansky, L. M., and H. M. Temin.** 1995. Lower in vivo mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase. J Virol **69:**5087-94.

243. **Mardon, G., and H. E. Varmus.** 1983. Frameshift and intragenic suppressor mutations in a Rous sarcoma provirus suggest src encodes two proteins. Cell **32:**871-9.

244. **Martin, K. A., R. Wyatt, M. Farzan, H. Choe, L. Marcon, E. Desjardins, J. Robinson, J. Sodroski, C. Gerard, and N. P. Gerard.** 1997. CD4-independent binding of SIV gp120 to rhesus CCR5. Science **278:**1470-3.

245. **Martin-Serrano, J., A. Yarovoy, D. Perez-Caballero, and P. D. Bieniasz.** 2003. Divergent retroviral late-budding domains recruit vacuolar protein sorting factors by using alternative adaptor proteins. Proc Natl Acad Sci U S A **100:**12414-9.

246. **Martin-Serrano, J., T. Zang, and P. D. Bieniasz.** 2001. HIV-1 and Ebola virus encode small peptide motifs that recruit Tsg101 to sites of particle assembly to facilitate egress. Nat Med **7:**1313-9.

247. **Martin-Serrano, J., T. Zang, and P. D. Bieniasz.** 2003. Role of ESCRT-I in retroviral budding. J Virol **77:**4794-804.

248. **Mascola, J. R., M. G. Lewis, G. Stiegler, D. Harris, T. C. VanCott, D. Hayes, M. K. Louder, C. R. Brown, C. V. Sapan, S. S. Frankel, Y. Lu, M. L. Robb, H. Katinger, and D. L. Birx.** 1999. Protection of Macaques against pathogenic simian/human immunodeficiency virus 89.6PD by passive transfer of neutralizing antibodies. J Virol **73:**4009-18.

249. **McBride, M. S., and A. T. Panganiban.** 1997. Position dependence of functional hairpins important for human immunodeficiency virus type 1 RNA encapsidation in vivo. J Virol **71:**2050-8.

250. **McCune, J. M., L. B. Rabin, M. B. Feinberg, M. Lieberman, J. C. Kosek, G. R. Reyes, and I. L. Weissman.** 1988. Endoproteolytic cleavage of gp160 is required for the activation of human immunodeficiency virus. Cell **53:**55-67.

251. **McDonald, D., L. Wu, S. M. Bohks, V. N. KewalRamani, D. Unutmaz, and T. J. Hope.** 2003. Recruitment of HIV and its receptors to dendritic cell-T cell junctions. Science **300:**1295-7.

252. **McDougal, J. S., J. K. Nicholson, G. D. Cross, S. P. Cort, M. S. Kennedy, and A. C. Mawle.** 1986. Binding of the human retrovirus HTLV-III/LAV/ARV/HIV to the CD4 (T4) molecule: conformation dependence, epitope mapping, antibody inhibition, and potential for idiotypic mimicry. J Immunol **137:**2937-44.

253. **McLaughlin, S., and D. Murray.** 2005. Plasma membrane phosphoinositide organization by protein electrostatics. Nature **438:**605-11.

254. **McMillan, N. A., R. F. Chun, D. P. Siderovski, J. Galabru, W. M. Toone, C. E. Samuel, T. W. Mak, A. G. Hovanessian, K. T. Jeang, and B. R. Williams.** 1995. HIV-1 Tat directly interacts with the interferon-induced, double-stranded RNA-dependent kinase, PKR. Virology **213:**413-24.

255. **Mehandru, S., M. A. Poles, K. Tenner-Racz, A. Horowitz, A. Hurley, C. Hogan, D. Boden, P. Racz, and M. Markowitz.** 2004. Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract. J Exp Med **200:**761-70.

256. **Mergia, A., K. E. Shaw, E. Pratt-Lowe, P. A. Barry, and P. A. Luciw.** 1991. Identification of the simian foamy virus transcriptional transactivator gene (taf). J Virol **65:**2903-9.

257. **Miller, M. A., M. W. Cloyd, J. Liebmann, C. R. Rinaldo, Jr., K. R. Islam, S. Z. Wang, T. A. Mietzner, and R. C. Montelaro.** 1993. Alterations in cell membrane permeability by the lentivirus lytic peptide (LLP-1) of HIV-1 transmembrane protein. Virology **196:**89-100.

258. **Miranda, K. C., T. Khromykh, P. Christy, T. L. Le, C. J. Gottardi, A. S. Yap, J. L. Stow, and R. D. Teasdale.** 2001. A dileucine motif targets E-cadherin to the basolateral cell surface in Madin-Darby canine kidney and LLC-PK1 epithelial cells. J Biol Chem **276:**22565-72.

259. **Mizutani, S., D. Boettiger, and H. M. Temin.** 1970. A DNA-depenent DNA polymerase and a DNA endonuclease in virions of Rous sarcoma virus. Nature **228:**424-7.

260. **Moebes, A., J. Enssle, P. D. Bieniasz, M. Heinkelein, D. Lindemann, M. Bock, M. O. McClure, and A. Rethwilm.** 1997. Human foamy virus reverse transcription that occurs late in the viral replication cycle. J Virol **71:**7305-11.

261. **Moore, R., M. Dixon, R. Smith, G. Peters, and C. Dickson.** 1987. Complete nucleotide sequence of a milk-transmitted mouse mammary tumor virus: two frameshift suppression events are required for translation of gag and pol. J Virol **61:**480-90.

262. **Morellet, N., N. Jullian, H. De Rocquigny, B. Maigret, J. L. Darlix, and B. P. Roques.** 1992. Determination of the structure of the nucleocapsid protein NCp7 from the human immunodeficiency virus type 1 by 1H NMR. Embo J **11:**3059-65.

263. **Morikawa, Y., D. J. Hockley, M. V. Nermut, and I. M. Jones.** 2000. Roles of matrix, p2, and N-terminal myristoylation in human immunodeficiency virus type 1 Gag assembly. J Virol **74:**16-23.

264. **Morikawa, Y., W. H. Zhang, D. J. Hockley, M. V. Nermut, and I. M. Jones.** 1998. Detection of a trimeric human immunodeficiency virus type 1 Gag intermediate is dependent on sequences in the matrix protein, p17. J Virol **72:**7659-63.

265. **Morita, E., and W. I. Sundquist.** 2004. Retrovirus budding. Annu Rev Cell Dev Biol **20:**395-425.

266. **Muesing, M. A., D. H. Smith, and D. J. Capon.** 1987. Regulation of mRNA accumulation by a human immunodeficiency virus trans-activator protein. Cell **48:**691-701.

267. **Munshi, U. M., J. Kim, K. Nagashima, J. H. Hurley, and E. O. Freed.** 2007. An Alix fragment potently inhibits HIV-1 budding: characterization of binding to retroviral YPXL late domains. J Biol Chem **282:**3847-55.

268. **Murakami, T., S. Ablan, E. O. Freed, and Y. Tanaka.** 2004. Regulation of human immunodeficiency virus type 1 Env-mediated membrane fusion by viral protease activity. J Virol **78:**1026-31.

269. **Murakami, T., and E. O. Freed.** 2000. Genetic evidence for an interaction between human immunodeficiency virus type 1 matrix and alpha-helix 2 of the gp41 cytoplasmic tail. J Virol **74:**3548-54.

270. **Murakami, T., and E. O. Freed.** 2000. The long cytoplasmic tail of gp41 is required in a cell type-dependent manner for HIV-1 envelope glycoprotein incorporation into virions. Proc Natl Acad Sci U S A **97:**343-8.

271. **Muriaux, D., J. Mirro, D. Harvin, and A. Rein.** 2001. RNA is a structural element in retrovirus particles. Proc Natl Acad Sci U S A **98:**5246-51.

272. **Murphy, J. E., and S. P. Goff.** 1989. Construction and analysis of deletion mutations in the U5 region of Moloney murine leukemia virus: effects on RNA packaging and reverse transcription. J Virol **63:**319-27.

273. **Murphy, J. E., and S. P. Goff.** 1994. Forced integration of Moloney murine leukemia virus DNA with a mutant integration site occurs through recombination with VL30 DNA. Virology **204:**458-61.

274. **Namy, O., S. J. Moran, D. I. Stuart, R. J. Gilbert, and I. Brierley.** 2006. A mechanical explanation of RNA pseudoknot function in programmed ribosomal frameshifting. Nature **441:**244-7.

275. **Newman, J. L., E. W. Butcher, D. T. Patel, Y. Mikhaylenko, and M. F. Summers.** 2004. Flexibility in the P2 domain of the HIV-1 Gag polyprotein. Protein Sci **13:**2101-7.

276. **Newman, J. T., T. J. Sturgeon, P. Gupta, and R. C. Montelaro.** 2007. Differential functional phenotypes of two primary HIV-1 strains resulting from homologous point mutations in the LLP domains of the envelope gp41 intracytoplasmic domain. Virology **367:**102-16.

277. **Nguyen, D. G., A. Booth, S. J. Gould, and J. E. Hildreth.** 2003. Evidence that HIV budding in primary macrophages occurs through the exosome release pathway. J Biol Chem **278:**52347-54.

278. **Nicholson, M. G., S. M. Rue, J. E. Clements, and S. A. Barber.** 2006. An internal ribosome entry site promotes translation of a novel SIV Pr55(Gag) isoform. Virology **349:**325-34.

279. **Niederman, T. M., B. J. Thielan, and L. Ratner.** 1989. Human immunodeficiency virus type 1 negative factor is a transcriptional silencer. Proc Natl Acad Sci U S A **86:**1128-32.

280. **Nisole, S., C. Lynch, J. P. Stoye, and M. W. Yap.** 2004. A Trim5-cyclophilin A fusion protein found in owl monkey kidney cells can restrict HIV-1. Proc Natl Acad Sci U S A **101:**13324-8.

281. **Nolan, K. M., A. P. Jordan, and J. A. Hoxie.** 2008. Effects of partial deletions within the human immunodeficiency virus type 1 V3 loop on coreceptor tropism and sensitivity to entry inhibitors. J Virol **82:**664-73.

282. **Ohlmann, T., M. Lopez-Lastra, and J. L. Darlix.** 2000. An internal ribosome entry segment promotes translation of the simian immunodeficiency virus genomic RNA. J Biol Chem **275:**11899-906.

283. **Ohno, H., R. C. Aguilar, M. C. Fournier, S. Hennecke, P. Cosson, and J. S. Bonifacino.** 1997. Interaction of endocytic signals from the HIV-1 envelope glycoprotein complex with members of the adaptor medium chain family. Virology **238:**305-15.

284. **Ono, A., S. D. Ablan, S. J. Lockett, K. Nagashima, and E. O. Freed.** 2004. Phosphatidylinositol (4,5) bisphosphate regulates HIV-1 Gag targeting to the plasma membrane. Proc Natl Acad Sci U S A **101:**14889-94.

285. **Ono, A., D. Demirov, and E. O. Freed.** 2000. Relationship between human immunodeficiency virus type 1 Gag multimerization and membrane binding. J Virol **74:**5142-50.

286. **Ono, A., and E. O. Freed.** 2004. Cell-type-dependent targeting of human immunodeficiency virus type 1 assembly to the plasma membrane and the multivesicular body. J Virol **78:**1552-63.

287. **Ono, A., and E. O. Freed.** 2005. Role of lipid rafts in virus replication. Adv Virus Res **64:**311-58.

288. **Orenstein, J. M., M. S. Meltzer, T. Phipps, and H. E. Gendelman.** 1988. Cytoplasmic assembly and accumulation of human immunodeficiency virus types 1 and 2 in recombinant human colony-stimulating factor-1-treated human monocytes: an ultrastructural study. J Virol **62:**2578-86.

289. **Oroszlan, S., and R. B. Luftig.** 1990. Retroviral proteinases. Curr Top Microbiol Immunol **157:**153-85.

290. **Ott, M., M. Schnolzer, J. Garnica, W. Fischle, S. Emiliani, H. R. Rackwitz, and E. Verdin.** 1999. Acetylation of the HIV-1 Tat protein by p300 is important for its transcriptional activity. Curr Biol **9:**1489-92.

291. **Owen, D. J., H. Setiadi, P. R. Evans, R. P. McEver, and S. A. Green.** 2001. A third specificity-determining site in mu 2 adaptin for sequences upstream of Yxx phi sorting motifs. Traffic **2:**105-10.

292. **Owens, R. J., J. W. Dubay, E. Hunter, and R. W. Compans.** 1991. Human immunodeficiency virus envelope protein determines the site of virus release in polarized epithelial cells. Proc Natl Acad Sci U S A **88:**3987-91.

293. **Pain, V. M.** 1996. Initiation of protein synthesis in eukaryotic cells. Eur J Biochem **236:**747-71.

294. **Panganiban, A. T., and H. M. Temin.** 1984. The retrovirus pol gene encodes a product required for DNA integration: identification of a retrovirus int locus. Proc Natl Acad Sci U S A **81:**7885-9.

295. **Parent, L. J., R. P. Bennett, R. C. Craven, T. D. Nelle, N. K. Krishna, J. B. Bowzard, C. B. Wilson, B. A. Puffer, R. C. Montelaro, and J. W. Wills.** 1995. Positionally independent and exchangeable late budding functions of the Rous sarcoma virus and human immunodeficiency virus Gag proteins. J Virol **69:**5455-60.

296. **Parren, P. W., P. Fisicaro, A. F. Labrijn, J. M. Binley, W. P. Yang, H. J. Ditzel, C. F. Barbas, 3rd, and D. R. Burton.** 1996. In vitro antigen challenge of human antibody libraries for vaccine evaluation: the human immunodeficiency virus type 1 envelope. J Virol **70:**9046-50.

297. **Pearl, L. H., and W. R. Taylor.** 1987. A structural model for the retroviral proteases. Nature **329:**351-4.

298. **Pelchen-Matthews, A., B. Kramer, and M. Marsh.** 2003. Infectious HIV-1 assembles in late endosomes in primary macrophages. J Cell Biol **162:**443-55.

299. **Pelchen-Matthews, A., G. Raposo, and M. Marsh.** 2004. Endosomes, exosomes and Trojan viruses. Trends Microbiol **12:**310-6.

300. **Perelson, A. S., A. U. Neumann, M. Markowitz, J. M. Leonard, and D. D. Ho.** 1996. HIV-1 dynamics in vivo: virion clearance rate, infected cell life-span, and viral generation time. Science **271:**1582-6.

301. **Pincus, S. H., K. Wehrly, and B. Chesebro.** 1989. Treatment of HIV tissue culture infection with monoclonal antibody-ricin A chain conjugates. J Immunol **142:**3070-5.

302. **Platt, E. J., K. Wehrly, S. E. Kuhmann, B. Chesebro, and D. Kabat.** 1998. Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1. J Virol **72:**2855-64.

303. **Pond, L., L. A. Kuhn, L. Teyton, M. P. Schutze, J. A. Tainer, M. R. Jackson, and P. A. Peterson.** 1995. A role for acidic residues in di-leucine motif-based targeting to the endocytic pathway. J Biol Chem **270:**19989-97.

304. **Poon, D. T., J. Wu, and A. Aldovini.** 1996. Charged amino acid residues of human immunodeficiency virus type 1 nucleocapsid p7 protein involved in RNA packaging and infectivity. J Virol **70:**6607-16.

305. **Pornillos, O., S. L. Alam, R. L. Rich, D. G. Myszka, D. R. Davis, and W. I. Sundquist.** 2002. Structure and functional interactions of the Tsg101 UEV domain. Embo J **21:**2397-406.

306. **Potempa, S., L. Picard, J. D. Reeves, D. Wilkinson, R. A. Weiss, and S. J. Talbot.** 1997. CD4-independent infection by human immunodeficiency virus type 2 strain ROD/B: the role of the N-terminal domain of CXCR-4 in fusion and entry. J Virol **71:**4419-24.

307. **Power, M. D., P. A. Marx, M. L. Bryant, M. B. Gardner, P. J. Barr, and P. A. Luciw.** 1986. Nucleotide sequence of SRV-1, a type D simian acquired immune deficiency syndrome retrovirus. Science **231:**1567-72.

308. **Puffer, B. A., L. J. Parent, J. W. Wills, and R. C. Montelaro.** 1997. Equine infectious anemia virus utilizes a YXXL motif within the late assembly domain of the Gag p9 protein. J Virol **71:**6541-6.

309. **Pumfery, A., L. Deng, A. Maddukuri, C. de la Fuente, H. Li, J. D. Wade, P. Lambert, A. Kumar, and F. Kashanchi.** 2003. Chromatin remodeling and modification during HIV-1 Tat-activated transcription. Curr HIV Res **1:**343-62.

310. **Quigley, J. P., D. B. Rifkin, and E. Reich.** 1971. Phospholipid composition of Rous sarcoma virus, host cell membranes and other enveloped RNA viruses. Virology **46:**106-16.

311. **Raposo, G., M. Moore, D. Innes, R. Leijendekker, A. Leigh-Brown, P. Benaroch, and H. Geuze.** 2002. Human macrophages accumulate HIV-1 particles in MHC II compartments. Traffic **3:**718-29.

312. **Ratner, L., W. Haseltine, R. Patarca, K. J. Livak, B. Starcich, S. F. Josephs, E. R. Doran, J. A. Rafalski, E. A. Whitehorn, K. Baumeister, and et al.** 1985. Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature **313:**277-84.

313. **Rein, A.** 1994. Retroviral RNA packaging: a review. Arch Virol Suppl **9:**513-22.

314. **Resh, M. D.** 2004. A myristoyl switch regulates membrane binding of HIV-1 Gag. Proc Natl Acad Sci U S A **101:**417-8.

315. **Roben, P., J. P. Moore, M. Thali, J. Sodroski, C. F. Barbas, 3rd, and D. R. Burton.** 1994. Recognition properties of a panel of human recombinant Fab fragments to the CD4 binding site of gp120 that show differing abilities to neutralize human immunodeficiency virus type 1. J Virol **68:**4821-8.

316. **Roof, P., M. Ricci, P. Genin, M. A. Montano, M. Essex, M. A. Wainberg, A. Gatignol, and J. Hiscott.** 2002. Differential regulation of HIV-1 clade-specific B, C, and E long terminal repeats by NF-kappaB and the Tat transactivator. Virology **296:**77-83.

317. **Rosen, C. A., J. G. Sodroski, and W. A. Haseltine.** 1985. The location of cis-acting regulatory sequences in the human T cell lymphotropic virus type III (HTLV-III/LAV) long terminal repeat. Cell **41:**813-23.

318. **Roth, M. G., R. V. Srinivas, and R. W. Compans.** 1983. Basolateral maturation of retroviruses in polarized epithelial cells. J Virol **45:**1065-73.

319. **Rous, B. A., B. J. Reaves, G. Ihrke, J. A. Briggs, S. R. Gray, D. J. Stephens, G. Banting, and J. P. Luzio.** 2002. Role of adaptor complex AP-3 in targeting wild-type and mutated CD63 to lysosomes. Mol Biol Cell **13:**1071-82.

320. **Rowell, J. F., P. E. Stanhope, and R. F. Siliciano.** 1995. Endocytosis of endogenously synthesized HIV-1 envelope protein. Mechanism and role in processing for association with class II MHC. J Immunol **155:**473-88.

321. **Roy, S., M. G. Katze, N. T. Parkin, I. Edery, A. G. Hovanessian, and N. Sonenberg.** 1990. Control of the interferon-induced 68-kilodalton protein kinase by the HIV-1 tat gene product. Science **247:**1216-9.

322. **Rudner, L., S. Nydegger, L. V. Coren, K. Nagashima, M. Thali, and D. E. Ott.** 2005. Dynamic fluorescent imaging of human immunodeficiency virus type 1 gag in live cells by biarsenical labeling. J Virol **79:**4055-65.

323. **Ruiz-Mateos, E., A. Pelchen-Matthews, M. Deneka, and M. Marsh.** 2008. CD63 is not required for production of infectious human immunodeficiency virus type 1 in human macrophages. J Virol **82:**4751-61.

324. **Russell, R. S., C. Liang, and M. A. Wainberg.** 2004. Is HIV-1 RNA dimerization a prerequisite for packaging? Yes, no, probably? Retrovirology **1:**23.

325. **Saad, J. S., J. Miller, J. Tai, A. Kim, R. H. Ghanam, and M. F. Summers.** 2006. Structural basis for targeting HIV-1 Gag proteins to the plasma membrane for virus assembly. Proc Natl Acad Sci U S A **103:**11364-9.

326. **Sakaguchi, K., N. Zambrano, E. T. Baldwin, B. A. Shapiro, J. W. Erickson, J. G. Omichinski, G. M. Clore, A. M. Gronenborn, and E. Appella.** 1993. Identification of a binding site for the human immunodeficiency virus type 1 nucleocapsid protein. Proc Natl Acad Sci U S A **90:**5219-23.

327. **Salzwedel, K., J. T. West, and E. Hunter.** 1999. A conserved tryptophan-rich motif in the membrane-proximal region of the human immunodeficiency virus type 1 gp41 ectodomain is important for Env-mediated fusion and virus infectivity. J Virol **73:**2469-80.

328. **Sandoval, I. V., and O. Bakke.** 1994. Targeting of membrane proteins to endosomes and lysosomes. Trends Cell Biol **4:**292-7.

329. **Sarnow, P., R. C. Cevallos, and E. Jan.** 2005. Takeover of host ribosomes by divergent IRES elements. Biochem Soc Trans **33:**1479-82.

330. **Sattentau, Q. J., and J. P. Moore.** 1991. Conformational changes induced in the human immunodeficiency virus envelope glycoprotein by soluble CD4 binding. J Exp Med **174:**407-15.

331. **Sattentau, Q. J., and J. P. Moore.** 1993. The role of CD4 in HIV binding and entry. Philos Trans R Soc Lond B Biol Sci **342:**59-66.

332. **Sattentau, Q. J., S. Zolla-Pazner, and P. Poignard.** 1995. Epitope exposure on functional, oligomeric HIV-1 gp41 molecules. Virology **206:**713-7.

333. **Sauter, M. M., A. Pelchen-Matthews, R. Bron, M. Marsh, C. C. LaBranche, P. J. Vance, J. Romano, B. S. Haggarty, T. K. Hart, W. M. Lee, and J. A. Hoxie.** 1996. An internalization signal in the simian immunodeficiency virus transmembrane protein cytoplasmic domain modulates expression of envelope glycoproteins on the cell surface. J Cell Biol **132:**795-811.

334. **Sayah, D. M., E. Sokolskaja, L. Berthoux, and J. Luban.** 2004. Cyclophilin A retrotransposition into TRIM5 explains owl monkey resistance to HIV-1. Nature **430:**569-73.

335. **Schmalzbauer, E., B. Strack, J. Dannull, S. Guehmann, and K. Moelling.** 1996. Mutations of basic amino acids of NCp7 of human immunodeficiency virus type 1 affect RNA binding in vitro. J Virol **70:**771-7.

336. **Schroder, H. C., M. Kelve, and W. E. Muller.** 1994. The 2-5A system and HIV infection. Prog Mol Subcell Biol **14:**176-97.

337. **Schrofelbauer, B., D. Chen, and N. R. Landau.** 2004. A single amino acid of APOBEC3G controls its species-specific interaction with virion infectivity factor (Vif). Proc Natl Acad Sci U S A **101:**3927-32.

338. **Schwartz, S., B. K. Felber, and G. N. Pavlakis.** 1991. Expression of human immunodeficiency virus type 1 vif and vpr mRNAs is Rev-dependent and regulated by splicing. Virology **183:**677-86.

339. **Shang, L., L. Yue, and E. Hunter.** 2008. Role of the membrane-spanning domain of human immunodeficiency virus type 1 envelope glycoprotein in cell-cell fusion and virus infection. J Virol **82:**5417-28.

340. **Shankarappa, R., J. B. Margolick, S. J. Gange, A. G. Rodrigo, D. Upchurch, H. Farzadegan, P. Gupta, C. R. Rinaldo, G. H. Learn, X. He, X. L. Huang, and J. I. Mullins.** 1999. Consistent viral evolutionary changes associated with the progression of human immunodeficiency virus type 1 infection. J Virol **73:**10489-502.

341. **Shehu-Xhilaga, M., S. Ablan, D. G. Demirov, C. Chen, R. C. Montelaro, and E. O. Freed.** 2004. Late domain-dependent inhibition of equine infectious anemia virus budding. J Virol **78:**724-32.

342. **Shehu-Xhilaga, M., S. M. Crowe, and J. Mak.** 2001. Maintenance of the Gag/Gag-Pol ratio is important for human immunodeficiency virus type 1 RNA dimerization and viral infectivity. J Virol **75:**1834-41.

343. **Sherman, P. A., M. L. Dickson, and J. A. Fyfe.** 1992. Human immunodeficiency virus type 1 integration protein: DNA sequence requirements for cleaving and joining reactions. J Virol **66:**3593-601.

344. **Shibata, R., T. Miura, M. Hayami, K. Ogawa, H. Sakai, T. Kiyomasu, A. Ishimoto, and A. Adachi.** 1990. Mutational analysis of the human immunodeficiency virus type 2 (HIV-2) genome in relation to HIV-1 and simian immunodeficiency virus SIV (AGM). J Virol **64:**742-7.

345. **Shkriabai, N., S. A. Datta, Z. Zhao, S. Hess, A. Rein, and M. Kvaratskhelia.** 2006. Interactions of HIV-1 Gag with assembly cofactors. Biochemistry **45:**4077-83.

346. **Silverman, R. H., and D. N. Sengupta.** 1990. Translational regulation by HIV leader RNA, TAT, and interferon-inducible enzymes. J Exp Pathol **5:**69-77.

347. **Simmen, T., A. Schmidt, W. Hunziker, and F. Beermann.** 1999. The tyrosinase tail mediates sorting to the lysosomal compartment in MDCK cells via a di-leucine and a tyrosine-based signal. J Cell Sci **112 (Pt 1):**45-53.

348. **Sokolskaja, E., D. M. Sayah, and J. Luban.** 2004. Target cell cyclophilin A modulates human immunodeficiency virus type 1 infectivity. J Virol **78:**12800-8.

349. **Sonigo, P., M. Alizon, K. Staskus, D. Klatzmann, S. Cole, O. Danos, E. Retzel, P. Tiollais, A. Haase, and S. Wain-Hobson.** 1985. Nucleotide sequence of the visna lentivirus: relationship to the AIDS virus. Cell **42:**369-82.

350. **Sonigo, P., C. Barker, E. Hunter, and S. Wain-Hobson.** 1986. Nucleotide sequence of Mason-Pfizer monkey virus: an immunosuppressive D-type retrovirus. Cell **45:**375-85.

351. **South, T. L., P. R. Blake, R. C. Sowder, 3rd, L. O. Arthur, L. E. Henderson, and M. F. Summers.** 1990. The nucleocapsid protein isolated from HIV-1 particles binds zinc and forms retroviral-type zinc fingers. Biochemistry **29:**7786-9.

352. **Spearman, P., R. Horton, L. Ratner, and I. Kuli-Zade.** 1997. Membrane binding of human immunodeficiency virus type 1 matrix protein in vivo supports a conformational myristyl switch mechanism. J Virol **71:**6582-92.

353. **Srinivas, S. K., R. V. Srinivas, G. M. Anantharamaiah, J. P. Segrest, and R. W. Compans.** 1992. Membrane interactions of synthetic peptides corresponding to amphipathic helical segments of the human immunodeficiency virus type-1 envelope glycoprotein. J Biol Chem **267:**7121-7.

354. **Strack, B., A. Calistri, S. Craig, E. Popova, and H. G. Gottlinger.** 2003. AIP1/ALIX is a binding partner for HIV-1 p6 and EIAV p9 functioning in virus budding. Cell **114:**689-99.

355. **Strack, B., A. Calistri, and H. G. Gottlinger.** 2002. Late assembly domain function can exhibit context dependence and involves ubiquitin residues implicated in endocytosis. J Virol **76:**5472-9.

356. **Sullivan, N., M. Thali, C. Furman, D. D. Ho, and J. Sodroski.** 1993. Effect of amino acid changes in the V1/V2 region of the human immunodeficiency virus type 1 gp120 glycoprotein on subunit association, syncytium formation, and recognition by a neutralizing antibody. J Virol **67:**3674-9.

357. **Summers, M. F., L. E. Henderson, M. R. Chance, J. W. Bess, Jr., T. L. South, P. R. Blake, I. Sagi, G. Perez-Alvarado, R. C. Sowder, 3rd, D. R. Hare, and et al.** 1992. Nucleocapsid zinc fingers detected in retroviruses: EXAFS studies of intact viruses and the solution-state structure of the nucleocapsid protein from HIV-1. Protein Sci **1:**563-74.

358. **Takeuchi, Y., M. Akutsu, K. Murayama, N. Shimizu, and H. Hoshino.** 1991. Host range mutant of human immunodeficiency virus type 1: modification of cell tropism by a single point mutation at the neutralization epitope in the env gene. J Virol **65:**1710-8.

359. **Takeuchi, Y., M. O. McClure, and M. Pizzato.** 2008. Identification of gammaretroviruses constitutively released from cell lines used for human immunodeficiency virus research. J Virol **82:**12585-8.

360. **Tan, K., J. Liu, J. Wang, S. Shen, and M. Lu.** 1997. Atomic structure of a thermostable subdomain of HIV-1 gp41. Proc Natl Acad Sci U S A **94:**12303-8.

361. **Tang, C., E. Loeliger, P. Luncsford, I. Kinde, D. Beckett, and M. F. Summers.** 2004. Entropic switch regulates myristate exposure in the HIV-1 matrix protein. Proc Natl Acad Sci U S A **101:**517-22.

362. **Tanzi, G. O., A. J. Piefer, and P. Bates.** 2003. Equine infectious anemia virus utilizes host vesicular protein sorting machinery during particle release. J Virol **77:**8440-7.

363. **Thali, M., A. Bukovsky, E. Kondo, B. Rosenwirth, C. T. Walsh, J. Sodroski, and H. G. Gottlinger.** 1994. Functional association of cyclophilin A with HIV-1 virions. Nature **372:**363-5.

364. **Thali, M., J. P. Moore, C. Furman, M. Charles, D. D. Ho, J. Robinson, and J. Sodroski.** 1993. Characterization of conserved human immunodeficiency virus type 1 gp120 neutralization epitopes exposed upon gp120-CD4 binding. J Virol **67:**3978-88.

365. **Tiley, L. S., M. H. Malim, H. K. Tewary, P. G. Stockley, and B. R. Cullen.** 1992. Identification of a high-affinity RNA-binding site for the human immunodeficiency virus type 1 Rev protein. Proc Natl Acad Sci U S A **89:**758-62.

366. **Toohey, K., K. Wehrly, J. Nishio, S. Perryman, and B. Chesebro.** 1995. Human immunodeficiency virus envelope V1 and V2 regions influence replication efficiency in macrophages by affecting virus spread. Virology **213:**70-9.

367. **Trkola, A., M. Purtscher, T. Muster, C. Ballaun, A. Buchacher, N. Sullivan, K. Srinivasan, J. Sodroski, J. P. Moore, and H. Katinger.** 1996. Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. J Virol **70:**1100-8.

368. **Trowbridge, I. S., J. F. Collawn, and C. R. Hopkins.** 1993. Signal-dependent membrane protein trafficking in the endocytic pathway. Annu Rev Cell Biol **9:**129-61.

369. **Valerie, K., and M. Rosenberg.** 1990. Chromatin structure implicated in activation of HIV-1 gene expression by ultraviolet light. New Biol **2:**712-8.

370. **Venable, R. M., R. W. Pastor, B. R. Brooks, and F. W. Carson.** 1989. Theoretically determined three-dimensional structures for amphipathic segments of the HIV-1 gp41 envelope protein. AIDS Res Hum Retroviruses **5:**7-22.

371. **Verdin, E., P. Paras, Jr., and C. Van Lint.** 1993. Chromatin disruption in the promoter of human immunodeficiency virus type 1 during transcriptional activation. Embo J **12:**3249-59.

372. **Verhey, K. J., and M. J. Birnbaum.** 1994. A Leu-Leu sequence is essential for COOH-terminal targeting signal of GLUT4 glucose transporter in fibroblasts. J Biol Chem **269:**2353-6.

373. **VerPlank, L., F. Bouamr, T. J. LaGrassa, B. Agresta, A. Kikonyogo, J. Leis, and C. A. Carter.** 2001. Tsg101, a homologue of ubiquitin-conjugating (E2) enzymes, binds the L domain in HIV type 1 Pr55(Gag). Proc Natl Acad Sci U S A **98:**7724-9.

374. **Vincent, M. J., L. R. Melsen, A. S. Martin, and R. W. Compans.** 1999. Intracellular interaction of simian immunodeficiency virus Gag and Env proteins. J Virol **73:**8138-44.

375. **Viscidi, R. P., K. Mayur, H. M. Lederman, and A. D. Frankel.** 1989. Inhibition of antigen-induced lymphocyte proliferation by Tat protein from HIV-1. Science **246:**1606-8.

376. **von Schwedler, U. K., M. Stuchell, B. Muller, D. M. Ward, H. Y. Chung, E. Morita, H. E. Wang, T. Davis, G. P. He, D. M. Cimbora, A. Scott, H. G. Krausslich, J. Kaplan, S. G. Morham, and W. I. Sundquist.** 2003. The protein network of HIV budding. Cell **114:**701-13.

377. **Voorhees, P., E. Deignan, E. van Donselaar, J. Humphrey, M. S. Marks, P. J. Peters, and J. S. Bonifacino.** 1995. An acidic sequence within the cytoplasmic domain of furin functions as a determinant of trans-Golgi network localization and internalization from the cell surface. Embo J **14:**4961-75.

378. **Vowels, J. J., and G. S. Payne.** 1998. A dileucine-like sorting signal directs transport into an AP-3-dependent, clathrin-independent pathway to the yeast vacuole. Embo J **17:**2482-93.

379. **Waterman, M. L., and K. A. Jones.** 1990. Purification of TCF-1 alpha, a T-cell-specific transcription factor that activates the T-cell receptor C alpha gene enhancer in a context-dependent manner. New Biol **2:**621-36.

380. **Weeks, K. M., C. Ampe, S. C. Schultz, T. A. Steitz, and D. M. Crothers.** 1990. Fragments of the HIV-1 Tat protein specifically bind TAR RNA. Science **249:**1281-5.

381. **Wehrly, K., and B. Chesebro.** 1997. p24 antigen capture assay for quantification of human immunodeficiency virus using readily available inexpensive reagents. Methods **12:**288-93.

382. **Wei, X., J. M. Decker, H. Liu, Z. Zhang, R. B. Arani, J. M. Kilby, M. S. Saag, X. Wu, G. M. Shaw, and J. C. Kappes.** 2002. Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. Antimicrob Agents Chemother **46:**1896-905.

383. **Weissenhorn, W., A. Dessen, S. C. Harrison, J. J. Skehel, and D. C. Wiley.** 1997. Atomic structure of the ectodomain from HIV-1 gp41. Nature **387:**426-30.

384. **West, J. T., P. B. Johnston, S. R. Dubay, and E. Hunter.** 2001. Mutations within the putative membrane-spanning domain of the simian immunodeficiency virus transmembrane glycoprotein define the minimal requirements for fusion, incorporation, and infectivity. J Virol **75:**9601-12.

385. **West, J. T., S. K. Weldon, S. Wyss, X. Lin, Q. Yu, M. Thali, and E. Hunter.** 2002. Mutation of the dominant endocytosis motif in human immunodeficiency virus type 1 gp41 can complement matrix mutations without increasing Env incorporation. J Virol **76:**3338-49.

386. **White, J. M.** 1992. Membrane fusion. Science **258:**917-24.

387. **Wilk, T., T. Pfeiffer, and V. Bosch.** 1992. Retained in vitro infectivity and cytopathogenicity of HIV-1 despite truncation of the C-terminal tail of the env gene product. Virology **189:**167-77.

388. **Wilkinson, T. A., K. Januszyk, M. L. Phillips, S. S. Tekeste, M. Zhang, J. T. Miller, S. F. Le Grice, R. T. Clubb, and S. A. Chow.** 2009. Identifying and characterizing a functional HIV-1 reverse transcriptase-binding site on integrase. J Biol Chem **284:**7931-9.

389. **Willey, R. L., F. Maldarelli, M. A. Martin, and K. Strebel.** 1992. Human immunodeficiency virus type 1 Vpu protein induces rapid degradation of CD4. J Virol **66:**7193-200.

390. **Willey, R. L., and M. A. Martin.** 1993. Association of human immunodeficiency virus type 1 envelope glycoprotein with particles depends on interactions between the third variable and conserved regions of gp120. J Virol **67:**3639-43.

391. **Wondrak, E. M., N. T. Nashed, M. T. Haber, D. M. Jerina, and J. M. Louis.** 1996. A transient precursor of the HIV-1 protease. Isolation, characterization, and kinetics of maturation. J Biol Chem **271:**4477-81.

392. **Woodward, C. L., S. Prakobwanakit, S. Mosessian, and S. A. Chow.** 2009. Integrase interacts with nucleoporin NUP153 to mediate the nuclear import of human immunodeficiency virus type 1. J Virol **83:**6522-33.

393. **Worthylake, D. K., H. Wang, S. Yoo, W. I. Sundquist, and C. P. Hill.** 1999. Structures of the HIV-1 capsid protein dimerization domain at 2.6 A resolution. Acta Crystallogr D Biol Crystallogr **55:**85-92.

394. **Wu, L., T. Zhou, Z. Y. Yang, K. Svehla, S. O'Dell, M. K. Louder, L. Xu, J. R. Mascola, D. R. Burton, J. A. Hoxie, R. W. Doms, P. D. Kwong, and G. J. Nabel.** 2009. Enhanced exposure of the CD4-binding site to neutralizing antibodies by structural design of a membrane-anchored human immunodeficiency virus type 1 gp120 domain. J Virol **83:**5077-86.

395. **Wyatt, R., P. D. Kwong, E. Desjardins, R. W. Sweet, J. Robinson, W. A. Hendrickson, and J. G. Sodroski.** 1998. The antigenic structure of the HIV gp120 envelope glycoprotein. Nature **393:**705-11.

396. **Wyma, D. J., J. Jiang, J. Shi, J. Zhou, J. E. Lineberger, M. D. Miller, and C. Aiken.** 2004. Coupling of human immunodeficiency virus type 1 fusion to virion maturation: a novel role of the gp41 cytoplasmic tail. J Virol **78:**3429-35.

397. **Wyss, S., C. Berlioz-Torrent, M. Boge, G. Blot, S. Honing, R. Benarous, and M. Thali.** 2001. The highly conserved C-terminal dileucine motif in the cytosolic domain of the human immunodeficiency virus type 1 envelope glycoprotein is critical for its association with the AP-1 clathrin adaptor [correction of adapter]. J Virol **75:**2982-92.

398. **Wyss, S., A. S. Dimitrov, F. Baribaud, T. G. Edwards, R. Blumenthal, and J. A. Hoxie.** 2005. Regulation of human immunodeficiency virus type 1 envelope glycoprotein fusion by a membrane-interactive domain in the gp41 cytoplasmic tail. J Virol **79:**12231-41.

399. **Yu, X., X. Yuan, Z. Matsuda, T. H. Lee, and M. Essex.** 1992. The matrix protein of human immunodeficiency virus type 1 is required for incorporation of viral envelope protein into mature virions. J Virol **66:**4966-71.

400. **Yuan, B., S. Campbell, E. Bacharach, A. Rein, and S. P. Goff.** 2000. Infectivity of Moloney murine leukemia virus defective in late assembly events is restored by late assembly domains of other retroviruses. J Virol **74:**7250-60.

401. **Yuan, X., X. Yu, T. H. Lee, and M. Essex.** 1993. Mutations in the N-terminal region of human immunodeficiency virus type 1 matrix protein block intracellular transport of the Gag precursor. J Virol **67:**6387-94.

402. **Yuste, E., J. D. Reeves, R. W. Doms, and R. C. Desrosiers.** 2004. Modulation of Env content in virions of simian immunodeficiency virus: correlation with cell surface expression and virion infectivity. J Virol **78:**6775-85.

403. **Zaitseva, L., P. Cherepanov, L. Leyens, S. J. Wilson, J. Rasaiyaah, and A. Fassati.** 2009. HIV-1 exploits importin 7 to maximize nuclear import of its DNA genome. Retrovirology **6:**11.

404. **Zapp, M. L., and M. R. Green.** 1989. Sequence-specific RNA binding by the HIV-1 Rev protein. Nature **342:**714-6.

405. **Zemel, A., A. Ben-Shaul, and S. May.** 2008. Modulation of the spontaneous curvature and bending rigidity of lipid membranes by interfacially adsorbed amphipathic peptides. J Phys Chem B **112:**6988-96.

406. **Zennou, V., D. Perez-Caballero, H. Gottlinger, and P. D. Bieniasz.** 2004. APOBEC3G incorporation into human immunodeficiency virus type 1 particles. J Virol **78:**12058-61.

407. **Zhou, W., L. J. Parent, J. W. Wills, and M. D. Resh.** 1994. Identification of a membrane-binding domain within the amino-terminal region of human immunodeficiency virus type 1 Gag protein which interacts with acidic phospholipids. J Virol **68:**2556-69.

408. **Zhou, W., and M. D. Resh.** 1996. Differential membrane binding of the human immunodeficiency virus type 1 matrix protein. J Virol **70:**8540-8.

409. **Zingler, K., and D. R. Littman.** 1993. Truncation of the cytoplasmic domain of the simian immunodeficiency virus envelope glycoprotein increases env incorporation into particles and fusogenicity and infectivity. J Virol **67:**2824-31.