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THE RELATIONSHIP BETWEEN T CELL SIGNALS AND HIV-1: THE POSITIVES AND THE NEGATIVES

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by

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ABSTRACT

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CD4+ T cell activation is a prerequisite for productive HIV-1 infection and replication. Therefore, the virus has evolved mechanisms to exploit host cellular factors to promote its own propagation. Relatively little is known regarding the interplay between proteins involved in T cell activation and HIV-1. The purpose of this work was to investigate the relationship between signals in the TcR/CD3/CD28 pathway and HIV-1 replication.

T cell stimulation through TcR/CD3 induces low level HIV-1 transcription. However, maximal HIV-1 expression requires the simultaneous ligation of a costimulatory receptor, such as CD28. Although the significance of transcription factors NF-κB, NF-AT, Sp1, and AP-1 in T cell activation-induced HIV-1 gene expression is well established, little is known regarding the specific *cis*-acting elements and the HIV long terminal repeat (LTR) region mediating the CD28 related enhancement of HIV-1 transcription. Using deletional analysis of the HIV-LTR in conjunction with electromobility shift assay (EMSA), we determined that the enhanced HIV-1 transcription in response to CD28 ligation was mediated through the binding of a specific protein to a potential CD28 response element (HIV-28RE) in the region spanning-182 to -205 of the HIV-LTR. This finding illustrates the positive regulation of HIV-1 expression by molecules in the T cell signaling pathway.

The tight link between HIV-1 transcription and T cell activation and its dependence on host transcription factors suggests that provirus activity would be silenced

as an infected cell transitioned from an activated to a resting state, such as during the process of anergy or memory induction. This is thought to be the underlying mechanism of HIV-1 postintegration latency. The population of resting memory CD4+ T cells harboring latent virus represents the primary barrier against eradication of the virus in HAART patients. The cellular mediators of the establishment of this reservoir are largely unknown due to the difficulty in the isolation of latent cells *in vivo* and the lack of available representative *in vitro* models. We have developed a novel *in vitro* system to identify the molecular events involved in silencing the HIV-1 provirus. This model utilizes a virus engineered to express placental alkaline phosphatase (PLAP) on its surface upon virus transcription in conjunction with the removal of this marker by Phospholipase C treatment. Measurement of PLAP re-expression is used as an indicator of the HIV-1 transcriptional response to various signal manipulations. Using this experimental approach, we demonstrated that suboptimal T cell signaling that activates the calcium pathway induces a reversible, long-term suppression of HIV-1 transcription that may represent a latent state. These findings indicate that signals in the TcR/CD28 pathway can negatively regulate HIV-1 gene expression.

The role of Lck, an early and integral member of the T cell activation pathway, was assessed via the infection of Lck deficient T cell lines. HIV-1 replication was consistently attenuated in the absence of Lck, suggesting that Lck was positively regulating HIV-1 expression. However, a series of experiments determined that Lck was not influencing HIV-1 transcription or the early stages of the virus life-cycle, but instead was mediating the later stages of the virus life cycle, namely HIV-1 assembly. HIV-1 particles accumulated in the intracellular compartments of cells lacking Lck, suggesting

that Lck was facilitating the progression of HIV-1 from the microvesicles to the plasma membrane for efficient virus release. This novel function of Lck did not involve its kinase activity but was mediated through a physical interaction between Lck and Gag.

These findings indicate that T cell signaling proteins can both positively and negatively regulate HIV-1 transcription, and can play important unforeseen roles in other stages of the virus life cycle, such as HIV-1 assembly. Insight regarding the interplay between T cell signals and HIV-1 will lead to a better understanding of how the virus exploits host proteins to promote its replication and will ultimately provide targets for future therapeutic intervention.

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LIST OF ABBREVIATIONS

Ab……...Antibody

AICD……...Activation induced cell death

AIDS……...Acquired Immunodeficiency Syndrome

AIP1……Atrophin 1 interacting protein 1

AKT…….”Ak” transforming

AP……...Activator protein

ATP……..Adenosine triphosphate

BAF…….Barrier to autointegration factor

BSA……...Bovine serum albumin

CIITA……..Class II transactivator

CA……...Capsid

CCR5…….CC chemokine receptor 5

cAMP……..cyclic AMP

Cbl……Casitas B-lineage lymphoma

CD……..Cluster of differentiation

CD28RE……...CD28 response element

Cdc42……..Cell division cycle 42

cDNA……..Complementary DNA

C/EBP β……...CCAAT/enhancer binding protein β

CHO……..Chinese hamster ovary

CpG……Cytodine and guanine separated by a phosphate

CREB…….cAMP response element-binding transcription factor

c-Rel……...Cellular reticuloendotheliosis oncogene

CRM-1…….Chromosome maintenance region 1

Csk…….....C-terminal src kinase

CTD……...Carboxyterminal domain

CTF/NF……..CCAAT-box binding transcription factor/nuclear factor 1

CTLA-4……...Cytotoxic T lymphocyte associated granule serine protease 4

CXCR4……...CXC chemokine receptor 4

c-Yes…….. Cellular Yamaguchi sarcoma oncogene

DGK……Diacylglycerol kinase

DMEM……..Dulbecco's modified Eagle's medium

DMSO……..Dimethylsulfoxide

DTT……..Dithiothreitol

EDTA……..Ethylenediaminetetraacetic acid

EGTA……..Ethyleneglycolbis(2-aminoethylether)N,N,N'N'-tetraacetic acid

ELISA……Enzyme-linked immunosorbent assay

EMSA…….Electromobility shift assay

ER…….Endoplasmic reticulum

ERK-1…….Extracellular response kinase

ESCRT……...Endosomal sorting complex required for transport

FCS……...Fetal calf serum

FITC……Fluorescein isothiocyanate

Fyn……..Feline yes-related novel gene

GATA3……GATA transcription factor 3

GCN5……General control nonderepressible

GFP……...Green fluorescent protein

gp……..Glycoprotein

GPI……..Glycosylphosphatidylinositol

GSK……...Glycogen synthase kinase 3

GRAIL……Gene related to anergy in lymphocytes

Grap2…….....GRB2 related adapter protein

GTP……..Guanine triphosphate

HAART……Highly active antiretroviral therapy

HAT……...Histone acetyltransferase

Hck……. .....Hemopoietic cell kinase

HDAC……Histone deacetylase

HEPES……...4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid

HIV-1……Human Immunodeficiency Virus type 1

HIV-LTR……Human Immunodeficiency Virus-Long terminal repeat

HMG-I(Y)……High mobility group protein- I(Y)

HP68……..Host protein 68

Hsp90……..Heat shock protein 90

HTLV-1……..Human T-lymphotropic virus

ICER……...Inducible cAMP early repressor

ICOS……..Inducible costimulator

IL……..Interleukin

IκB……...Inhibitory κB

IN……Integrase

INI1……..Integrase interactor 1 protein

IRES……..Internal ribosomal entry site

IRF……..Interferon regulatory factor

IST……..Inducer of short transcripts

ITAM…….....Immunoreceptor tyrosine-based activation motif

Itk……...IL-2 inducible T cell kinase

J-Lat…… Jurkat- latent

JNK…….Jun N-terminal kinase

LAT……...Linker for activation of T cells

Lck……..Lymphokine-specific protein tyrosine kinase

LEDGF (PSIP1)…… .....PC4 and SFRS1 interacting protein 1

LEF1……...Lymphoid enhancer-binding factor 1

LFA-1……Leukocyte function associated antigen 1

Luc……Luciferase

MA……..Matrix

MAPK……...Mitogen activated protein kinase

MEK1……..Mitogen activated ERK-activating kinase

MEKK……MEK kinase

MFI…….Mean fluorescent intensity

MHCII…….Major histocompatibility complex II

MHR……....Major homology region

mRNA…….Messenger RNA

MVB……..Multivesicular body

NBT…….Nitroblue tetrazolium

NC……Nucleocapsid

NF-AT……Nuclear factor of activated T cells

NF-κB……...Nuclear factor κB

NF-MATp35……...Nuclear factor of mitogenic activated T cells

NP-40……....Nonidet P-40

NRE……..Negative regulatory element

Nuc0……....Nucleosome 0

Nuc1……Nucleosome 1

P300/CBP…….p300/CREB-binding protein

PBS……...Phosphate-buffered saline

PCAF…….p300/CREB-binding protein (CBP)-associated factor

PCR……..Polymerase chain reaction

PD-1……...Programmed cell death protein 1

PHA……...Phytohemagglutinin

PMSF……...Phenyl-methylsulfonyl fluoride

PI3K……...Phosphatidylinositol-3-kinase

PIC……....Preintegration complex

PI(4,5)P2……Phosphatidylinositol (4,5) biphosphate

PKC…….Protein kinase C

PKR……Protein kinase R

PLAP…….Placental alkaline phosphatase

PLC…….Phospholipase C

PMA……..Phorbol myristate acetate

POSH…….Plenty of SH3s

PP1……...Protein phosphatase inhibitor 1

PR…….Protease

PTEFb…… Transcriptional elongation factor b

RBF-2…….Retinoblastoma family protein 2

RE…….Response element

RNA……Ribonucleic acid

RPMI…….Roswell Park Memorial Institute

RSV……Rous sarcoma virus

RT……Reverse transcriptase

RTC……...Reverse transcriptase complex

RT-PCR……Reverse transcriptase polymerase chain reaction

Scid-hu……Severe combined immunodeficiency-human

SDS……..Sodium dodecyl sulfate

SDS-PAGE……..Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SH2……...Src-homology 2

SH3……...Src-homology 3

SLP-76……SH2 domain-containing leukocyte protein

STAT5……Signal transducer and activator of transcription

SU……..Surface

TAFII250……TBP-associated factor 250

TAR……...Transactivating region

TBP……TATA binding protein

TCF11 (NFE2L1)……...Nuclear factor (erythroid-derived 2)-like 1

TcR……T cell receptor

TFIID (TAF1)…….....TBP associated factor

TGN……...Trans golgi network

Thy-1……..Thymus cell antigen 1

TM……Transmembrane

TNFα……....……...Tumor necrosis factor α

Tob…….Transducer of ERBB2

TsA…….Trichostatin A

Tsg101……..Tumor Susceptibility gene 101

UBP-2……..Ubiquitin specific protease-2

UD…….Unique domain

USF/TFE-3……..Upstream stimulator factor/transcription factor E -3

UTR……Untranslated region

VLP……...Virus-like particle

Vps28……Vacuolar protein sorting 28

VSV-G……Vesicular stomatitus virus glycoprotein

YY1……...Yin and yang 1

ZAP70……...ζ-associated protein 70

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Chapter 1

Literature Review

Human Immunodeficiency Virus type 1 (HIV-1) was identified in 1984 as the etiologic agent of Acquired Immunodeficiency Syndrome (AIDS). In 25 years, over 60 million people worldwide have been infected with HIV-1, of which 20 million have died (UNAIDS/WHO), making it the most devastating disease that humankind has ever faced. Despite substantial advancements in the treatment of AIDS, 14,000 individuals are still being infected on a daily basis worldwide (UNAIDS/WHO). In fact, AIDS is predicted to become the world's most burdensome disease by 2030, largely due to a lack of preventative efforts by many affected countries (Hopkin 2006). Thus, the enormous global health burden imposed by this disease necessitates the implementation of strategies aimed specifically at the prevention of HIV-1 infection. Unfortunately, no effective HIV-1 vaccines have been developed thus far. Therefore, because HIV-1 is not a self-sufficient virus and is known to be highly dependent on host cell factors for its replication and survival, research is beginning to focus on targeting the biological mechanisms that promote HIV-1 infection and replication rather than the virus itself. The $CD4^+$ T cell is a primary target of HIV-1 and the dependence of virus expression on T cell activation and signaling is well established. However, few of the specific components of the T cell signaling pathway that influence HIV-1 replication have been elucidated. Thus, this review will focus on the identification of cellular proteins in the T

cell activation pathway that regulate the HIV-1 life-cycle and discuss the potential mechanisms involved.

1.1 $CD4^+$ T Cells are Important for HIV-1 Infection and the Progression to AIDS

The $CD4^+$ T cell is a primary target of HIV-1 and as such, plays a central role in HIV-1 infection and disease progression to AIDS. The susceptibility of a cell to HIV-1 infection is determined by the expression of the primary virus receptor, CD4, as well as the chemokine receptors CCR5 and CXCR4 on the cell surface. As exposure to HIV-1 occurs predominantly at the mucosal surface, the mucosal $CD4^+$ $CCR5^+$ memory T cells are the principal targets for and source of HIV-1 infection and replication at the onset of infection. Infection results in a profound depletion of this T cell subset and its peripheral blood counterpart, which culminates in an overall decline in the $CD4^+$ T cell population throughout the first few weeks of infection. Despite the partial recovery of this population following the initial phase of infection, chronic HIV-1 infection is characterized by a heightened state of virally-induced immune activation and dysfunction, leading to a slow, progressive decay of the $CD4^+$ T cell compartment (Douek 03). During this time, activated $CD4^+$ T cells are the primary source of virus replication in the peripheral blood (Schnittman 89), and remain so throughout the course of infection (Stevenson 03). Eventually, peripheral blood $CD4^+$ T cells reach a critically low level of 200 cells/mm^3 of blood, which marks the onset of full-blown AIDS and often the emergence of opportunistic infections (Douek 03).

A small population of resting, memory $CD4^+$ T cells harboring latent HIV-1 provirus represents the primary barrier against the eradication of HIV-1 in patients

undergoing HAART therapy. Thus, due to its pivotal role in infection and disease progression, understanding the interplay between HIV-1 and the $CD4^+$ T cell is imperative to controlling virus replication and the incidence of AIDS.

1.2 HIV-1 replication is contingent on T cell activation

HIV-1 infection and replication are linked to the metabolic and activation state of the target cell (Stevenson 90). For example, HIV-1 infection of activated T cells is productive and efficient, as illustrated by reports of increased HIV-1 replication in T cell lines following stimulation with known T cell activators, including phytohemagglutinin (PHA) and phorbol ester (PMA) (Zagury 86, Harada 86) In contrast, resting T cells are refractory to HIV-1 infection. IL-2 stimulation can overcome this block in infection (Unutmaz 99) and facilitate the entry of a temporally labile post-fusion HIV-1 complex into naïve T cells (Woods 97). However, additional activation signals are required in order for reverse transcription and integration to occur (Unutmaz 01). HIV-1 has evolved to counteract this barrier in that the viral proteins Nef and Tat are transcribed prior to integration and are able to induce T cell activation and increase HIV-1 replication (Wu 01). Nef has also been shown to interact with host cellular proteins that regulate signal transduction, such as Src family kinases, in order to promote a cellular environment that is conducive to virus replication (Greenway 03, Renkema 00). Similarly, the viral envelope protein, gp120, can activate signaling and expedite productive infection of suboptimally activated T cells via induction of NF-AT, TFIID, and plasma membrane localization of syntaxin and Cdc42 (Cicala 02, Kinter 03), as well as activation of AP-1

and NF-κB via Lck, Raf1, MEK1 and ERK-1 (Briant 98). These data underscore the significance of T cell activation for HIV-1 infection and replication.

1.3 The HIV-1 Life Cycle

HIV-1 initially enters the cell through a receptor-specific, fusion-dependent mechanism. The engagement of the viral envelope protein (Env) subunit gp120 (SU) to its receptor, CD4, induces a conformational change in Env that allows it to bind chemokine coreceptors, in particular CCR5 or CXCR4, and exposes the gp41 (TM) Env subunit. The gp41 protein mediates fusion of the viral and cellular membranes, facilitating entry of the encapsulated duplicate single-stranded, positive sense RNA viral genome. Once in the cytoplasm, the viral capsid uncoats, releasing the viral RNA, which forms a reverse transcription complex (RTC) with the virally encoded reverse transcriptase (RT) and integrase (IN) enzymes, and the viral matrix (MA) and Vpr proteins (Bukrinsky 04). The HIV-1 RNA genome is converted to cDNA via RT, which enters the nucleus in the linear form as a component of a preintegration complex (PIC), along with MA, IN, Vpr, host importins, and the SWI/SNF component INI1 (Turelli 01). The PIC furnishes HIV-1 with the unique ability to import its genomic material into the nucleus in the absence of nuclear breakdown, allowing for infection of non-dividing cells. The viral cDNA then integrates preferentially into active euchromatin and is recognized as a provirus (Schroeder 02). Transcription of the HIV-1 provirus is executed by the viral protein Tat in conjunction with a number of host cellular factors. The viral protein Rev transports singly and unspliced viral mRNA from the nucleus into the cytoplasm for subsequent packaging and release via budding from the lipid rafts in the plasma membrane. After

exiting the cell, the HIV-1 structural protein Gag undergoes cleavage by the viral protease (PR) enzyme, altering the configuration of the virion and inducing maturation into an infectious particle (Fig. 1.1, 1.2)

. Fig. **1-1**

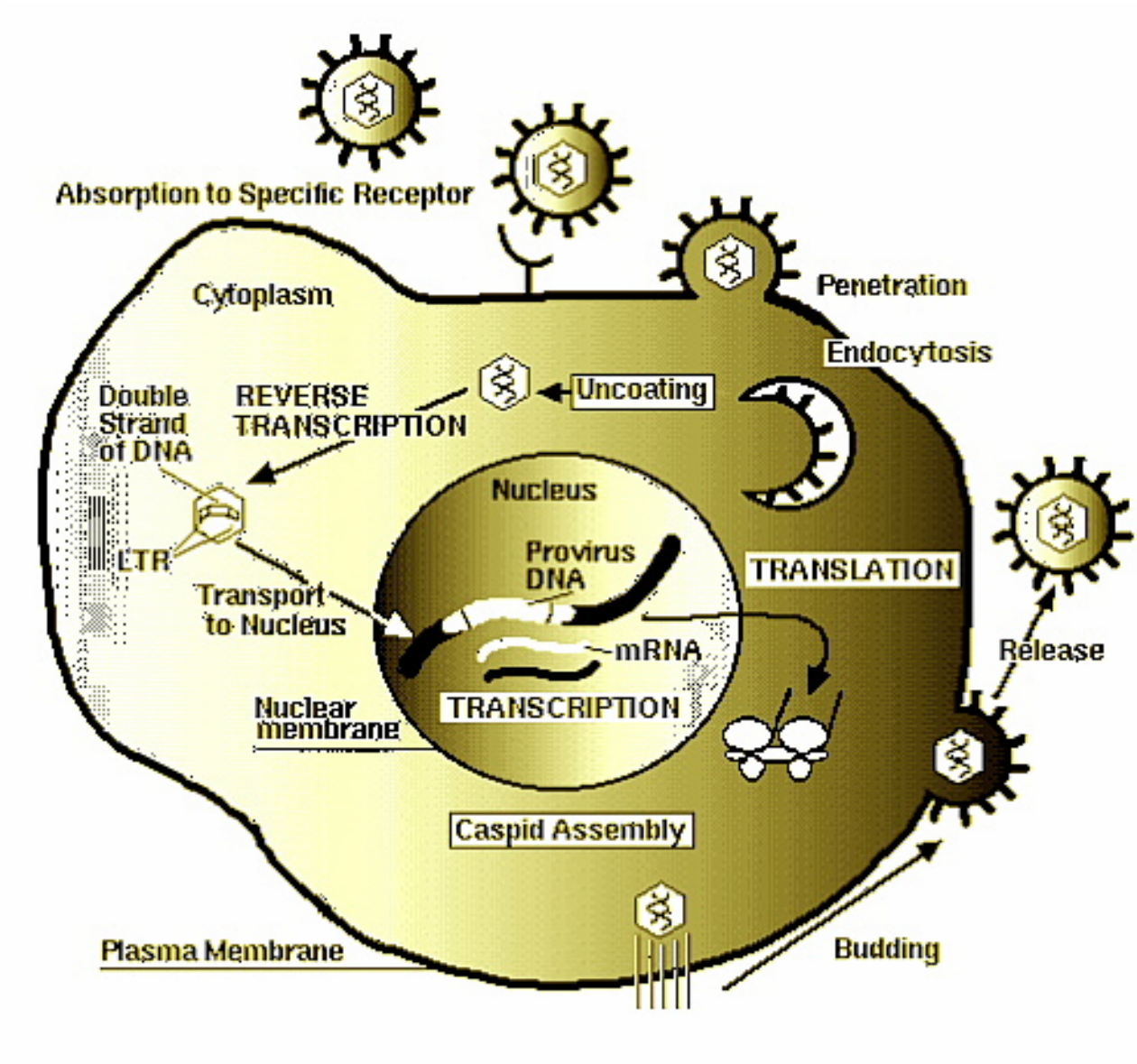


Figure **1-1**: Schematic of the HIV-1 Life Cycle

Source: http://www.rhodes.edu/biology/glindquester/viruses/pagespass/hiv/retrovirus.jpg

Fig. 1-2

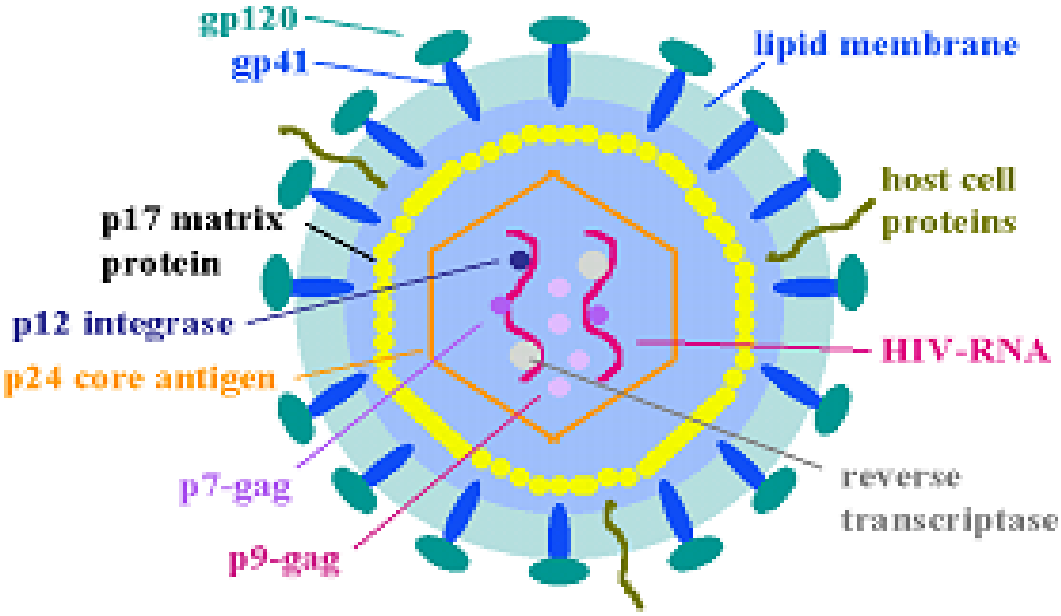


Figure **1-2**: Structure of HIV-1

1.4 T cell activation regulates HIV-1 gene expression

Proviral transcription is the aspect of HIV-1 replication that has been most extensively studied regarding the role of inducible host proteins. Integrated HIV-1 provirus remains unexpressed in quiescent $CD4^+$ T cells (Seshamma 92, Wong 97, Finzi 97, Adams 94, Chun 97). HIV-1 gene expression is dependent on the interaction of host

cell transcription factors with *cis*-acting elements on the viral promoter, the HIV-1 long terminal repeat (HIV-LTR) (Al Harthi 98). The composition of the HIV-LTR closely resembles that of the IL-2 promoter in that it contains many of the same regulatory elements, such as NF-κB, NF-AT, and AP-1 binding sites (Fig. 1.3), thus it is responsive to mitogenic signals similar to those inducing T cell activation (Siekevitz 87). Specifically, the HIV-LTR is comprised of 3 regions: a U3 region consisting of a core promoter (nt -78 to -1), core enhancer (nt -105 to -79), and a modulatory region (nt -454 to -104) which encompasses a negative regulatory element (NRE); an R region containing the transcriptional start site and the transactivation response (TAR) element (nt +1 to +60); and a U5 region (Pereira 00) (Fig. 1.4). The basal promoter region consists of the initiator (Inr), the TATA box, and three Sp1 motifs. Transcription factors such as NF-κB and NF-AT, and TCF-1a/LEF-1 and Ets1 associate with the proximal and distal enhancer region, respectively. The modulatory element contains diverse binding sequences including NF-AT1, USF/TFE-3, C/EBPβ, cyclic AMP (cAMP) response element-binding protein (CREB), and nuclear hormone receptors (Garcia 94, Kingsman 96, Pereira 00, Van Lint 04). Although it has received relatively little attention, the 5' untranslated region (5'-UTR) also contains the inducer of short transcripts (IST) and target sequences for crucial transcription factors such as LSF/YY1, UBP-2, CTF/NF1, AP-1, NF-κB, NF-AT, IRF, and Sp1 and is a proposed downstream enhancer element. Importantly, a nucleosome that is displaced upon T cell activation is also located in this region (Fig. 1.5) (Al-Harthi 98, Van Lint 04).

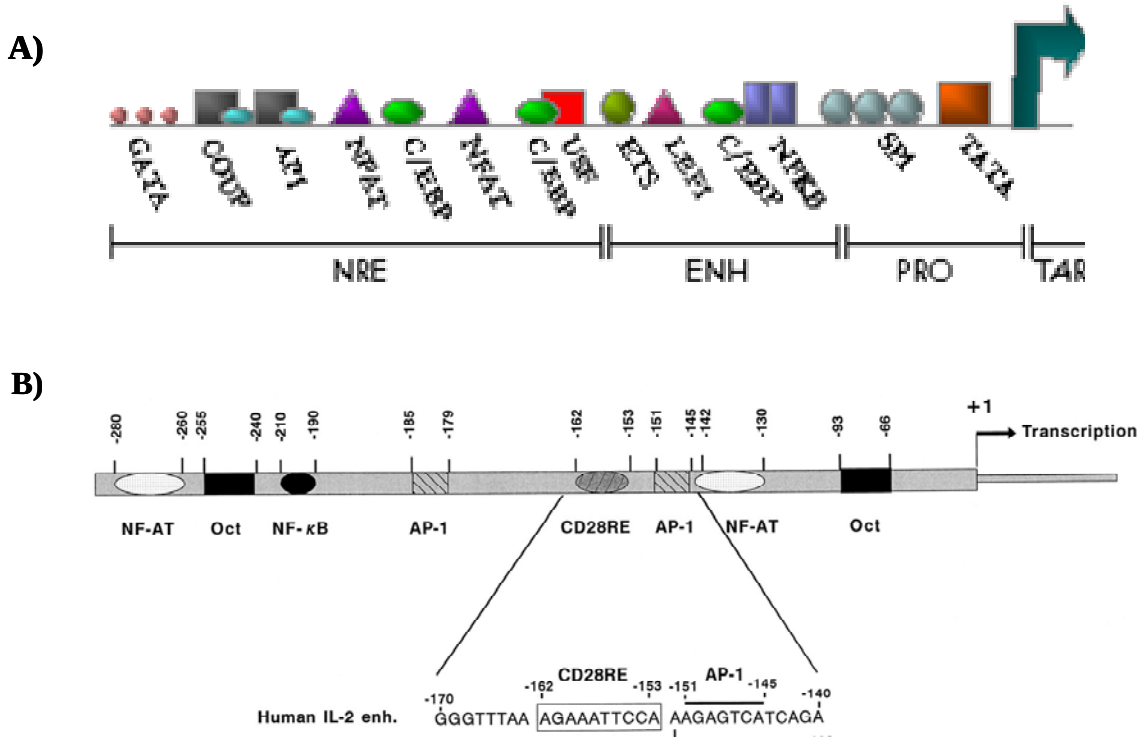
Seminal research demonstrated a robust increase of HIV-1 gene expression following T cell stimulation with PMA and PHA that was mediated by the enhancer element of the HIV-LTR (Tong-Starksen 87, Siekevitz 87). In addition, these investigations discovered that the combination of mitogens and the viral transactivation protein Tat synergized to induce HIV-1 transcription, suggesting that mitogenic stimulation mediated HIV-1 expression at the transcriptional level and that Tat was likely to exert its effect via a post-transcriptional mechanism (Tong-Starksen 87, Siekevitz 87). It is now known that provirus transcription is poorly processive and usually terminates prematurely in the absence of Tat. Tat binds to a specialized RNA stem-loop structure in the TAR element and principally functions to promote elongation via the recruitment of the P-TEFb complex and the subsequent phosphorylation of the carboxyterminal domain (CTD) of RNA polymerase II. Furthermore, NF-κB is induced upon T cell activation and cooperates with Tat to promote HIV-1 expression. In fact, NF-κB can recruit pTEFb and promote HIV-1 transcription in the absence of Tat (Barboric 01). More recent studies indicate that both Tat function and overall HIV-1 transcription are dependent on the NF-κB binding sites in the HIV-LTR, suggesting that NF-κB is required for enhanced HIV-1 expression following T cell activation (Alcami 95, Nabel 87). However, HIV-1 transcription is independent of NF-κB in cells that exhibit low basal nuclear NF-κB or high spontaneous LTR activity, suggesting that NF-κB activation is sufficient but not necessary for optimal virus expression and growth under certain circumstances (Alcami 95, Chen 97). Similarly, HIV isolates containing mutations in the NF-κB binding site replicate efficiently, suggesting that NF-κB is dispensible for HIV-1 replication in

activated cells (Leonard 89, Ross 91). Alternatively, it is possible that this modification induces changes that enhance virus replication. It is interesting to note that the p50 subunit of NF-κB is present on the HIV-LTR in resting cells, but that PMA stimulation is required for the induction of the p50-p65 heterodimers that are associated with increased HIV-1 transcription in T cells (Alcami 95). Based on the known significance of NF-κB activation in HIV-1 transcription, this finding may represent one potential mechanism to explain the relationship between T cell activation and HIV-1 gene expression.

Although NF-κB has traditionally been considered as the most critical factor for HIV-1 transcription in T cells, much of this evidence was derived from studies in which the tandem NF-κB motifs in the HIV-LTR had been mutated. It has since been demonstrated that, like NF-κB, NF-ATc, an inducible Rel-family transcription factor that is vital for T cell activation, can bind these sequences and synergize with Tat to induce HIV-1 gene expression and replication in response to T cell signaling (Kinoshita 97, Pessler 04, Cron 00, Fortin 01, Robichaud 02, Barbeau 01, Cron 01). Further support for the role of NF-AT in HIV-1 transcription is provided by the observation that cyclosporin A, a calcineurin-dependent NF-AT inhibitor, ablated the observed enhancement in HIV-1 LTR activity (Markovitz 92, Siekevitz 87). It should be noted that cyclosporin A potentially inhibits NF-κB as well as NF-AT, thus these findings may be at least partially reflective of an NF-κB effect. Prior to this discovery, considerable interest surrounded the role of NF-AT in HIV-1 transcription based on the significance of NF-AT in T cell activation coupled with the presence of a putative NF-AT binding motif in the negative regulatory element (NRE) of the HIV-1 LTR. However, this NFAT binding site was

determined to either be dispensable for (Markovitz 92) or negatively regulate HIV-1 gene expression (Macian 99, Lu 90). Together, these data imply that the differential effects of NF-AT1 activation on the HIV-LTR vary with the location of the target binding sequence in the HIV-LTR and its interaction with neighboring factors. For example, physical interactions between Ets and either NF-κB or NF-AT proteins mediate the inducible expression of T cell specific genes and viruses, including HIV-1 and HIV-2 (Bassuk 97).

Fig. 1-3



Civil, A. et al. J. Biol. Chem. 1996;271:8321-8327

Figure **1-3**: Comparison between HIV-1 and IL-2 promotors. A. Schematic of HIV-LTR. B. Schematic of IL-2 promotor and CD28 response element

Fig. 1-4

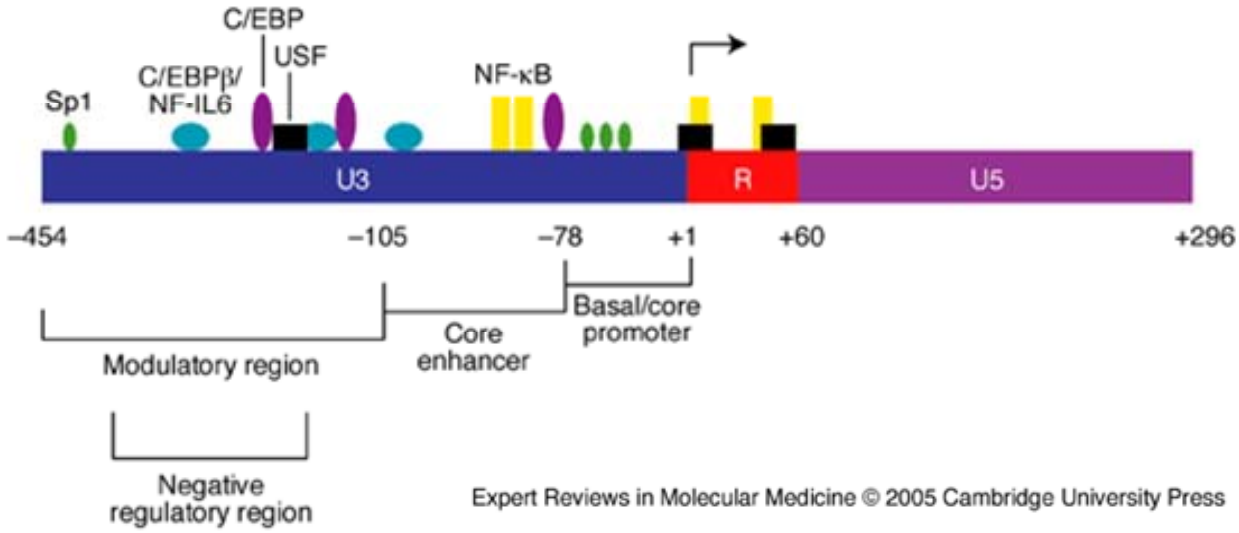


Figure **1-4**: Composition of the HIV-1 LTR. The HIV-1 LTR contains 3 regions- U3, R, and U5, and 4 functional elements- the modulatory region, negative regulatory region, core enhancer, and basal/core promoter.

Despite the vital role of transcription factors in the induction of HIV-1 gene expression in response to T cell activation, their presence alone is not sufficient for this response to occur. As a lentivirus, HIV-1 must integrate into the host genome prior to transcriptional initiation. Although the specific site of HIV-1 integration is random, research indicates that HIV-1 integration occurs most often in areas of active euchromatin (Schroder 02). However, conflicting results were reported using a clonally selected cell population (Jordan 03). Independent of the integration site, two nucleosomes, nuc 1 and nuc 0, are precisely positioned with respect to regulatory sequences within the 5' HIV-LTR (Fig

1.5). The arrangement of these nucleosomes defines two large nucleosome free regions, one containing the core promoter and enhancer regions and the other the primer binding site. The majority of *cis*-acting regulatory sequences in these nucleosome free sections are occupied under basal conditions, precluding binding site accessibility as a limiting factor in HIV-1 transcriptional activation. The two nucleosome free areas are separated by nuc1, which is positioned immediately downstream of the transcriptional start site and is rapidly remodeled upon T cell activation. The location of nuc1 relative to the transcriptional start site and its rapid destabilization in response to T cell signals implicates chromatin structure as an additional mechanism regulating HIV-1 expression. Furthermore, it suggests that the presence of nuc1 maintains the provirus in a latent state and that its repositioning is imperative for transcriptional activation (Lusic 03, Mahmoudi 06, Sadowski 05).

Nuc1 remodeling is accomplished through two primary mechanisms. First, the N-terminal tails of histone proteins can be post-translationally modified via acetylation, phophorylation, or methylation, inducing deterioration of the histone-DNA contacts and promoting transcription. An additional means of modifying chromatin structure is through a physical alteration, disruption, or repositioning of the nucleosome, which interrupts histone domain-DNA contacts and subsequently increases DNA accessibility. This process is initiated by ATP-dependent protein complexes, such as SWI/SNF. The T cell signaling-induced nuc1 remodeling that accompanies HIV-1 transcriptional activation is thought to be mediated by the viral protein Tat. Tat is known to interact with basal transcription factors including TBP, TAFII250, and RNA Polymerase II, and to recruit cellular kinases such as pTEFb. In addition, Tat promotes the assembly of

histone acetyltranferase (HAT) complexes that include p300/CBP, PCAF, and GCN5 on the HIV-LTR, which acetylate histone tails, as well as transcription factors and Tat itself, facilitating nuc1 remodeling and ultimately HIV-1 transcription (Mahmoudi 06, Lusic 03, Deng 00, Quivy 02, Van Lint 96). Tat also recruits SWI/SNF complex, which synergizes with p300 and is critical for Tat-mediated HIV-1 transcriptional activation (Mahmoudi 06). Nucleosome remodeling can also be Tat-independent. For example, the concurrent binding of transcriptional activators Sp1, NF-κB, LEF-1, and USF cooperatively induced the release of N-terminal histone tails from nucleosomal DNA (Angelov 00). Similarly, the binding of RBF-2 to the conserved RBEIII element of the HIV-LTR in response to TCR induced Ras and MAPK signaling was suggested to promote chromatin reorganization and activate HIV-1 transcription. This response was dependent on the presence of the cofactor TFII-I at the HIV-LTR (Sadowski 05). Therefore, the T cell-dependent activation of HIV-1 transcription is regulated by the recruitment of factors that influence transcriptional initiation and elongation, as well as chromatin reorganization. To summarize, T cell signals induce HIV-1 transcription via the binding of transcriptional activators, such as NF-κB p65, to *cis*-acting sequences in the HIV-LTR enhancer. These transcriptional activators, such as NF-κB1, LEF1, and USF, along with the constitutive factor Sp1, can disrupt the interaction between N-terminal histone tails and result in nucleosome remodeling. Cellular activation also promotes chromatin reorganization and nuc1 remodeling via Tat mediated recruitment of and interaction with HAT complexes and SWI/SNF. It is likely that the acetylation of transcription factors associated with the LTR by HAT complexes, such as p300/CREB, can induce chromatin reorganization as well. Finally, Tat recruits the pTEFb complex to the HIV-LTR, which

modifies the CTD of RNA Polymerase II, effectively increasing its processivity and promoting transcriptional elongation.

Fig. 1-5

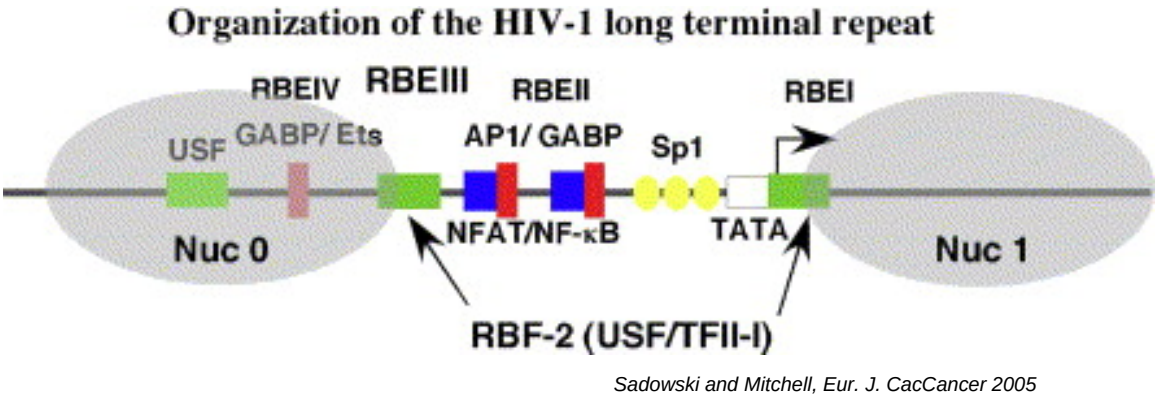


Figure **1-5**: Chromatin organization of the HIV-LTR

1.5 Regulation of T cell activation and HIV-1 gene expression by T cell signaling

As with mitogen stimulation, T cell activation via the TcR complex is an effective stimulus for HIV-1 transcription. Antigen presenting cells activate T cells through ligation of the TCR/CD3 receptor complex with peptide-presenting MHCII. However, engagement through this receptor alone fails to elicit T cell proliferation, differentiation, or IL-2 production, and often induces apoptosis or a reversible unresponsive state of the T cell known as anergy (Jenkins 87). Optimal T cell activation by an antigen presenting cell requires a second, costimulatory signal, in addition to that received through the TCR/CD3 complex (Lafferty 75). The best characterized costimulatory receptor is CD28, which interacts with ligands, CD80 and CD86 on the antigen presenting cell. Simultaneous stimulation through both the TCR/CD3 complex and CD28 initiates a

signaling cascade which culminates in the binding of regulatory factors to *cis*-acting elements on the IL-2 promoter which induce IL-2 transcription and production (Fig. 1.3).

The mechanism of integration of CD28 costimulatory signals into the TcR/CD3 signaling pathway remains unclear. CD28 stimulation both qualitatively and quantitatively sustains, amplifies, and modifies the TCR/CD3 signaling pathway, resulting in a lowering of the threshold necessary for T cell activation. More specifically, CD28 has been said to initiate qualitative signals, independent of those originating from TcR/CD3, such as the inhibition of Rap1 activation, which results in an enhancement of TcR-induced ERK activation (Carey 00, Kim 06), and AKT mediated activation of the CD28 response element (Kane 01). The activation of phosphatidylinositol 3-kinase (PI-3K) has also been exclusively attributed to CD28 signaling (Clavreul 00, Rudd 03), but contradictory reports have suggested that the role of PI-3K in CD28 signaling remains inconclusive (Alegre 01, Cefai 98). Nevertheless, the majority of research indicates that the contribution of CD28 signaling to the TcR/CD3 pathway is quantitative in nature. In other words, CD28 stimulation serves to duplicate or modify TcR/CD3-induced signals. . The point at which these two pathways converge remains to be elucidated, but it is thought that CD28 signals augment those of CD3 to induce IL-2 expression. The point of CD28 integration into the TcR/CD3 pathway has been suggested to be the autophosphorylation of Lck (Kim 06, Holdorf 02) and its phosphorylation of the TCR ζ chain (Tuosto 98). These data imply that this point of convergence may occur much earlier in the T cell activation pathway than previously thought. As with qualitative signaling, PI-3K has been implicated in mediating costimulatory signaling (Kim 06, Michel 02). PI-3K, a receptor proximal protein, has been shown to amplify the CD3

transcriptional response via prolonging the retention of nuclear NF-AT due to the activation of AKT and subsequent inhibition of GSK3 (Diehn 02). More recently, conflicting findings indicate both PI-3K and the phosphorylation of GSK3 to be unaffected by costimulation (Kim 06). Similarly, a role for ZAP70 in CD28 signaling has been proposed (Tuosto 98) but is currently disputed (Michel 01). A number of studies identify Vav phosphorylation as the integration point for the CD3 and CD28 pathways (Kim 06, Dennehy 07, Michel 02, Tuosto 98), yet differ regarding the proteins specified as facilitating this role of Vav1. One investigation employed a mass spectrometry-based proteomics approach to determine that CD28 potentates CD3 signals through a PLCγ, Shc, Grap 2 pathway (Kim 06). In contrast, superagonistic CD28 antibodies (Ab) were used to demonstrate that assembly of the SLP-76- PLCγ -Vav-Itk signalosome sustains calcium signaling and defines the level at which the CD3 and CD28 pathways converge (Dennehy 07), supporting earlier research findings (Michel 01). Proposed downstream targets of Vav1 phosphorylation include p38 MAP kinase (Kim 06), JNK (Su 94), c-Fos (Li 01, Nandiwada 06), AP-1 (Nandiwada 06, Jung 95), phosphorylated Jun, and NF-κB via phosphorylation of IκB (Zhou 02, Jung 95).

An additional mechanism for the fine tuning of the T cell activation threshold by CD28 is that of Cbl-b degradation (Zhang 02, Li 04) Cbl-b is an E3 ubiquitin ligase that negatively regulates CD28 signaling, therefore its ubiquitination and subsequent down-regulation result in an enhancement of T cell signaling. The identification of a critical CD28 response element (CD28RE) (Fig. 1.3) similar in sequence to the NF-κB consensus motif located at position -174 to -146 in the IL-2 promoter has provided further insight into the regulation of CD28 signaling. This region is positively influenced by known c-

Rel and CREB family members, as well as NF-MATp35 and MEKK (Butscher 98, Civil 96, Tao 02) and negatively regulated by LOK (Tao 02). Lastly, in addition to potentiating TCR/CD3 signals and promoting T cell activation at the transcriptional level, CD28 has been implicated in increasing T cell survival signals via the upregulation of Bcl_{xL} (Chambers 99) and Akt, cytoskeletal rearrangement mediated by Cdc42 and subsequent clustering of lipid rafts (Salazar-Fontana 03, Miceli 01), cell cycle progression via increasing G_1 cell cycle kinases and down-regulating $p27_{kip}$ (Chambers 99), and stable epigenetic alterations of the IL-2 promoter (Thomas 05). Interestingly, CD28 costimulation initiates the induction and binding of the p300/CBP HAT protein complex to both the CD28RE (Butscher 98, 01) and the HIV-LTR enhancer via AP1 activation (Nandiwada 06), and the activity of this complex is associated with increased histone acetylation and chromatin reorganization.

The presence of several common regulatory elements in the IL-2 and HIV-1 promoters indicates that HIV-1 exploits host proteins associated with T cell activation to promote its own transcription. Similar to IL-2 production, maximal HIV-LTR activity and virus replication require stimulation through both CD3 and CD28 (Tong-Starksen 89), despite the ability of CD3 and CD28 signaling alone to activate HIV-1 transcription (Tong-Starksen 89, Smithgall 95, Asjo 93). These findings suggest that factors elicited downstream of CD28 promote HIV-1 transcription. The mechanism by which CD28 enhances CD3-induced HIV-1 transcription is unknown. Research indicates that Vav and Rac1 are upstream mediators of this response (Cook 2003). Similarly, the Tec kinase Itk has been shown to enhance HIV-1 gene expression (Schiralli and Readinger, unpublished). In addition, NF-κB, NFATc, Sp-1 and AP-1 have been implicated as

critical transcription factors for HIV-1 expression in T cells (Nabel 87, Lu 89, Alcami 95, Ross 91, Parrott 91, Al-Harthi 98, Henderson 00, Van Lint 97, Ganesh 03, Li 00, Kinoshita 97, Tong-Starksen 89). However, the particular transcription factors and the region of the HIV-LTR specifically targeted by CD3 + CD28 signaling have not been elucidated. Together, these data demonstrate that T cell activation via signals from the simulataneous ligation of the TCR/CD3 and CD28 receptors positively regulate HIV-1 transcription.

Fig. **1-6**

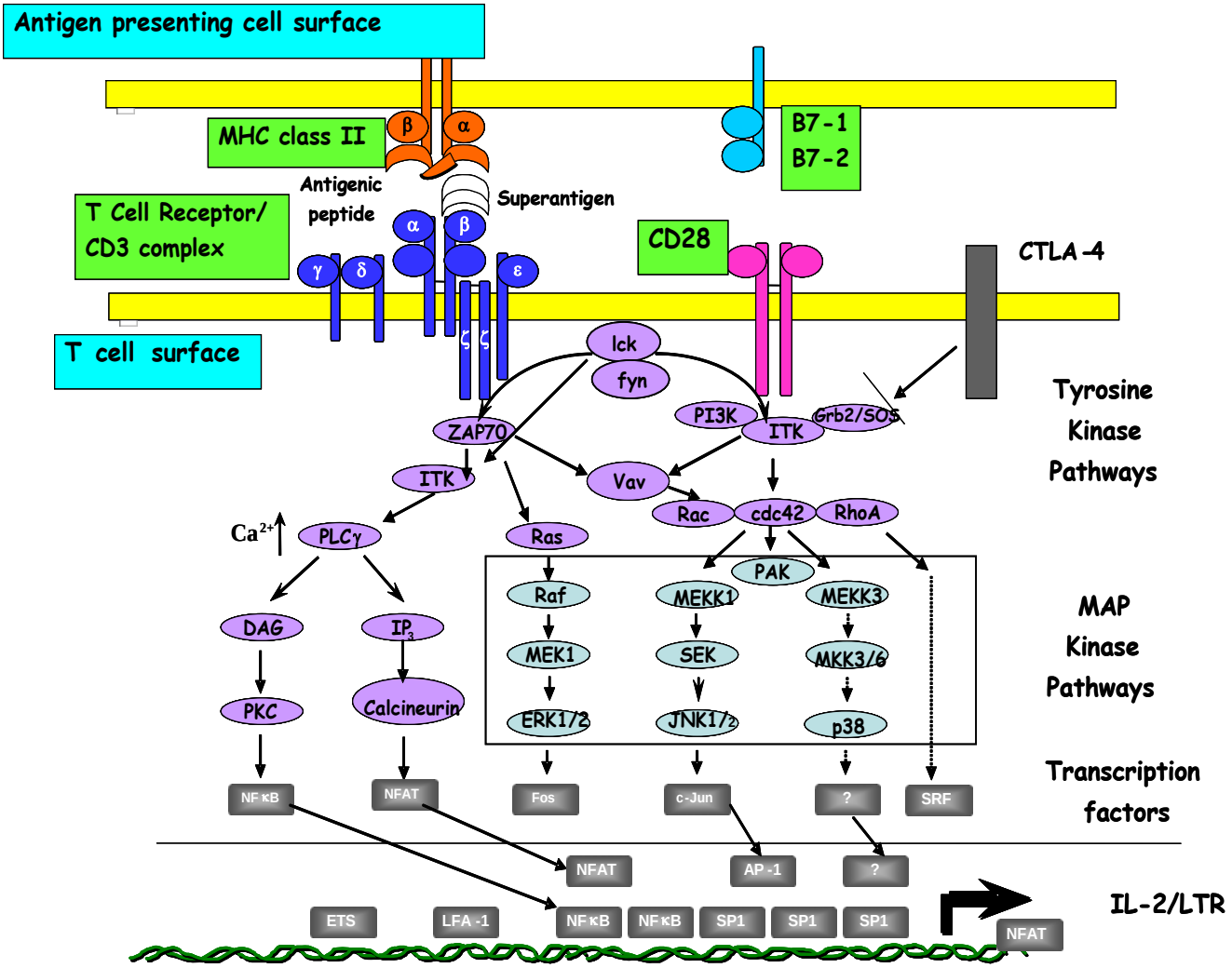


Figure **1-6**: T cell signaling pathway in response to antigen presentation
(courtesy of Dr. A. August, The Pennyslvania State University)

1.6 Signal Quality Determines Cellular Phenotype

Typically, T cell stimulation via antigen presentation results in optimal T cell activation and T cell differentiation, maturation and IL-2 production. However, not all signals are equal, meaning that the cellular response to antigenic stimulation is a function of the strength and duration of the applied stimulus. For example, complete T cell activation

and IL-2 production characteristically require simultaneous engagement of TCR/CD3 and a costimulatory receptor (Lafferty 75), such as CD28 (Civil 99). However, robust signals emanating from the TCR/CD3 complex alone can compensate for deficient costimulation and generate a full T cell response (Teh 97). In contrast, weak overall stimulation or standard stimulation through TCR/CD3 in the absence of costimulation induces either apoptosis, or a state of clonal anergy (Jenkins 87). This is thought to occur in two stages: the first is suboptimal activation, which is followed by a prolonged, unresponsive state of the cell in which it becomes refractory to consequent antigenic stimulation. The unifying mechanism for induction of this type of tolerance is elevated intracellular free calcium. This is presumed to result in an imbalance in NF-AT activation relative to its binding partner, AP-1, and the induction of anergy associated genes, such as Cbl-b, Itch and GRAIL E3 ligases and Tsg101 (Macian 02, Mueller 04, Heissmeyer 04). Similarly, a strong agonist signal elicited by ionomycin, a calcium ionophore, promoted increased nuclear entry of NFATp (NFATC2), which inhibited IL-4 transcription. This was in contrast to the induction of a relatively higher concentration of NFATc (NFATC1) entering the nucleus and subsequent IL-4 production in response to a weak stimulus (Brogdon 02). This type of anergy is predominantly observed in previously activated cells, is maintained by a Ras/MAPK block, and is reversible upon IL-2 or mitogenic stimulation and cellular proliferation (Macian 02, Schwartz 03, Wells 01, Colombetti 02, Huang 03). Additional scenarios involving T cell signaling molecules that have been implicated in the development or maintenance of an anergic state include a palmitoylation associated reduction in the presence of LAT in lipid rafts (Hundt 06), an uncoupling of Lck and CD45 in lipid rafts (Fujimaki 01, Thomas 03), the association of

TCR zeta and Fyn (Boussiotis 96), excess diacylglycerol kinase (DGK) activity and defective Ras signaling (Zha 06), and expression of Tob, an anti-proliferative gene family member (Tzachanis 01). An NF-AT binding partner, inducible cAMP early repressor (ICER) has also been implicated in anergy induction and attenuation of IL-2 gene expression, possibly through uncoupling p300/CBP (Bodor 00). Furthermore, ICER has been shown to inhibit Tax mediated transcription of HTLV-1, and thus, may play a role in viral maintenance and persistence (Newbound 00). These findings suggest that T cell anergy and quiescence are actively maintained states and must be overcome to achieve optimal T cell activation (Tzachanis 01).

A similar process in T cells is that of memory induction. Memory T cells survive in a resting state for extended periods of time following an initial antigen encounter and are capable of mounting a heightened immune response upon subsequent antigen exposure. Although the mechanism is unclear, it is thought that memory is conferred through some type of signal received either during or following the primary immune response. Along these lines, exposure to IL-2 during primary infection has been shown to play a role in promoting the generation of $CD8^+$ T cell memory (Williams 06). Furthermore, the majority of models incorporate a transition from either a fully or suboptimally activated state to a resting state (Champagne 01, Harbertson 02). Memory T cells are thought to have a greater affinity for specific peptide than their naïve counterparts, and one explanation for this is that the quality of the signal received by low versus high affinity cells during primary antigen encounter determines the cells fate of either activation-induced cell death (AICD) or conversion to a memory cell (Valitutti 96). Furthermore, specific or enhanced signals due to increased avidity through interaction

with accessory molecules, namely CD2, LFA-1, CD28, CTLA-4, ICOS, and PD-1, at the synapse has been suggested to drive memory T cell selection (Bachmann 99, Parra 97, Wulfing 98, Coyle 01). Currently, the precise molecular mechanisms mediating the generation of memory are unknown. However, as with anergy induction, the nature of the signal received through the TCR/CD3 and CD28 or an uncharacterized pathway seems to be important in conferring a reversibly prolonged resting state on T cells. The known dependence of HIV-1 replication and transcription on T cell activation and associated host cellular proteins suggests that HIV-1 provirus activation would be silenced along with cellular metabolism during anergy or memory generation, and that each of these processes would be regulated by similar factors. In this regard, signaling molecules within the TCR/CD3 and CD28 activation pathway would have a negative influence on HIV-1 transcription and replication.

1.7 HIV-1 Latency in T cells

Similar to the processes of anergy and memory induction, it is thought that HIV-1 gene expression is extinguished as an actively infected $CD4^+$ cell transitions from the G_1 to G_0 stage of the cell cycle and obtains a memory phenotype (Persaud 03). This quenching of provirus activity is believed to be the result of a replicative block at the transcriptional level (Van Lint 04, Hermankova 03) and suggests that the virus takes advantage of biological processes such as anergy and memory induction and the factors involved in order to promote a latent infection. Unlike other viruses, such as herpesvirus, HIV-1 does not typically exist in a latent state. In fact, less than 1% of the T cell pool contains transcriptionally silent HIV-1 provirus (Finzi 97, Fondere 04, Chun 97,

Hermankova 03). Other cell types, such as macrophages, serve as latent reservoirs, although little is known regarding the significance of this population in HIV-1 infection and pathogenesis. The establishment of the memory T cell latent reservoir occurs very rapidly after infection, as it was not prevented by the administration of antiretroviral treatment 10 days after the onset of primary HIV-1 infection symptoms (Chun 98). The pool of long-lived memory $CD4^+$ T cells harboring a dormant, integrated copy of the HIV-1 genome that can be readily reactivated is the best characterized and most significant of all latent reservoirs (Wong 97, Finzi 97, Chun 97, Fondere 04, Muller 02, Pierson 00). Despite its small size, this cell population presents serious clinical implications to HIV-1 patients undergoing highly active antiretroviral therapy (HAART). The expense and long-term toxicity of the HAART regimen coupled with the prediction that the virus contained in these cells remains stable for up to 60 years (Zhang 99, Mascio 03, Williams 05) renders this population of latently infected cells a major obstacle in the abolition of HIV-1 in HAART patients.

Unfortunately, the biochemical events underlying the establishment of this reservoir have yet to be elucidated. However, as with anergic and memory T cells, the quality of the stimulus seems to be critical for determining the consequent signaling cascade and its ultimate effect on gene expression. For example, T cell activation through TCR/CD3 and CD28 has been shown to either enhance or suppress HIV-1 replication, depending on the strength of the applied signal (Mengozzi 01, Barker 98). Although the mediators of this response were not determined, several lines of evidence suggest that they are similar to those involved in anergy induction and/or memory generation. First, memory T cells comprise the primary reservoir of latent virus in T

cells. Furthermore, HIV-1 gene expression is tightly linked to cellular activation, indicating that comparable signals inhibit both processes.

To date, the primary barrier to the identification of the specific molecular mechanisms driving the generation of HIV-1 postintegration latency is the difficulty of isolating these cells *in vivo*, and the lack of representative *in vitro* models available. The existing models, including cell lines such as U1, ACH-2, and J-Lat, that contain integrated dormant HIV-1 provirus, and the Scid-Hu chimeric mouse infection model, are best suited for the study of the reactivation or maintenance of HIV-1 latency. Research using these systems implicated a myriad of cytokines, including IL-7, IL-10, TNFα, IL-2, and IL-6, in the induction of HIV-1 replication from latently infected cells (Scripture-Adams 02, Rabbi 98, Ghose 01, Zanussi 00). This effect has been shown to be independent of cell cycle progression, at least in the case of TNFα (Tobiume 98). However, the signaling intermediates of this response are unknown. One investigation demonstrated that PMA or cytokine stimulation of HIV-1 replication from latently infected cells could be attributed to a MAPK induced AP-1/NF-κB interaction (Yang 99). Similarly, reactivation of silenced HIV-1 provirus via CpG oligodeoxynucleotides was mediated by activation of NF-κB (Scheller 04). The presence of AP-1 and NF-κB binding sites in both the core enhancer and the 5'-untranslated regions (Al Harthi 98) of the HIV-LTR lends further support to the likelihood that these factors contribute to the reactivation of HIV-1 latency. In addition, recent studies used a SCID-hu (Thy/Liv) latency model to implicate NF-AT, PKC, NF-κB, Lck and prostratin in the reactivation of latent provirus (Brooks 03). The authors suggested that these findings provide evidence

that latency may be maintained by a reduced availability of transcriptional activators in quiescent cells. In support of this hypothesis, recruitment of TFIIH to the HIV-LTR in conjunction with NF-κB has been suggested to be rate limiting in the reactivation of latent HIV-1 provirus in a Jurkat cell model (Kim 06). Also consistent with these findings are the suggestions that HIV-1 postintegration latency is regulated by a withdrawal of stimulatory factors, namely IL-2 or by an enhancement in proteasome mediated degradation of host proteins (Krishan 04). Furthermore, the demonstration that the inhibition of NF-κB or NF-AT signaling by Murr1 or Cyclosporin A, respectively, attenuated HIV-1 expression in T cells, confirmed the significance of these proteins in HIV-1 provirus transcription and suggested that interference with activating factors suppresses HIV-1 transcriptional activity (Ganesh 03, Li 00, Greene 04). This hypothesis is further supported by data suggesting that the viral protein Nef represses HIV-1 transcription, potentially promoting HIV-1 latency, via interfering with the association of transcriptional activators with their binding motifs in the HIV-LTR (Guy 90, Mori 90) These data, coupled with the known dependence of HIV-1 proviral integration and gene expression on T cell signaling, and the presence of cellular transcription factor binding sites in the HIV-LTR, indicate that T cell signals may be involved in the generation and maintenance of HIV-1 postintegration latency. Furthermore, these signals may be similar to those involved in T cell anergy or memory generation. It is important to note that additional factors, including viral proteins such as Nef (Tobiume 02, Fujinaga 95), gp120-CD4 signals (Cicala 02, Kinter 03, Briant 98), and cell cycle proteins (Kundu 97) have been implicated in the regulation of T cell activation and latency. Therefore, the potential role of these molecules in the reactivation, generation, and maintenance of HIV-

1 postintegration latency cannot be discounted. However, this has yet to be proven, and the factors involved in this process remain largely unknown. Furthermore, it should be noted that signals other than those involved in T cell activation have also been shown to reactivate latent HIV-1 provirus, including IRF-1 (Sgarbanti 02) and STAT5 (Selliah 2006).

Investigations regarding the reactivation of silenced provirus have also revealed a level of complexity in the regulation of HIV-1 postintegration latency that exceeds previous conceptions and involves more than merely an absence of inducible positive regulatory factors. For example, a repressive chromatin environment has been implicated in the transcriptional silencing of the HIV-1 provirus. There are several potential mechanisms by which this can occur. It had been suggested that HIV-1 preferentially integrates into regions of heterochromatin (Jordan 03). However, conflicting data indicated that integrated HIV-1 provirus is typically located within active genes (Schroder 02, Han 04), leading to the authors suggestion that latency may be regulated via transcription interference (Han 04). In addition, the presence of a potentially repressive nucleosome, nuc 1, located just downstream of the transcriptional start site has been the focus of intensive research involving the regulation of postintegration latency. This nucleosome is rapidly displaced upon transcriptional activation, implicating its contribution to the generation or maintenance of a latent state of the provirus. The remodeling of this nucleosome has been ascribed to both Tat-dependent and –independent mechanisms, as previously discussed. Evidence suggests that the condensed chromatin structure associated with transcriptionally silent HIV-1 provirus can be maintained via post-translational mechanisms. Acetylation of lysine residues within the

N-terminal tail of nucleosome-associated histones by histone acetyltransferase enzymes (HATs) has been correlated with chromatin disruption and gene activation. In contrast, proteins possessing deactylase activity (HDACs) have been implicated in transcriptional silencing (Wu 00, Gregory 01). A significant role for histone acetylation in the regulation of HIV-1 transcriptional activity has been demonstrated by the use of chemical histone deacetylase inhibitors, such as trichostatin A (TSA) (Quivy 02) , as well as the identification of proteins associated with the inactive HIV-LTR that inhibit HIV-1 expression via the recruitment of HDAC's. Examples of these inhibitory factors include p50 homodimers (Williams 05), AP-4 (Imai 06), NFAT (Baksh 02, Mouzaki 00), RBF-2 (Sadowski 05), YY1 and LSF (Coull 00). Other cellular proteins have been reported to inhibit the HIV-LTR via mechanisms that are independent of HDAC recruitment. For instance, cRel attenuates p65-induced HIV-1 transcription through competitive binding to the HIV-LTR with p50/p65 heterodimers (Doerre 93). Other examples include CIITA (Sarol 02, Accolla 02), ICER (Newbound 00), and IRF-8 (Sgarbanti 02). Importantly, this data underscores that the presence of inhibitory factors may be one of several means of silencing HIV-1 proviral transcription

Cellular proteins are not the only factors that have been implicated in the promotion of HIV-1 proviral latency. Initial research indicated that HIV-1 postintegration latency could be explained by a defect in the viral protein Rev (Pomerantz 90). Rev functions to transport viral RNA from the nucleus to the cytoplasm. However, contradictory data from Hermankova et. al. suggested that latency is instead regulated at the transcriptional level. Transcriptional silencing in many of the existing latent cell line models, such as U1, is attributed to a Tat mutation, as evidenced by the presence of

promoter-proximal RNA transcripts. Thus, based on the role of Tat in recruitment of the pTEFb complex and subsequent phosphorylation of the CTD of RNA polymerase II, it has been suggested that postintegration latency is regulated at the level of elongation (Adams 94). This hypothesis is supported by evidence that p65 is required for efficient elongation (West 01) and that RNA polymerase pausing mediates the repression of gene expression in U1 cells (Zhang, unpublished). Tat also functions to assemble a transcriptional coactivator complex consisting of HAT and other proteins on the HIV-LTR, suggesting that a lack of acetylation and related alterations in chromatin structure may be responsible for latent HIV-1 provirus in U1 cells. These data suggest a model wherein cellular activation induces histone acetylation, resulting in the remodeling of nuc1 and increasing DNA accessibility to transcriptional activators. Induction of the p65 subunit of NF-κB promotes interactions with Sp1 on the HIV-LTR and the simultaneous binding of these factors to the HIV-LTR initiates transcription, leading to the recruitment of Tat, followed by pTEFb and HAT protein complexes to the HIV-LTR. Therefore, HIV-1 latency can be regulated at any step during this process

Proposed mechanisms for the establishment of HIV-1 transcriptional silencing that have received relatively less attention include the induction of natural antiviral defenses either via PKR or HIV double stranded RNA (Muto 99). It has also been suggested that latent HIV-1 provirus development occurs during the process of thymopoeisis upon infection of T cell progenitors (Brooks 01). Regardless, the relevance and application of data from cell line models to latent HIV-1 in primary, quiescent cells *in vivo* is unclear. Furthermore, the presence of previously integrated HIV-1 provirus in these cells precludes the evaluation of the involvement of the identified factors in the

generation of HIV-1 latency. Thus, the development of an *in vitro* system which allows for the elucidation of the biochemical events mediating the establishment of HIV-1 post-integration latency, such as T cell signals, is critical for understanding the post-integration silencing and the eventual elimination of this reservoir.

1.8 Host cellular factors mediate progression through the HIV-1 life cycle

Of the stages in the HIV-1 life cycle, HIV-1 transcription has been the best characterized with respect to the involvement of cellular factors. However, it is becoming increasingly clear that HIV-1 exploits host proteins at every stage of its replication cycle. For example, NFATc has been shown to overcome a block in reverse transcription (Kinoshita 97). Interestingly, this illustrates that a specific T cell signal can participate in more than one stage of the HIV-1 life cycle. Likewise, PI3 kinase activity is required after viral entry and reverse transcription, but prior to integration (Francois 03). Importantly, these findings underscore the ability of HIV-1 to manipulate host proteins involved in T cell activation for functions other than the promotion of transcription. Host factors mediating provirus integration include LEDGF (Llano 06), BAF (Chen 98), HMG I(Y) (Farnet 97), p300/CBP (Cereseto 05), and the INI1 subunit of the SWI/SNF complex (Turelli 01). It is likely that these proteins assist with the selection of an optimal integration site. Additionally, it is clear that cellular activation is vital for proviral integration, implying that T cell signaling molecules may contribute to this process, although none have been elucidated. A cellular nuclear export protein, CRM-1, is usurped by the viral protein Rev to promote the transport of HIV-1 RNA from the nucleus to the cytoplasm for translation and packaging (Neville 97). The

identification of host factors that regulate the assembly and release of the virus is currently an area of intensive research. A discussion of this process and its regulation is below. Together, these findings emphasize that host cellular protein participation at each stage of the virus life-cycle is vital to HIV-1 replication. Interestingly, many of these proteins are also important mediators of T cell activation, although the exact role of these molecules in HIV-1 replication remains poorly defined.

1.9 HIV-1 assembly

HIV-1 assembly is driven by the viral protein Gag (Fig. 1.7). In fact, Gag is such an integral component of the virus assembly process that it is sufficient for the production of non-infectious virus-like particles (VLP's). Gag is translated in the cytoplasm as a precursor protein, p55 (Gag_{p55}), and is comprised of four protein domains: matrix (MA) (p17), which is essential for Gag membrane binding; capsid (CA) (p24) which constitutes the viral core and has been implicated in Gag multimerization;. nucleocapsid (NC) (p7) which is responsible for binding the viral RNA genome and Gag multimerization; and p6, a proline-rich peptide, that is critical for viral budding (Demirov 02) (Fig. 1.7). Gag also contains two spacer peptides, p1 and p2, located between CA-NC and NC-p6, respectively (Resh 05). In addition to the four protein domains, Gag_{p55} contains three functional domins, M (membrane binding), I (interaction), and L (late), which are central to virus assembly and budding. An N-terminal myristate and cluster of basic residues in MA combine to represent the M domain, both of which are essential for Gag membrane targeting and binding. The myristate group inserts hydrophobically into the lipid membrane, while the basic residues form electrostatic interactions with acidic PI(4,5)P2

in the membrane, thereby enhancing Gag binding affinity (Muriaux 04) In addition, the highly basic domain of MA has been implicated in Gag plasma membrane targeting (Ono 00). Membrane binding occurs through a "myristol switch" mechanism as follows: the myristate group is sequestered in a hydrophobic pocket of MA in monomeric Gag. Upon Gag multimerization, the myristate group is exposed, resulting in Gag membrane binding. The membrane lipids PI(4,5)P2 have been suggested to initiate the "myristol switch" upon membrane binding and Gag multimerization (Saad 06). Gag multimerization is mediated predominantly by the I domain, which includes the C-terminal third of CA, p2, and NC. The NC domain contributes to Gag multimerization via its binding to both viral and nonspecific RNA, which serves as a bridge for Gag-Gag interactions. In addition, the I domain was recently reported to contain an internalization signal that appears to play a significant role in HIV-1 assembly and release (Lindwasser 04). Finally, the L domain has been shown to bind the cellular protein Tsg101 via its PTAP motif. This interaction, together with ubiquitin, mediates "pinching off" phase of HIV-1 budding in T cells (Demirov 02, Schubert 00, Goila-Gaur 03, Demirov 02, Goff 03, Garrus 01, Strack 00, Patnaik 00, Gottwein 05). In close proximity to the PTAP sequence is an LXXLF motif that associates with another cellular protein, AIP1 (Strack 03). Tsg101 interacts with Vps 28, a member of the protein sorting machinery, to form the ESCRTI complex that is transiently recruited to endosomal membranes. AIP1 is a component of the ESCRTII complex, which is recruited in conjunction with ESCRTIII by ESCRTI to instigate multivesicular body (MVB) biogenesis(Fig. 1.7). AIP1 connects the ESCRTI and ESCRTII complexes through its interaction with both Tsg101 and Gag. In doing this, Gag exploits the cellular protein sorting machinery (Langelier 06, von Schwedler

03) by mimicking the Tsg101 target hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) in order to promote HIV-1 assembly and budding (Muriaux 04, Amara 03). The viral protease enzyme (PR) cleaves Gag_{p55} into its four individual proteins components during or just after budding, which triggers the formation of the virus capsid by the CA protein and results in a condensed core and overall conformational change and the maturation from a noninfectious to an infectious virus particle (Fig. 1.7).

Once synthesized Gag multimerizes and is targeted to the site of viral assembly. However, the location at which virus assembly occurs and the molecular mechanism by which Gag is targeted to the assembly site remains unclear. It has been proposed that Gag forms small oligomers, possibly dimers, in the cytosol and that this process might be mediated by the NC-RNA interaction or C-terminal cysteine linkage (Alfadhli 05, Nermut 03). Once dimerized, a conformational change occurs which allows for the assembly of these small oligomers (Alfadhli 05). However, an additional environmental cue is essential for assembly into larger-order complexes and this occurs primarily through plasma membrane binding (Alfadhli 05, Nermut 03).

Early electron microscopy data led to the belief that HIV-1 assembly occurred exclusively at the plasma membrane (Muriaux 04). However, research demonstrating the presence of HIV-1 particles within the golgi apparatus and vesicles of T cell syncytia hinted that this may not be the case (Grief 91, Dowsett 87) It has since been discovered that HIV-1 takes advantage of the MHC Class II (MHCII) pathway in macrophages, and that virus assembly and budding typically occurs in the MHCII and CD63-enriched MVB's, using a process similar to that of internal vesicle formation in these structures These vesicles then fuse with the plasma membrane and release virus via an exosomal

pathway (Raposo 02, Pelchen-Matthews 03, Ono 04). Subsequent live cell imaging and electron microscopy data revealed that HIV-1 interacts with the host cellular protein sorting machinery and assembles on the membranes of MVB's in all cell types, not just macrophages as previously thought (Sherer 03, Nydegger 03). A recent investigation defined the cellular pathway taken by Gag after its synthesis as being initially distributed throughout the cytoplasm prior to its accumulation in the perinuclear compartment. Gag localization in the perinuclear compartment then becomes more concentrated, at which time it passes transiently through an MVB-like structure on its way to the plasma membrane in T cells. The authors proposed that Gag traverses the MVBs in all cell types and that the determination of the assembly site is regulated by the extent and kinetics of Gag interaction with intracellular compartments (Perlman 06).

Once at the plasma membrane, Gag preferentially buds from lipid raft domains (Nguyen 00, Lindwasser 01, Ono 01). Lipid rafts are detergent resistant sections of the cell surface that are enriched in cholesterol, sphingolipids, and glycosylphosphatidylinositol (GPI)-linked proteins and have been demonstrated to play a central role in T cell activation. Indeed, the composition of the viral membrane is similar to that of lipid rafts in its expression of proteins such as Thy-1, CD59, GM1, and CD45 and Gag has been shown to colocalize with these markers using confocal microscopy (Nguyen 00). The viral protein Nef contributes to this process via increasing the synthesis and transport of cholesterol to lipid rafts, thereby promoting viral infectivity (Zheng 03). Similarly, the interaction between HIV-1 Gag and gp41 Env proteins is crucial for virus association with detergent resistant membrane domains (Bhattacharya 06). In contrast to these data, Ding et. al. reported that Gag forms detergent-resistant

complexes at the plasma membrane that were distinct from traditional lipid rafts, lacked classical lipid raft marker proteins, and were unaffected by cholesterol extraction (Ding 03). Interestingly, a recent investigation determined that Gag, in conjunction with Tsg101 and Vps28, is targeted to and exits from discrete plasma membrane domains in T cells that are enriched for the exosomal and endosomal proteins CD9, CD63, CD81, and CD82 (Booth 06, Nydegger 06). This finding further supports the hypothesis that Gag passes through intracellular compartment prior to being targeted to the plasma membrane of T cells.

As previously mentioned, it is well known that HIV-1 Gag associates with intracellular compartments and acquires cellular sorting proteins on its way to the plasma membrane, where it assembles and buds from T cells. The differential location of HIV-1 assembly in T cells and macrophages implies that a cell-specific protein regulates the determination of the viral assembly site. Early evidence for the involvement of host factors in HIV-1 assembly was derived from a study in murine cells in which aberrantly assembled virions were detected in the intracellular vesicles (Mariani 00). The authors concluded that the primary block in HIV-1 replication in these cells was at the level of virus assembly, (Mariani 00). A subsequent investigation comparing postintegration HIV-1 replication in murine, mink, and human cells reported that the absent cellular factor required for efficient HIV-1 assembly and release in murine cells was present in both mink and human cells (Koito 03, Koito 03) A number of host factors have recently been implicated in the HIV-1 assembly process. For example, protein kinase C (PKC) was shown to mediate the "myristoyl-protein switch" and Gag targeting to the membrane via phosphorylation of MA (Yu 95). Similarly, PI(4,5)P2, which has been associated

with the "myristol switch" has also been implicated in HIV-1 Gag membrane targeting (Ono 04). Furthermore, the basic residues in the NC domain of HIV-1 Gag, which were previously reported to promote assembly via a nonspecific RNA interaction, were demonstrated to transiently bind to a cellular ATPase, HP68 , representing an additional regulatory mechanism for NC-mediated assembly (Zimmerman 02, Dooher 03, Lingappa 06). More recently, adaptor proteins AP-2 and AP-3 were demonstrated to be involved in HIV-1 assembly and release via interaction with MA. Specifically, AP-2 restricts HIV-1 egress to distinct microdomains (Batonick 05), and AP-3 mediates Gag transport to MVB's (Dong 05). Interestingly, a novel role for the Golgi network in virus assembly and release was implicated from the finding that POSH, a trans-Golgi network human ubiquitin ligase, was essential for Gag targeting to the plasma membrane (Alroy 05). These results, together with the dependence of virus release on the E2-like ligase Tsg101 (Goila-Gaur 03, Demirov 02, Goff 03, Garrus 01), underscore the importance of the previously determined role of ubiquitin in HIV-1 assembly and release (Strack 00, Patnaik 00, Gottwein 05). Finally, more general roles for actin (Sasaki 95, Wilk 99), fatty acids (Lindwasser 02), cholesterol and intracellular calcium (Lindwasser 04) in HIV-1 assembly and release have been demonstrated.

Together, these data prompted a modified model proposed by Resh (Resh 05) in which Gag is synthesized and simultaneously myristoylated on polysomes in the cytosol (Fig. 1.7). Gag may traverse the Golgi network and be ubiquitinated by POSH prior to its targeting to the MVB's by the Gag dileucine-motif and the MA-AP-3 interaction, and anchoring to the membrane by myristate. Gag interacts with the host ESCRT machinery and a potentially a number of different host proteins within the endosomal pathway.

From there, Gag targeting to the plasma membrane is regulated by MA, membrane lipids, and AP-3. Once at the plasma membrane, binding is mediated by myristate, the N-terminal basic clusters, PIP(4,5)P2, and PKC and Gag is limited to plasma microdomains by AP-2. In T cells, the majority of Gag is located at the plasma membrane at steady state. Multimerization proceeds via the basic residues in NC and possibly HP-68, followed by budding with the help of Tsg101 and ubiquitin. Despite the identification and association of these host proteins with Gag targeting and assembly, the precise molecular mechanisms mediating Gag targeting to the assembly site are unknown. It is likely that unidentified cellular factors participate in this process.

Fig. 1-7

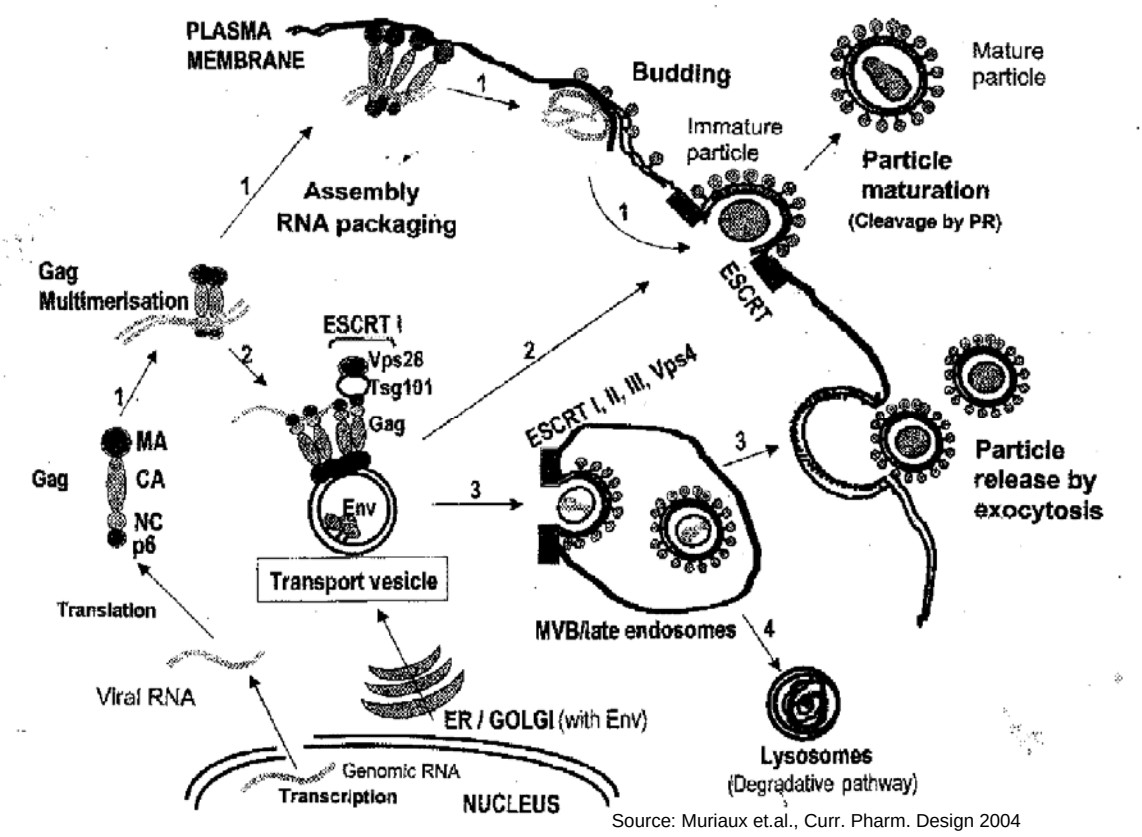


Figure **1-7**: Schematic of HIV-1 Assembly

1.10 The role of Lck in HIV-1 replication

One cellular protein that potentially influences HIV-1 assembly and release is Lck. Lck is a T-cell specific member of the first tier of Src family kinases and is triggered early on in the T cell activation process via the CD2, IL-2R, CD4, or TCR/CD3/CD28 pathways through its association with the CD4 and IL2 receptors. Lck is composed of an SH1

(kinase) domain, an SH2 domain, and an SH3 domain that are conserved among all Src family kinase members. In addition, the N-terminus of Lck contains a unique domain harboring two cysteine residues, C3 and C5, which serve as substrates for palmitoylation and mediate membrane targeting. In addition, Lck is myristoylated on its N-terminus and the C-terminal tail contains a tyrosine residue, Y505, which mediates inhibitory phosphorylation by Csk (Fig. 1.8). In its inactive form, Lck Y505 is phosphorylated, mediating the binding of this residue to the proteins SH2 domain, resulting in a closed conformation. Upon T cell activation, TCR/CD3 clustering occurs, bringing CD4 in close proximity to CD45, which dephosphorylates Y505 and activates Lck. Lck then phosphorylates the ITAMS in the CD3 zeta chain, initiating a signaling cascade that promotes T cell activation. Alternatively, autophosphorylation of Y394 can activate Lck, potentiating the signal. The significance of Lck in T cell activation and development is evidenced by the arrest in T cell development prior to the double negative stage and the robust depletion of developed T lymphocytes observed in Lck deficient mice (Molina 92, Boggon 04).

Fig. 1-8

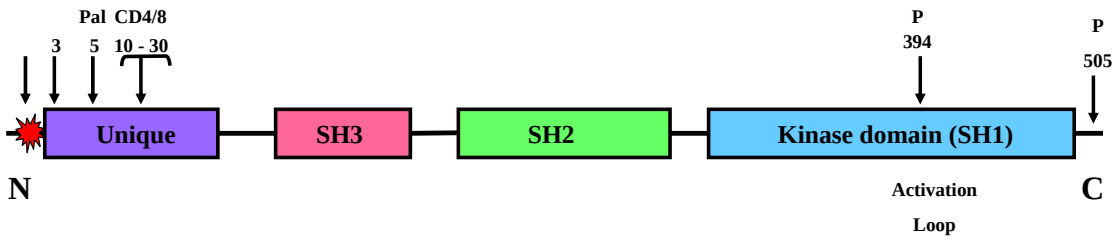


Figure **1-8**: Structure of Lck

With respect to HIV-1, Lck is concentrated in lipid rafts, the site of HIV-1 egress, and constitutively binds CD4, the primary HIV-1 receptor, in a non-covalent manner. Although Lck is primarily located in plasma membrane lipid rafts, it has also been detected in the golgi network (Hu 01) and the endosomal compartment (Blanchard 02, Marie-Cardine 96). In fact, a recent report detected an accumulation of Lck in the

endosomal compartment of HIV-infected relative to uninfected cells (Thoulouze 06). In addition, Lck has been associated with many aspects of HIV-1 infection. For example, Lck activity is enhanced as an early response to HIV-1 infection (Phipps 96). Furthermore, it is known to interact with the viral protein Nef through its SH2 and SH3 domains, and this interaction was demonstrated to be critical for Nef-induced enhancement of HIV-1 replication (Cheng 99). Lck has also been implicated in syncytia formation (Yoshida 92, Briand 97), which is a proposed mechanism for HIV-1-induced virus transmission and $CD4^+$ cell death. In addition, dysregulation of Lck protein has been observed in HIV-1 infected patients (Cayota 94). Specifically, HIV-1 infection has been associated with reductions in Lck and increases in Fyn, and these alterations have been linked to anergy and HIV-1 progression.

A more direct role for Lck in HIV-1 replication has been suggested by reports of its negative regulation of HIV-1 transcription (Tremblay 94). However, this conclusion was derived solely from experiments using CD4-Lck binding mutants, thus, the function of Lck was never directly assessed in this study. These findings were challenged by data suggesting that an alternative signaling partner for CD4, not Lck, is responsible for the CD4-induced suppression of HIV-1 transcription (Coudronniere 98). Furthermore, Lck has been implicated in CD43-induced HIV-1 gene expression (Barat 02). Interestingly, Lck activation was implicated in the reactivation of HIV-1 from latency (Brooks 03). Clearly, additional research is needed to characterize the role of Lck, as well as other TCR/CD3/CD28 signals in HIV-1 transcriptional regulation. Collectively, this information implicates Lck as a mediator of HIV-1 replication. To date, the only direct examination of the role of Lck in HIV-1 replication reported Lck kinase activity to

influence virus at a postintegration stage, ultimately delaying productive HIV-1 infection. However, after three days of infection, viral replication was independent of kinase activity. The authors concluded that the initial level of endogenous Lck kinase activity inhibits some aspect of the viral life cycle, but that eventually, the virus, or a viral protein such as Nef, may target Lck to facilitate HIV-1 replication (Yousefi 03).

More recently, Lck was identified inside the HIV-1 virion (Ott 00), implying a role for this protein in HIV-1 assembly or release. In support of this, a chimeric Fyn (10) Gag protein, comprised of the N-terminal sequence of Fyn fused to the remainder of Gag, exhibited a heightened affinity for plasma membrane "barges" and an associated two- to four-fold enahancement in VLP release (Lindwasser 01). Furthermore, Lck interacts with several ubiquitin-binding proteins (Scott 04, Umebayashi 03), and ubiquitin is critical for HIV-1 assembly and release.

It is interesting to note that Src family kinases have been implicated in influencing the replication of a number of viruses, including hepatitis B virus (Klein 1997; Klein 1999), vaccinia virus (Newsome 2004) and mouse polyoma virus (Messerschmitt 1997). Furthermore, herpesvirus saimiri encodes a protein, Tip, which recruits Lck into endosomal vesicles (Park 2002). In addition, HIV-1 Vif has been shown to interact with the Src kinase Hck in macrophages (Hassaine 2001). Finally, it has been suggested that c-Yes contributes to RSV budding and release via its role in the transit of the assembled West Nile virion from the ER through the cellular secretory pathway (Hirsch 2005). Based on these data, we hypothesize that Lck regulates HIV-1 Gag targeting to the plasma membrane in T cells.

1.11 Concluding Remarks

As a primary target of HIV-1, the $CD4^+$ T cell plays a pivotal role in disease progression and pathogenesis by serving as both a key source of viral replication and as a latent viral reservoir. Activated T cells provide an environment that is conducive to virus replication while quiescent T cells promote the suppression of HIV-1 transcription, signifying that HIV-1 expression is directly correlated to the activation state of the T cell. Thus, it is likely that HIV-1 has evolved to usurp the cellular activation machinery in order to promote is own propagation and survival. We were interested in examining the various mechanisms by which HIV-1 exploits these proteins to effectively drive its status toward either active replication or latency. Our studies focused on the interplay between HIV-1 and T cell signaling proteins, specifically those in the pathway emanating from the CD3 and CD28 receptors. Previous work (Cook 03) demonstrated an enhancement of HIV-1 replication following T cell costimulation via CD28 and determined this response to partially be mediated by the receptor proximal proteins Vav and Rac. In addition, the transcription factor NF-κB was shown to play a role in the positive regulation of HIV-1 by CD28 signals. We expanded upon these findings by defining the sequence of the HIV long terminal repeat (LTR) between -182 and -205 as a potential response element for the CD28-induced increase in HIV-1 transcriptional activity. This region of the HIV-1 promoter was also associated with the binding of specific, unidentified factors in response to simultaneous CD3 and CD28 stimulation. A computerized transcription factor database search identified the histone acetyltransferase (HAT) p300/CBP as one of

several candidates binding to this region of the HIV-1 LTR, which could be indicative of post-translational transcription factor modification in response to CD28 signaling. In general, these findings illustrate that proteins in the CD3+CD28 pathway can positively regulate HIV-1 transcription and replication.

In contrast, the negative regulation of HIV-1 transcription results in a latent proviral state and minimal HIV-1 replication. We hypothesized that signals emanating from the CD3 and/or CD28 receptors can suppress HIV-1 transcription under certain circumstances. To study this, we developed a novel *in vitro* model which will be useful for the elucidation of factors mediating HIV-1 transcriptional silencing. Using this system, we demonstrated that stimulation of the calcium component of the T cell activation pathway can reversibly inhibit HIV-1 gene expression and may represent a latent proviral state. Further studies are needed to identify the biochemical mediators of this response. Together with the previous results, these data indicate that T cell signals can differentially effect HIV-1 transcription depending upon the cellular environment. Specifically, full T cell activation positively regulated HIV-1 transcription while incomplete T cell signaling negatively regulated virus transcription.

It is becoming increasingly clear that proviral transcription is not the only aspect of HIV-1 replication that is dependent on host cellular factors. For example, the inducible T cell specific protein NF-AT is known to overcome a block in reverse transcription. Importantly, this suggests that T cell signaling proteins can participate in stages of the virus life cycle other than transcription. The identification of cellular mediators of HIV-1 assembly has recently become an area of intensive research. Virus assembly and release occurs at the multivesicular bodies (MVB's) in macrophages and at

the plasma membrane in T cells. The different locations of virus packaging and egress between these two populations imply that a cell-specific factor regulates the determination of the assembly site. Based on its cellular distribution and previous associations with HIV-1, we examined the role of the T cell specific protein, Lck, in HIV-1 assembly and budding. We determined that Lck facilitates virus assembly by physically interacting with Gag and targeting it to the plasma membrane for efficient packaging and release. Surprisingly, this effect of Lck on HIV-1 assembly is mediated by the adapter function of its unique domain and not its kinase activity. These findings indicate that T cell signals can regulate stages of HIV-1 replication other than transcription.

In summary, T cell signals emanating from the CD3 and CD28 receptors can both positively and negatively regulate HIV-1 transcription. In addition to transcription, these T cell signaling proteins can also mediate other stages of the virus life cycle, such as HIV-1 assembly. Insight into the interplay between T cell signals and HIV-1 may heighten our appreciation of how the virus exploits host proteins for its replication and survival. This information may provide novel targets for preventative strategies against HIV-1. Furthermore, this knowledge may reveal novel functions of known host proteins, such as the adapter function of Lck and its potential role in protein trafficking.

Chapter 2

Identification of a CD28 Responsive Element in the HIV-1 Long Terminal Repeat (LTR)

2.1 Introduction.

HIV-1 replication in T cells is directly correlated with the activation state of the host cell. For example, integrated HIV-1 provirus replicates at extremely low levels or remains unexpressed in quiescent CD4+ T cells. In contrast, HIV-1 infection is productive and efficient in activated T cells, suggesting that T cell signals positively regulate HIV-1 expression. Two signals are required for complete T cell activation (Lafferty 75) - ligation of TcR/CD3 alone is insufficient for cell proliferation, differentiation, or cytokine production, including IL-2, and often results in apoptosis or T cell anergy (Jenkins 87). In contrast, stimulation through both TcR/CD3 and a costimulatory receptor, the best characterized of which is CD28, results in optimal T cell activation and IL-2 production (Civil 95). The point at which these two pathways converge remains to be elucidated, but it is thought that CD28 signals augment those of CD3 to induce IL-2 expression, and that this effect is primarily mediated through PI3 kinase (Hutchcroft 96) and NF-κB (Zhou 02), although JNK (Su 94), Zap70, p95vav (Tuosto 98), PLCγ1 (Michel 01), and NFAT (Diehn 02) may also play a role. In addition, a critical CD28 response element (CD28RE) similar in sequence to the NF-κB consensus motif located at position -174 to -146 in the IL-2 promoter has been identified. This region is positively regulated by known c-Rel and CREB family members, as well

as NF-MATp35 and MEKK (Butscher 98, Civil 96, Tao 02) and negatively regulated by LOK (Tao 02).

The presence of several common regulatory elements in the IL-2 and HIV-1 promoters indicates that HIV-1 exploits host proteins associated with T cell activation to promote its own transcription. This is supported by reports of enhanced HIV-1 promoter activity in T cell lines following either PMA or PMA+PHA treatment (Tong-Starksen 87, 89). Similar to IL-2 production, maximal HIV-LTR activity and virus replication require stimulation through both CD3 and CD28 (Tong-Starksen 89). The mechanism by which CD28 enhances CD3-induced HIV-1 transcription is unknown. Research indicates that Vav and Rac1 are upstream mediators of this response (Cook 2003). In addition, NF-κB, NFATc, Sp-1 and AP-1 have been implicated as critical transcription factors for HIV-1 expression in T cells (Nabel 87, Lu 89, Alcami 95, Ross 91, Parrott 91, Al-Harthi 98, Henderson 00, Van Lint 97, Ganesh 03, Li 00, Kinoshita 97). However, the particular transcription factors and the region of the HIV-LTR specifically targeted by CD3 + CD28 signaling have not been elucidated. The similarities in the composition and regulation of the IL-2 promoter and the HIV-LTR suggest that the HIV-LTR might contain a CD28RE analogous to that of the IL-2 promoter. Therefore, we sought to identify the transcription factors and respective binding sites on the HIV-1 LTR responsible for the induction of HIV-1 transcription following CD3+CD28 stimulation. We demonstrated that the enhanced HIV-1 transcription observed following CD3+CD28 signaling is mediated by the specific binding of one or more unique, unidentified proteins to a potential composite CD28 response element (HIV-28RE) extending from position -182 to -205 of the HIV-LTR.

2.2 Materials and Methods.

Cells and Plasmids. Human acute T cell leukemia cell line Jurkat E6-1 (WT), obtained from ATCC (Manassas, VA) was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.2 M L-glutamine. Chinese Hamster Ovary cells (CHO) expressing Fc receptors (CHO-Fc) (gift from I. Mellman, Yale University, New Haven, CT) were cultured in DMEM supplemented with 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.2 M L-glutamine. Primary mononuclear cells were isolated from whole blood using a Ficoll/Histopaque gradient (Sigma-Aldrich, St. Louis, MO). Macrophages were removed by adherence to plastic and $CD4^+$ T cells were positively selected from the non-adherent population using a $CD4^+$ isolation kit (Dynal, Oslo, Norway).

Activation of T cells. Jurkat cells were washed and serum starved from 4 h-overnight prior to activation. CHO-Fc cells were plated at 2 x 10^5 cells/well in a 24-well plate, incubated 12 h to allow adherence, treated with mitomycin C (Sigma-Aldrich, St. Louis, MO) at 10 μg/mL, and incubated in the absence of serum for 2 h prior to use. Jurkat cells (1 x 10^6 cells/well) were activated via co-culture with mitomycin C- treated CHO-Fc cells and mouse anti-human CD3 (0.1 μg/mL) and CD28 (1 μg/mL) Abs (BD Pharmingen, San Diego, CA). Following stimulation, Jurkats cells were harvested and assayed for reporter gene expression as described below.

Transient Transfections and Luciferase Assay. Jurkat (7.5 x 10^6) cells were washed once with and subsequently resuspended in 250 μL [20mM] Hepes/RPMI. 15 μg LTR Luc, -205LTR Luc, or -158LTR Luc were transfected by electroporation with a BTX Electro Square Porator T820 (215 V, 65 msec, low voltage, 1 pulse). Cells were cultured in 5% FCS/RPMI for 24 h prior to lysis and measurement of luciferase activity using a commercial luciferase assay kit (Promega, Madison WI). Briefly, 1.0 x 10^6 cells were lysed in 1x Reporter Lysis Buffer (Promega, Madison, WI) and supernatants were collected. 20 μL of cell extract was added to 100 μL luciferase substrate (Promega, Madison, WI). Luciferase activity was measured using a TD-20/20 luminometer (Turner BioSystems, Sunnyvale, CA).

Nuclear Extract Preparations and Electromobility Shift Assays (EMSA). Nuclear extracts from either Jurkat T cells or primary CD4+ T cells were prepared as described previously (Schreiber 89) by lysing 1.0-2.0 x 10^6 cells with 10% nonidet NP-40 in Buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1mM DTT, 0.5 mM PMSF). The extracts were recovered in 50 μL Buffer C (20 mM HEPES (pH 7.9), 0.4 M NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT, 1 mM PMSF). 50 ng of annealed HIV-28RE (5'-CATGTGATGAAATGCTAGGCGGCTGTCAAACCTCCACTCTAACACTTC-3'), HIV-28RE1 (5'-CATGTGATGAAATGCTAGGCGGCT-3'), HIV-28RE2 (5'-GTCAAACCTCCACTCTAACACTTC-3'), NF-κB (5'-AGCTAAGGGACTTTCCGCTGGGGACTTTCCAGG-3', and 5'-

AGCTCCTGGAAAGTCCCCAGCGGAAAGTCCCTT-3'), or TCF11 (5'-GTCAAACCTCCAC-3') DNA were end filled with [α-^{32}P] dCTP using bacterial Klenow fragment (Promega, Madison, WI). The DNA probe was used at a specific activity of 10^8-10^9 cpm/μg and incubated with 5 μg of nuclear extract samples in a reaction mixture containing 3 μg dI-dC (Amersham Pharmacia Biotech, Arlington Heights, IL), 0.25 M HEPES (pH 7.5), 0.6 M KCl, 50 mM $MgCl_2$, 1 mM EDTA, 7.5 mM DTT and 9% glycerol for 20 min at 25°C. 50-fold excess of unlabeled HIV-28RE, HIV-28RE1, HIV-28RE2, NF-κB, Sp1, TCF11, or GATA3 binding site DNA was used as either a specific or non-specific competitor. The samples were run on a 6% polyacrylamide gel and visualized by autoradiography.

2.3 Results.

The HIV-LTR region between -158 and -205 is a putative CD28 response element. In order to identify the region of the HIV-LTR that is responsible for the known increase in proviral transcription following CD3+CD28 (3+28) stimulation, we performed mutational analysis using several HIV-LTR deletion constructs linked to a luciferase reporter gene. Full length HIV-LTR Luc, -205LTR Luc (lacking most of the negative regulatory element (NRE)), or -158LTR Luc constructs (comprised of promoter and enhancer regions) (Fig. 2.1A) were transfected into Jurkat cells which were subsequently stimulated with various combinations of CD3 and/or CD28 antibodies and LTR activity was determined by measuring luciferase activity. CD3 ligation of cells transfected with the HIV-LTR construct resulted in a threefold increase in HIV-1 transcription. As

expected, HIV-1 expression was enhanced even further, to 5 times that of unstimulated cells, following 3+28 stimulation. The transcriptional response of the -205LTR Luc to all stimulation conditions was equal or greater to that of the full length HIV-LTR Luc construct (Fig. 2.1B). In contrast, both absolute luciferase activity and luciferase activity relative to unstimulated cells was blunted following 3+28 but not CD3 stimulation alone in the cells transfected with -158LTR Luc compared to HIV-LTR Luc (Fig. 2.1C). The -158 LTR activity was approximately 50% of that observed for the LTR following 3 + 28, suggesting that the region of the HIV-LTR spanning -158 to -205 may be a 28RE (HIV-28RE) involved in the CD28-induced enhancement of CD3- stimulated HIV transcription .

Fig. 2-1

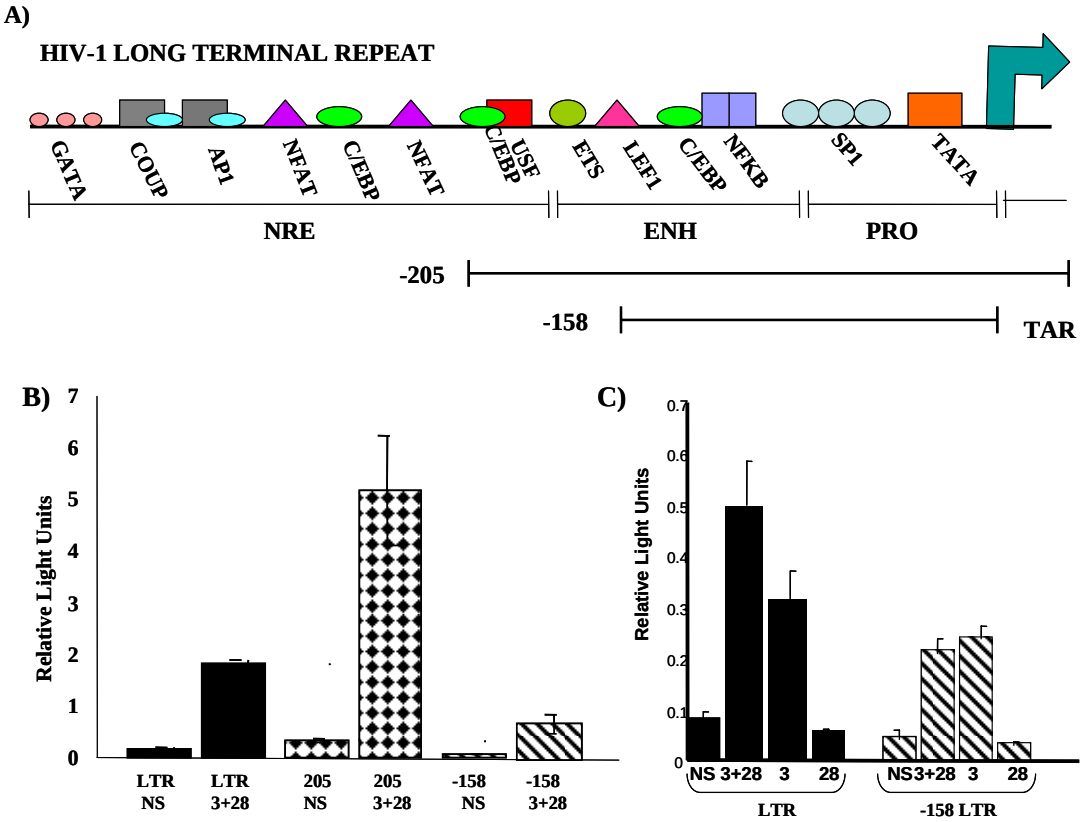


Figure **2-1**: Identification of a potential CD28 Response Element in the HIV-LTR A) Schematic of HIV-LTR and deletion constructs used for mutational analysis. B) Jurkat cells were transfected via electroporation with either full length, -205 HIV LTR Luc or -158 HIV LTR Luc DNA and recovered overnight in the absence of serum. Following 12 h inubation with CHO-Fc cells alone or presenting antibodies against CD3+CD28, luciferase activity was assessed an an indicator of HIV-1 transcriptional activity. C) Jurkat cells were transfected via electroporation with either full length or -158 HIV LTR Luc DNA and recovered overnight in the absence of serum. Following 12 h stimulation with CHO-Fc cells presenting antibodies against either CD3, CD28, or CD3+CD28, luciferase activity was assessed as an indicator of HIV-1 transcriptional activity

Specific factors induced by CD3+CD28 stimulation bind -182 to -205 on HIV-LTR.

Numerous transcription factors are induced and bind to a CD28 response element in the IL-2 promoter to stimulate IL-2 expression in response to T cell stimulation through CD3

and CD28 (Butscher 01, Civil 96, Butscher 98). To evaluate 3+28-induced transcription factor binding to the potential HIV-28RE, a probe was designed representing the region spanning -158 to -205 of the HIV-LTR (HIV-28RE; Fig. 2.2A) for use in EMSA analysis of 3 + 28 stimulated cells. Consistently, 3 unique proteins or protein complexes (Figs. 2.2B and D, complexes A,B,C) bound full length HIV-28RE in all experiments using Jurkat cell extracts. The two upper-most bands (complexes A and B) were only partially inhibited by specific cold competitor (SC), indicating the presence of complexes containing both specific and non-specific proteins induced during this response (Fig. 2.2B). We were most interested in the lower-most band (complex C) due to the fact that it was completely ablated by unlabeled HIV-28RE in both Jurkat and primary CD4+ cells (Figs. 2.2B and C), suggesting the presence of a specific factor binding to the 28RE in response to 3+28 ligation. To delineate the region of the HIV-LTR corresponding to the lower, more specific EMSA band (complex C), the 28RE probe was split into two, smaller probes, one from positions ¯158-¯182 and the other ¯182-¯205 of the HIV-LTR (Fig. 2.2A) which were used to evaluate transcription factor binding using unstimulated and 3+28 stimulated Jurkat cells. EMSA analysis revealed a differential binding pattern between the two probes; the slower migrating higher molecular weight complexes (complexes A and B) bound to the probe spanning the region of the HIV-LTR from -158 to -182, whereas the faster migrating complex (complex C), bound the probe representing the HIV-LTR sequence from -182 to -205 (Fig. 2.2D). These data localize the potential HIV-28RE to -182 to -205 of the HIV-LTR and indicate the presence of specific transcription factors bound to this region following both mitogenic and non-mitogenic T cell stimulation.

Fig. 2-2

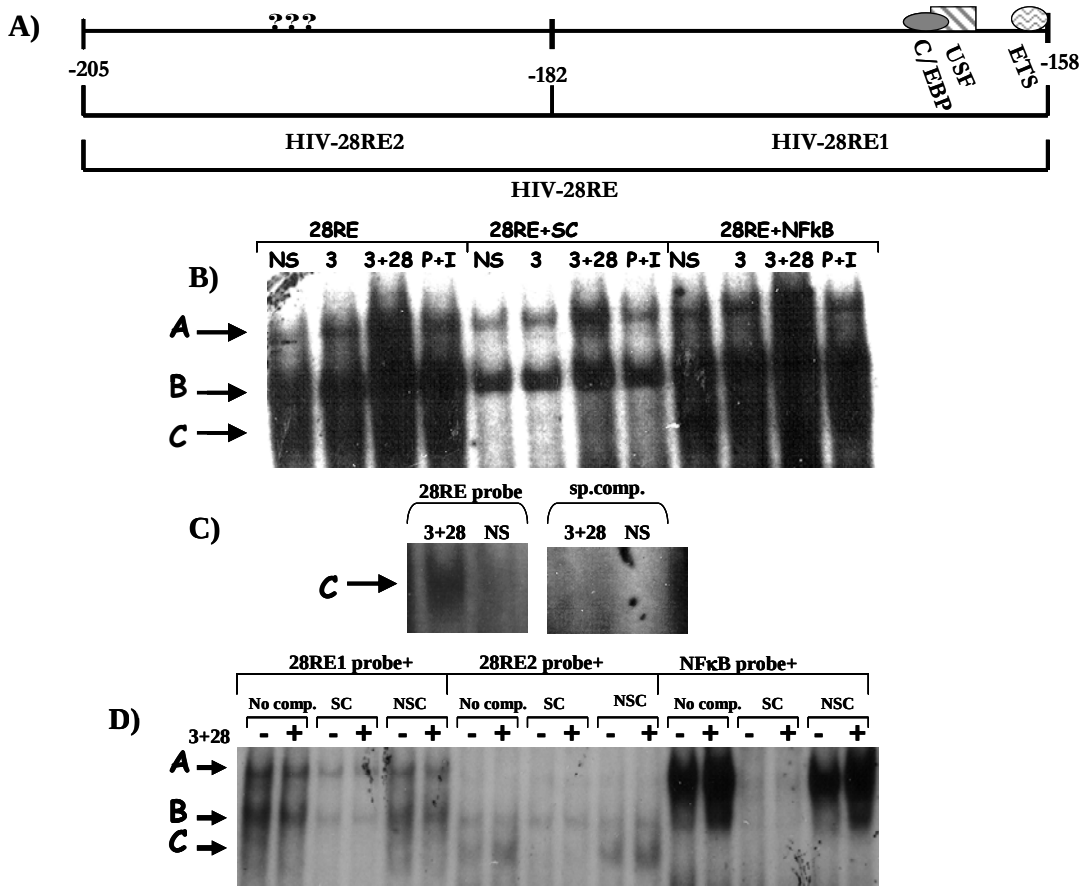


Figure **2-2**: **Specific Factors bind ¯182 to ¯205 on HIV-LTR in response to CD3+CD28 stimulation**. A) Schematic of HIV-28RE, HIV-28RE1, and HIV-28RE2 probes used in EMSA analysis. B) EMSA was conducted using nuclear extracts from Jurkat cells stimulated as indicated and incubated with an oligonucleotide probe spanning the -158 to -205 region of the HIV-LTR (HIV-28RE) C) EMSA was conducted using nuclear extracts prepared either from unstimulated or CD3+CD28 stimulated primary CD4+ cells incubated with an oligonucleotide probe spanning the -158 to -205 region of the HIV-LTR (HIV-28RE). Extracts were incubated with no competitor, cold, specific 28RE competitor (SC), or nonspecific cold competitor (NSC; either NFκB or Sp1). C) EMSA were performed on nuclear extracts prepared from Jurkat cells stimulated as indicated and incubated with either an oligonucleotide probe spanning the -158 to -182 (HIV-28RE1), or -182 to -205 (HIV-28RE2) region of the HIV-LTR, or the NFκB consensus binding site which was used as a positive control for 3 + 28 stimulation. Extracts were incubated with no competitor, 100-fold excess specific cold Competitor (SC), or 100-fold excess cold, non-specific NFκB or Sp1 consensus binding site competitor (NSC).

GATA3 or TCF11 do not bind the potential HIV-28RE. In an attempt to identify specific regulatory elements within the HIV-28RE, a computer search using MatInspector software was conducted for both the ¯158 to ¯182 HIV-28RE and ¯182 to ¯205 HIV-28RE sequences. This analysis implicated CEBPβ and GATA3 as potential factors binding the former and TCF11 as a candidate for binding the latter HIV-28RE probe (Fig. 2.3) Although C/EBP is expressed in T cells, it appears that it is dispensable for HIV-1 transcription. Thus, we tested the possibility of either GATA3 or TCF11, a member of the CNC subfamily of bZIP transcription factors implicated in the oxidative stress response (Myhrstad 01) binding the full length HIV-28RE sequence. To address this question, the consensus sequence for the binding sites of each of these proteins were used as cold competitors in EMSA using the HIV-28RE as a probe and extracts from Jurkat cells. Neither TCF11 nor GATA3 cold competitor diminished any of the 3 complexes bound to HIV-28RE under any stimulation condition compared to 28RE cold competitor (Fig. 2.4A). We evaluated whether proteins that bind to the TCF11 consensus site are expressed in unstimulated, 3-, or 3+28-stimulated Jurkat cells by performing a gel shift assay using the TCF11 consensus binding sequence as a probe. No unique complexes bound the TCF11 probe (Fig. 2.4B), supporting the conclusion that the identity of the protein specifically binding the HIV-1 28RE is not likely to be TCF11.

Fig. **2-3**

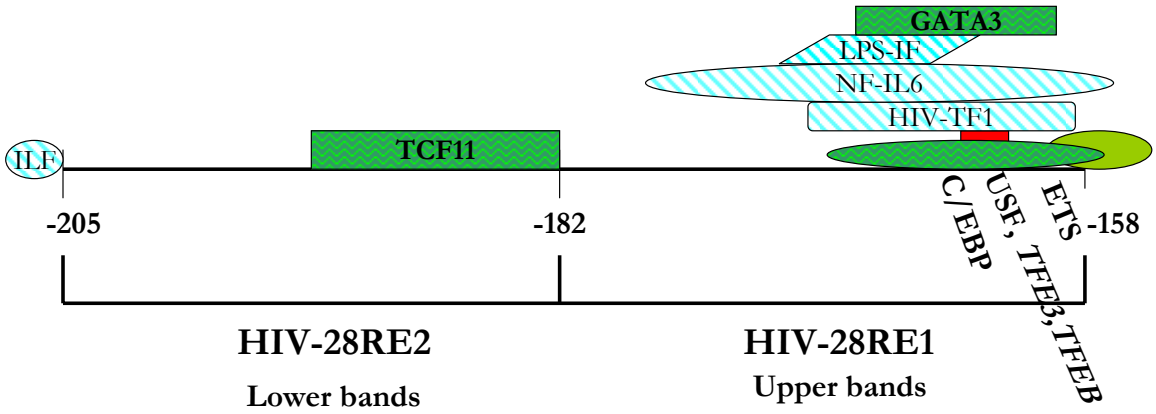


Figure **2-3**: **Schematic of MatInspector- predicted transcription factor binding to HIV-28RE1 and HIV-28RE2**. A computer search for transcription factors predicted to bind the HIV-28RE sequence was performed using MatInspector Software and the results diagramed above.

Fig. 2-4

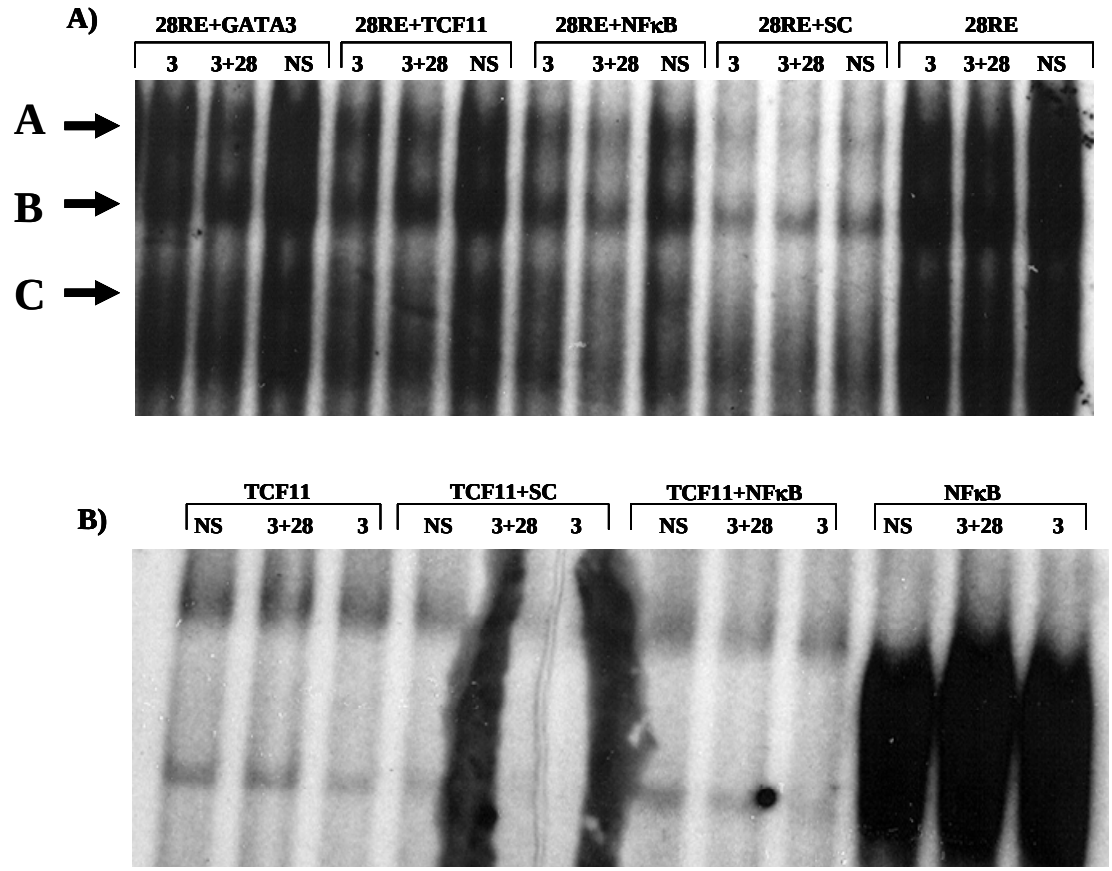


Figure **2-4**: GATA3 or TCF11 do not bind the HIV-28RE. A) EMSA were conducted using either unstimulated, CD3-, or CD3+CD28-stimulated Jurkat nuclear extracts. Extracts were incubated with no competitor, cold, specific HIV-28RE competitor (SC), cold, non-specific NFκB consensus binding site competitor, cold, GATA3 consensus binding site competitor, or cold, TCF11 consensus binding site competitor. B) EMSA was performed using either unstimulated, CD3-, or CD3+CD28-stimulated WT Jurkat nuclear extracts incubated with an oligonucleotide probe representing the TCF11 or NFκB consensus binding sequence. Extracts were incubated with either 100-fold excess cold, specific competitor (SC), or 100-fold excess cold, non-specific NFκB consensus binding site DNA.

Fig. 2-5

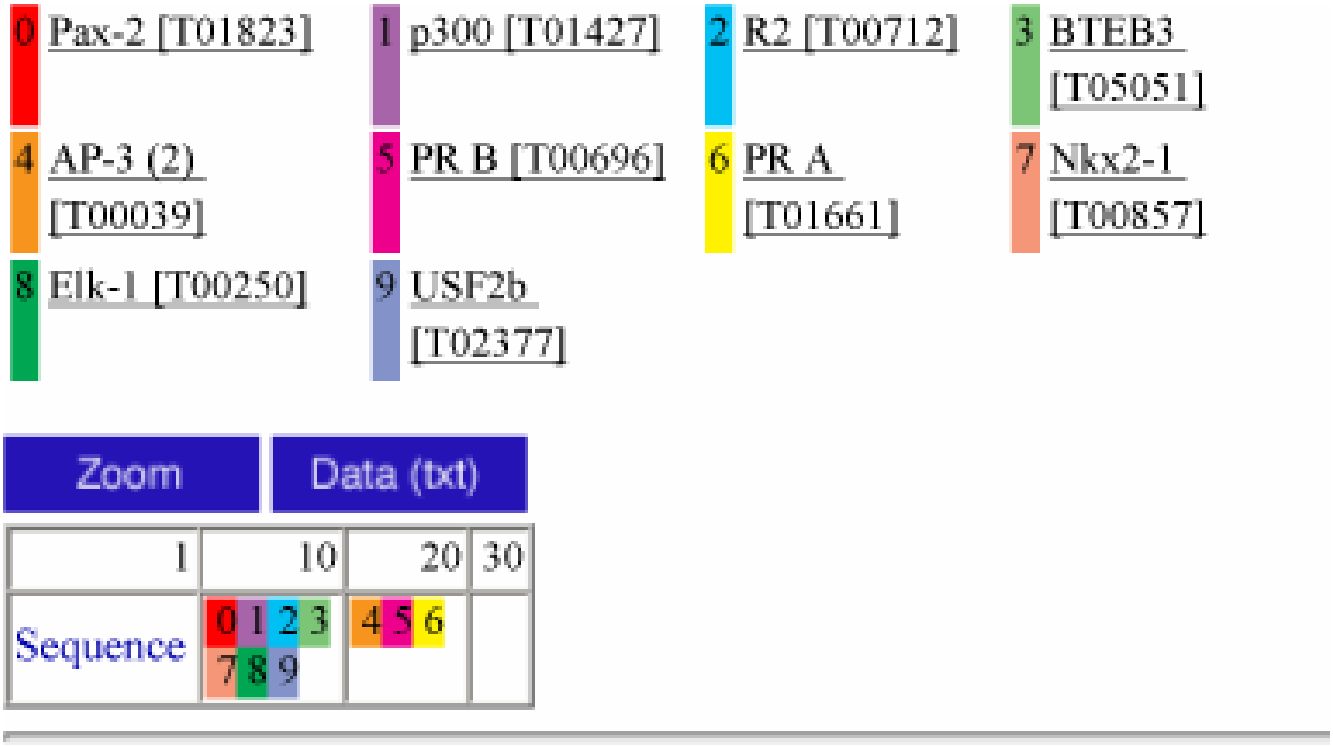


Figure **2-5**: **Schematic of PROMO- predicted transcription factor binding to HIV-28RE2** A computer search for transcription factors predicted to bind the HIV-28RE2 sequence was performed using PROMO Software and the results diagramed above.

2.4 Discussion.

We have defined the sequence spanning positions -182 to -205 of the HIV-LTR as a potential CD28 response element (HIV-28RE) based on the enhancement of HIV-1 transcription mediated by the full length sequence in conjunction with the presence of specific proteins bound to this region following 3 + 28 stimulation. The similarities between IL-2 and HIV-1 transcription regulation make it enticing to speculate that the HIV-28RE is analogous to the CD28RE-TRE found in the IL-2 promoter. Interestingly,

a recent transcription factor binding search of the HIV-LTR sequence from -182 to -205 (HIV-28RE2) using PROMO software revealed p300, a histone aceytltransferase (HAT) that participates in remodeling chromatin, as a prospective candidate binding to this element (Fig. 2.5). Full T cell responsiveness and IL-2 gene induction in response to CD28 signaling has been attributed to CREB family transcription factor binding and p300/CREB- binding protein coactivator (p300/CBP) recruitment to the CD28 composite response element (Butscher 98), suggesting a similarity between the regulation of the HIV-28RE and the IL-2 CD28RE-TRE. Similarly, p300/CBP mediates activation-induced HIV-1 transcription via its role in nuc1 remodeling (Marzio 98) and acetylation of Tat (Kiernan 99) and NF-κB (Furia 02). Thus, it is not inconceivable that CD28 signaling would induce p300/CBP binding to the HIV-LTR.

It is interesting to note that the HIV-1 transcriptional response to CD3 signaling is comparable in the cells transfected with the full length LTR Luc and the -158 LTR Luc constructs. This data suggests that CD3-induced HIV-1 transcription is mediated through the region of the HIV-LTR spanning from the transcription start site to -158 and thus, its regulation is independent from that of CD28. Despite this finding, proteins do appear to be associated with the full length HIV-28RE probe in response to CD3 stimulation. It is possible that a complex is recruited to the HIV-28RE following CD3 stimulation, but that it is insufficient for the induction of transcription in the absence of CD28 signaling. However, a more likely explanation is that transcription factor binding to the HIV-28RE in response to CD3 ligation is an artifact of the constitutively high activity of Jurkat cells, as evidenced by the considerable amount of NF-κB binding under both unstimulated and

CD3 stimulation conditions. Future studies using CD3 stimulated primary cells are needed to discriminate between these possibilities.

In conclusion, our data suggest that CD3 stimulation is sufficient and necessary for the induction of low level HIV-1 transcription. However, simultaneous ligation of CD28 with CD3 amplifies CD3 signals to generate optimal HIV-1 expression and this response is associated with specific factors bound to the region of the HIV-LTR between -182 and -205 (HIV-28RE). Several possibilities exist to explain this phenomenon: 1) CD28 signaling promotes the binding of additional, unique transcription factors to the HIV-28RE which are independent of those induced by CD3, 2) CD28 potentates CD3 signaling to induce transcription factor binding to the HIV-28RE, or 3) CD3 signaling is sufficient for maximal transcription factor binding, but CD28 signals are required to post-translationally modify these proteins, possibly via p300 recruitment, in order to achieve maximal HIV-1 transcription. Further research is needed to address these potential mechanisms of the CD28-induced enhancement of HIV-1 transcription.

Chapter 3

An *in vitro* system for studying the suppression of HIV-1: Activation of calcium signaling inhibits HIV transcription

3.1 Introduction

Recent statistics indicate a continuing rise in the incidence of HIV-1 infection in the United States. Nearly 1 million U.S. residents are currently living with HIV-1, with approximately 40,000 new infections occurring each year. The increased prevalence of HIV-1 infection in the U.S. can largely be explained by the extended survival of HIV-1 patients since the advent of highly active antiretroviral therapy (HAART) in 1996. This treatment has proven effective in reducing plasma viremia to undetectable levels, which initially inspired optimism for complete viral eradication. However, the discovery of latent HIV-1 reservoirs persisting in both adult and child HAART patients has drastically reduced the likelihood of HAART-induced viral elimination. The best characterized and most significant of these reservoirs is a small pool of long-lived memory $CD4^+$ T cells harboring a dormant, integrated copy of the HIV-1 genome that can be readily reactivated upon cessation of therapy. The expense and long-term toxicity of the HAART regimen coupled with the prediction that the virus contained in these cells remains stable for up to 60 years renders this population of latently infected cells a major obstacle in the abolition of HIV-1 in HAART patients.

It is thought that the post-integration latency in these memory $CD4^+$ T cells arises during the transition of an activated infected $CD4^+$ T cell from the G_1 to G_0 stage of the cell cycle, and is the result of a replicative block at the transcriptional level. Because

HIV-1 replication is known to be tightly linked to cellular activation and require host transcription factors, it is likely that viral gene expression is silenced as the cell becomes metabolically inactive and returns to a resting state. This process is similar to that of anergy or memory induction, suggesting that the virus takes advantage of one of these biological processes and the factors involved in order to promote a latent infection.

Unfortunately, the specific molecular mechanisms underlying the establishment of post-integration latency have yet to be elucidated, largely due to the difficulty in isolating these cells *in vivo*, and the lack of representative *in vitro* models available. The existing models, including cell lines such as U1, ACH-2, and J-Lat, that contain integrated dormant HIV-1 provirus, and the Scid-Hu chimeric mouse infection model, are best suited for the study of the reactivation or maintenance of HIV-1 latency. Research using these systems has suggested that the recruitment of HDACs by transcription factors at the HIV-LTR may be one mechanism regulating the maintenance of HIV-1 latency in T cells (Wu 00, Gregory 01). Importantly, this data underscores that the presence of inhibitory factors may be one of several means of silencing HIV-1 proviral transcription However, the relevance and application of data from cell line models to latent HIV-1 in primary, quiescent cells *in vivo* is unclear. Furthermore, the presence of previously integrated HIV-1 provirus in these cells precludes the evaluation of the involvement of the identified factors in the generation of HIV-1 latency. Thus, we have developed a system which allows for the analysis of events that extinguish HIV transcription. Using this model, we have determined that incomplete signaling that stimulates the calcium pathway of T cell activation is capable of inducing long-term suppression of HIV-1 transcription that may represent a latent provirus. Furthermore, this experimental

approach will facilitate the identification of additional biochemical events and factors that repress HIV-1 transcription and establish and maintain provirus latency.

3.2 Materials and Methods.

Cell lines and plasmids. Human acute T cell leukemia cell line Jurkat E6-1, obtained from ATCC (Manassas, VA) was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.2 M l-glutamine. Human embryonic kidney cells 293T (ATCC) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.2 M l-glutamine. The HIV-1 infectious cDNA clone pHXBnPLAP-IRES-Nef+ (HIVPLAP) was obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH and has been described by Chen et al (Chen 96). This construct was generated by inserting placental alkaline phosphatase (PLAP) in place of the Nef gene and Nef expression restored through its reinsertion with an upstream IRES elelment.

Generation of infectious virus and infections. The generation, collection, and infection with conditioned media containing vesicular stomatitis virus glycoprotein (VSV-G) pseudotyped HIV-1 by transient transfection of 293T cells using the CaPO4 method has been previously described (Cook, 03). Conditioned media was filtered with 0.45 μm syringe filter (Whatman, Clifton, NJ) prior to infection. For HIV-PLAP viruses, infection was assessed by flow cytometry using murine anti-PLAP antibody (Sigma-Aldrich) and FITC-conjugated anti-mouse antibody (BD Pharmingen, San Diego, CA).

Enrichment of infected cell populatons using PLAP. At 3-6 days post-infection, PLAP expressing Jurkats were quantified by flow cytometry and subsequently isolated via positive selection with magnetic beads (Dynal) coated with anti-PLAP antibody as previously described (Cook 03). Bead bound cells were incubated with phospholipase C (PLC) (0.25U/10^6 PLAP positive cells) in 1% FCS RPMI at 37°C for 1h to sever cell surface PLAP and associated beads from HIV-PLAP infected cells. The resulting cells were washed once to remove PLC and recovered for use. Re-expression of cell surface PLAP on infected cells was determined via flow cytometry as described above. In some experiments, cells were treated with Ionomycin (500 nM) (Sigma Aldrich, St. Louis, MO) or DMSO (Sigma Aldrich, St. Louis, MO) immediately following PLC treatment.

3.3 Results.

In vitro model to evaluate HIV-1 transcriptional status. Due to the lack of available systems for analyzing the generation of HIV-1 post-integration latency, we developed an *in vitro* model which would allow us to identify mediators of HIV-1 transcriptional silencing. Briefly, Jurkat cells were infected with an $HIV_{HXB.2}$ clone that was engineered to express a cell surface marker, placental alkaline phosphatase (PLAP) upon viral transcription. HIV-PLAP infected cells were positively selected using an anti-PLAP antibody and magnetic beads and subsequently incubated with PLC to remove PLAP from the cell surface. PLAP re-expression was monitored via flow cytometry at various timepoints following PLC treatment (Fig.3.2). Exposure of infected cells to PLC effectively cleaved PLAP from the surface, as demonstrated by the diminished number of PLAP positive cells following PLC treatment. (Mixed infected cell population-

MFI=5.48 vs. PLC treated cells- MFI=4.18). After 20 hours, a reappearance of cell surface PLAP was observed (MFI=7.10) (Fig.3.2), indicating that provirus transcription remained active and that this system could be used to monitor HIV-1 transcriptional status. It is important to note that PLAP positive population at 20 h post-PLC treatment is a single peak indicative of high enrichment efficiency

Fig. **3-1**

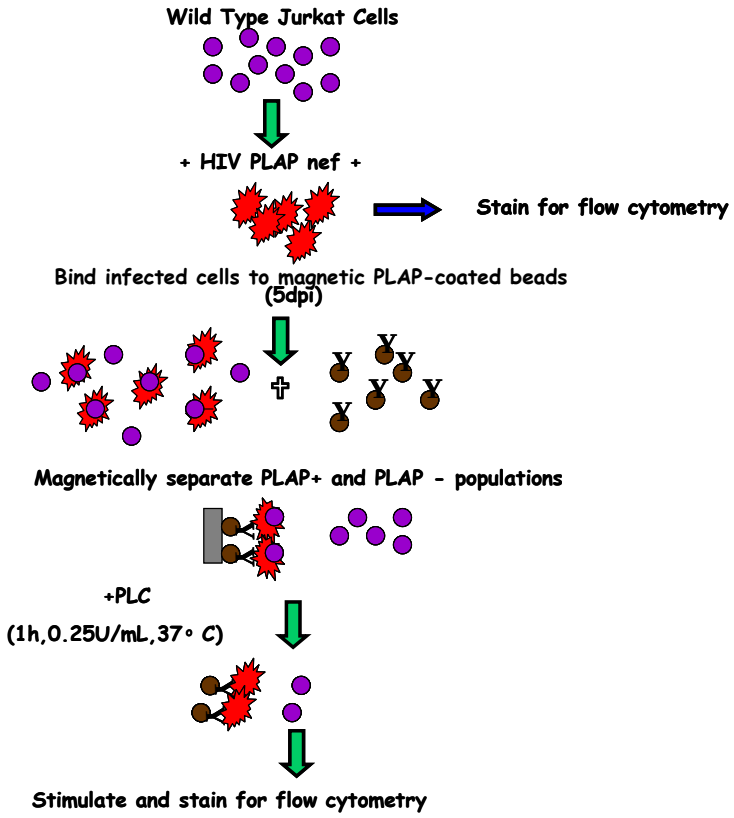


Figure **3.1-** Schematic of protocol for *in vitro* model to evaluate HIV-1 transcriptional status

Fig. **3-2**

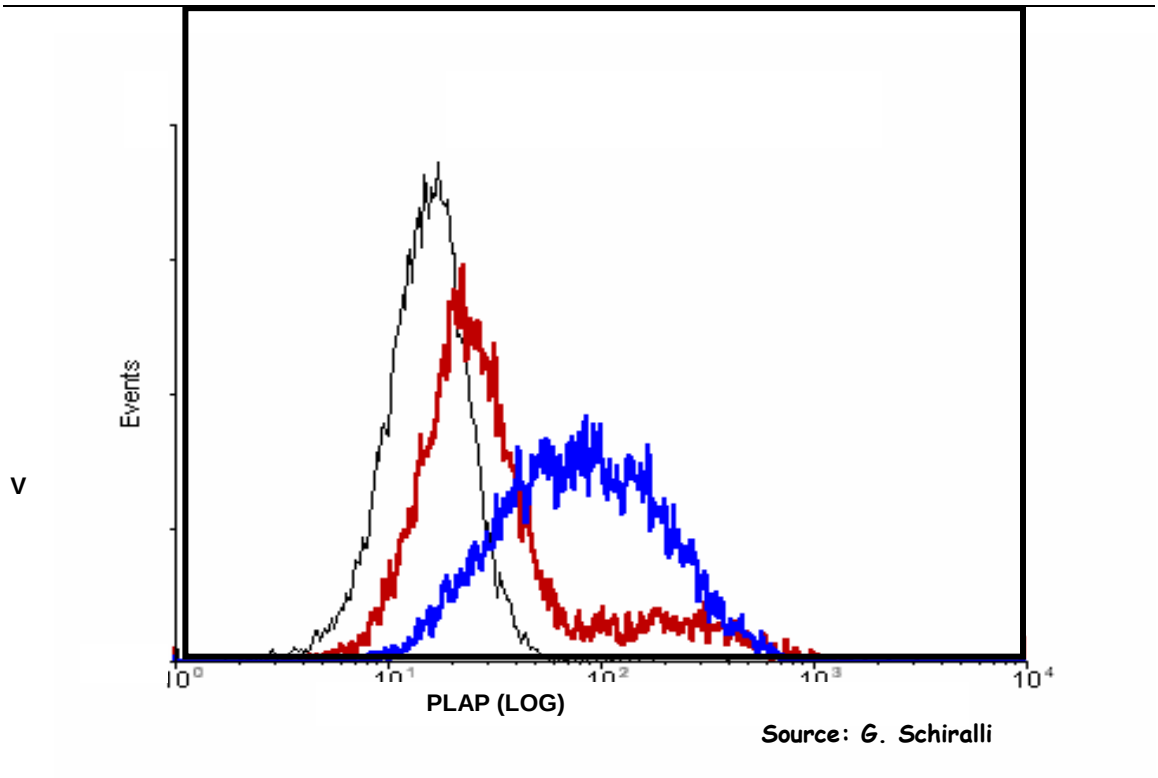


Figure **3-2**: Enrichment for HIV infected cells**.** Jurkat cells were infected with HIV-PLAP and the percentage of PLAP-expressing cells quantified via flow cytometry (red line). PLAP positive Jurkats were then isolated and surface PLAP removed via PLC treatment (black line). Surface PLAP expression was reassessed in these cells at 20h post PLC exposure (blue line). These data are courtesy of Gillian Schiralli and represent data of 3 experiments performed by Strasner and Schiralli

Calcium signaling suppresses HIV-1 transcription. We were interested in determining whether this experimental system would allow for the detection of changes in HIV-1 transcription in response to various signaling events. Complete T cell activation requires signaling through TcR/CD3, which initiates the Ca^{++} pathway and culminates in NFAT activation in conjunction with costimulatory signals, such as those delivered by CD28,

which stimulate the GTPase Rac and MAPK signaling. Recent studies have indicated that inappropriate signals may be responsible for driving T cells into an unresponsive state (Macian 02, Zha 06, Huang 03, Colombetti 02). Since efficient HIV-1 transcription requires full T cell activation, we hypothesized that incomplete activation, such as Ca^{++} signaling alone, may repress HIV-1 expression. Thus, immediately following cleavage of surface PLAP by PLC, infected Jurkats were incubated with Ionomycin, a calcium ionophore, to mimic incomplete T cell activation and determine if HIV-1 expression was suppressed. HIV-1 transcription, monitored by PLAP expression, was not inhibited after 18 h of exposure to DMSO, a vehicle control. This was illustrated by the increase in the number of PLAP positive cells relative to the immediate post PLC treatment negative control. In contrast, Ionomycin treatment resulted in a reduction of PLAP re-expression compared to DMSO, such that the intensity of PLAP staining was comparable to that observed in the PLC- treated cells (Fig. 3.3A). To determine if this was simply a transient response to Ionomycin, after an 18 h incubation, Ionomycin was removed and cells remained in culture for an additional 25 h in the absence of ionomycin prior to quantification of surface PLAP. PLAP re-expression was blunted in infected cells that had been exposed to Ionomycin as compared to their DMSO-treated counterparts (Fig. 3.3B). Together, these data imply that this experimental system is capable of detecting changes in HIV-1 transcription activity, and that incomplete signaling induced long-term negative change in HIV-1 transcriptional status in a population of infected cells.

Fig. 3-3

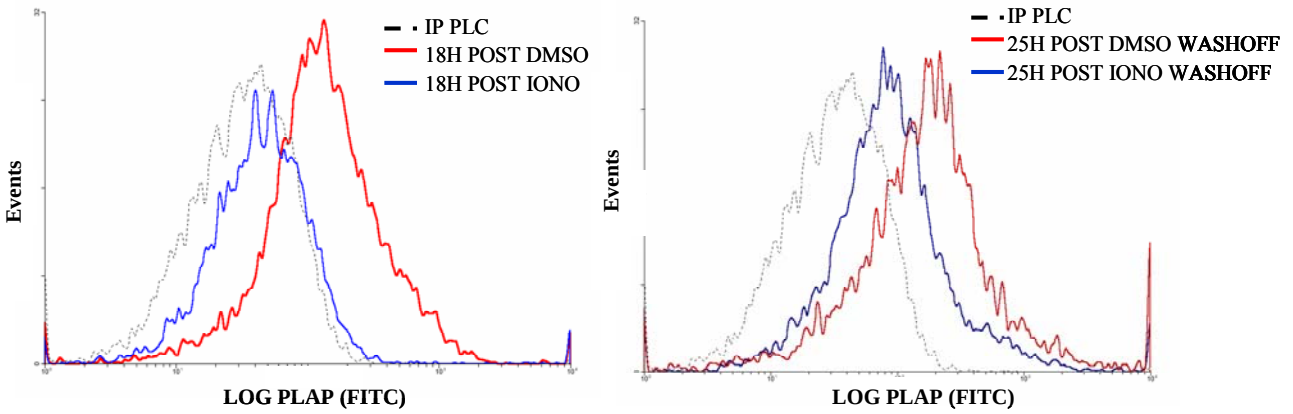


Figure **3-3**: Calcium signaling suppresses HIV-1 transcription**.** A) PLAP positive Jurkats were isolated and surface PLAP removed via PLC treatment. Following PLAP cleavage, one population of infected Jurkats was immediately stained with αPLAP for quantification of PLAP expression (black line) via flow cytometry. The remaining infected cells were treated with either DMSO (red line) or ionomycin (blue line) for 18 h prior to measurement of surface PLAP. B) After 18 h of treatment, ionomycin (10μM) or DMSO was removed from the culture supernatant and infected cells from A) ren in culture for an additional 25 h prior to flow cytometry measurement of PLAP expression. Cells stained with anti-PLAP immediately post PLC treatment (black histogram) were included as a negative control. Cell viability was comparable in cells treated with DMSO and those treated with Ionomycin.

Transcriptionally suppressed HIV-1 can be reactivated. By definition, HIV-1 latency is a reversible state of transcriptionally silenced provirus, thus, activity of a latent provirus should be restored by cellular activation. To determine whether the suppression induced by ionomycin could be reversed, infected cells that had been exposed to ionomycin for 17 h (Fig. 3.4A) and remained in culture for an additional 3 days (Fig. 3.4B) were stimulated with PMA and ionomycin. Upon activation, the reduction in PLAP expression observed in ionomycin- versus DMSO-treated cells was reversed and PLAP levels were now comparable to that of the vehicle control (Fig. 3.4C). Therefore, mitogenic T cell activation can overcome the transcriptional suppression resulting from the partial

signaling induced by ionomycin, suggesting that aspects of proviral transcription latency are being recapitulated.

Fig. **3-4**

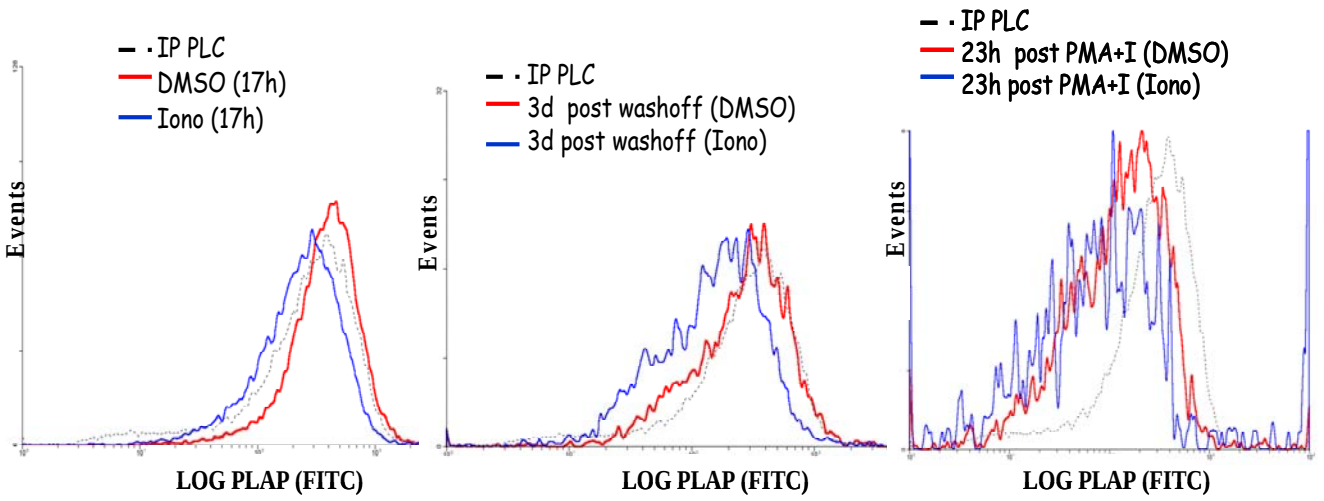


Figure **3-4** Transcriptional silencing induced by calcium signaling is reversible**.** A) PLAP positive Jurkats were isolated and surface PLAP removed via PLC treatment. Following PLAP cleavage, one population of infected Jurkats was immediately stained with αPLAP for quantification of PLAP expression (black line) via flow cytometry. The remaining infected cells were treated with either DMSO (red line) or ionomycin (blue line) for 17 h prior to measurement of surface PLAP. B) After 17h of treatment, ionomycin or DMSO was removed from the culture supernatant and infected cells from A) remained in culture for an additional 3 d prior to flow cytometry measurement of PLAP expression. C) Cells were stimulated with PMA (10 ng/mL) + Ionomycin (1 μM) for 23 h after removal of stimulus (DMSO=red line, Ionomycin=blue line). PLAP expression was determined via flow cytometry. Cells stained with anti-PLAP immediately post PLC treatment (black line) were included as a negative control

3.4 Discussion

A small population of resting memory $CD4^+$ cells harboring replication competent, latent HIV-1 provirus represents the primary barrier against eradicating HIV-1 in HAART patients. Currently, the molecular mechanisms contributing to the establishment of these latent reservoirs are unknown, largely due to the inability to study latently infected cells

in vivo coupled with the lack of available *in vitro* models. Thus, the introduction of an *in vitro* model that would facilitate the elucidation of factors regulating the generation and maintenance of HIV-1 latency is imperative to better understand and ultimately eliminate this reservoir. In this study, we have developed a novel *in vitro* system utilizing HIV-PLAP in conjunction with PLC treatment to actively monitor the quenching of HIV-1 transcription and potentially identify proteins involved in this process via signal manipulation.

Using this model, we have determined that calcium activation, in the absence of additional signaling, results in a reversible, long-term transcriptionally silent state of the HIV-1 provirus that resembles that of HIV-1 latency. This type of signaling has also been shown to attenuate IL-2 production and induce an anergic state in T cells via an imbalanced activation of NFAT relative to its binding partner, AP-1, and the subsequent up-regulation of anergy associated genes, including E3 ligases. In addition to the IL-2 promoter, NFAT has been reported to bind to the HIV-LTR at two tandem NF-κB sites in the proximal enhancer as well as an NFAT consensus sequence in the negative regulatory element (NRE) (Kinoshita 97, Pessler 04, Cron 00, Fortin 01, Robichaud 02, Barbeau 01, Cron 01). This binding has been implicated in both the activation (Macián 99) and suppression of HIV-1 transcription (Macián 99) as well as in the reactivation of latent HIV-1 provirus (Brooks 03). Interestingly, recent evidence suggests that one mechanism regulating HIV-1 latency is via chromatin remodeling resulting from the recruitment of HDACs by inhibitory factors present on the HIV-LTR (Coull 00, Williams 05, Imai 06). This information coupled with reports of NFAT's ability to recruit HDACs (Baksh 02) raises the possibility that the ionomycin-induced suppression of HIV-1 transcription

observed in our system is mediated by NFAT. Studies are currently underway to address this hypothesis.

Several weaknesses are associated with the results from the current investigation. First, although the flow cytometry data indicates that HIV-1 transcription is being suppressed, it remains unclear whether this translates into a reduction in replication as would be expected in a latently induced state. Therefore, future studies will include simultaneous measurements of virus replication as well as confirmation of the flow cytometric analysis via RT-PCR. Finally, these data have been derived from experiments involving Jurkat T cell lines. These cells are known to be highly active and exhibit extensive proliferation, therefore, a true latent state may not be inducible in these cells. In addition, a transcriptionally silent HIV-1 provirus in Jurkat cells may not be representative of the *in vivo* situation in quiescent, primary cells. Thus, an ultimate goal would be the incorporation of $CD4^+$ cells into this system.

In conclusion, we have designed a novel, *in vitro* model that will be useful for the elucidation of factors mediating the establishment of HIV-1 latency. This system has been successful in identifying a signaling pathway that is capable of reversibly suppressing HIV-1 transcription in T cells. Future research will focus on dissecting this and other pathways to determine more specific proteins and mechanisms involved in the silencing of HIV-1 expression in T cells.

Chapter 4

The Src Kinase Lck is Required for Efficient Assembly of HIV-1 at the Plasma Membrane

4.1 Introduction

HIV-1 assembly and release are driven by the viral protein Gag. Expression of Gag in the absence of any other viral factors is sufficient for the formation and release of virus-like particles (Freed 98). In T cells, HIV-1 Gag interacts with the Golgi membrane (Ono 00) and traffics through the late endosomal compartment on the way to the plasma membrane, the primary site of viral assembly and release (Ono 04). In contrast, HIV-1 is both assembled at and buds into multivesicular bodies (MVBs) in macrophages (Pelchan-Matthews 03). The observation that HIV-1 assembles at different sites in T cells and macrophages suggests that cell specific factors are operative in Gag targeting. Although several cellular proteins have been determined to be critical for HIV-1 budding (Garrus 01, Strack 03, von Schwelder 03), the pathways and cellular factors involved in regulating Gag trafficking during virus assembly have only recently begun to be identified (Alroy 05, Dong 05, Ono 04, Nydegger 03).

Lck, a lymphoid specific Src kinase found predominantly in T cells, plays a critical role in T cell activation. The majority of Lck is associated with CD4 and plasma membrane lipid rafts, however, it is also found associated with the Golgi network (Hu 01) and in the endosomal compartment (Blanchard 02, Marie-Cardine 96). Lck interacts with several ubiquitin-binding proteins which have critical functions for protein trafficking (Scott 04, Umebayashi 03) and HIV-1 release (Patnaik 00, Strack 00), although a role in

the secretory pathway has not been established. In the context of HIV-1, Lck binds the viral protein Nef, which has been implicated in altering the structure and function of the endosomal compartment (Cheng 99, Stumptner-Cuvelette 03). Lck is also activated following HIV-1 infection (Phipps 96) and mediates syncytium formation (Briand 97, Yoshida 92). Furthermore, Lck protein levels were reported to be altered in some HIV-1 patients (Cayota 94, Trebak 98), and it has been proposed that increased Lck activity following T cell stimulation leads to reactivation of latent HIV-1 (Brooks 03). Finally, Lck is present in the HIV-1 virion (Ott 00), implying an important role in the late stages of the viral life cycle. Based on these observations and its presence at critical sites of HIV-1 assembly and release, we hypothesized that Lck is a cellular regulator of HIV-1 Gag targeting. We report here that Lck assists in directing Gag to the plasma membrane and participates in efficient production of HIV-1 from T cells.

4.2 Materials and Methods

Cells and Plasmids. Human acute T cell leukemia cell line Jurkat E6-1, obtained from ATCC (Manassas, VA) and J.CaM1.6 (JCaM) (Straus 92), an Lck-deficient line derived from Jurkat were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.2 M l-glutamine. JCaM-Lck cells were generated by limiting dilution and G418 (Sigma-Aldrich) selection of JCaM cells (7.5×10^6) transfected with 15 μg pMEX-Lck by electroporation with a BTX Electro Square Porator T820 (215 V, 65 msec, low voltage, 1 pulse). Pooled cells and several JCaM-Lck clones were used for analysis. Primary mononuclear cells were isolated from whole blood using a Ficoll/Histopaque gradient (Sigma-Aldrich, St. Louis,

MO). Macrophages were separated by adherence to plastic and $CD4^+$ T cells were positively selected from the non-adherent population using a $CD4^+$ isolation kit (Dynal, Oslo, Norway). Human embryonic kidney cells 293T (ATCC) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.2 M l-glutamine. The HIV-1 infectious cDNA clone pHXBnPLAP-IRES-Nef+ (HIVPLAP) was obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH and has been described by Chen et al (Chen 96). This construct was generated by inserting placental alkaline phosphatase (PLAP) in place of the Nef gene and Nef expression restored through its reinsertion with an upstream IRES elelment. pBS-HXB2 was obtained from Dr. N. Landau (Scripps, San Diego CA). The LC1, LC2, LC1/2, and UD GFP mutant constructs were generously provided by Dr. Marie-José Bijlmakers (University College London, London, UK). The W97ALck GFP mutant was from Dr. Marietta Harrison (Purdue University, IN). The K154, F505, and R273 mutant Lck constructs were kindly provided by Dr. Jonghwa Won (Mogam BioTechnology Research Institute, Gyunggido, Korea). The mouse pCIneo-c-Src construct was from Dr. Josée Lavoie (Université Laval, Québec, Canada).

Generation of infectious virus and infections. The generation, collection, and infection with conditioned media containing vesicular stomatitis virus glycoprotein (VSV-G) pseudotyped HIV-1 by transient transfection of 293T cells using the $CaPO_4$ method has been previously described (Cook 03). Conditioned media was filtered through a 0.45μm

syringe filter (Whatman, Clifton, NJ) prior to infection. For HIV-PLAP viruses, infection was assessed by flow cytometry using murine anti-PLAP antibody (Sigma-Aldrich) and FITC-conjugated anti-mouse antibody (BD Pharmingen, San Diego, CA). HIV replication was measured by p24 ELISA (PerkinElmer, Atlanta, GA) per manufacturer's instructions. Infected $CD4^+$ T cells were sorted for PLAP expression using magnetic beads (Dynal) coated with anti-PLAP antibody, as previously described (Cook 03) and Src kinases were inhibited using 10 μM PP1 for 4 d prior to p24 measurement.

Integrated HIV-1 provirus was detected using a previously described linear Alu-based nested PCR technique (Butler 01). Briefly, 250 ng genomic DNA isolated from infected cells was amplified with primers specific for the HIV LTR and chromosomal Alu repeats (Alu3: 5'AGGCAAGCTTTATTGAGGCTTAAGC3', Alu5:5'TCCCAGCTACTCGGGAGGC TGAGG 3'), respectively. Following an initial incubation at 94°C for 3 min, 22 cycles of amplification were conducted using the following conditions: 94°C for 30 seconds, 70°C for 30 seconds, and 70°C for 5 min. A final incubation at 72°C for 10 min was performed, the resulting product diluted 10 fold, 50 fold or 250 fold as indicated and subjected to a second round of amplification using a primer set internal to the HIV LTR (NI-3: 5' GCCACTCCCCIGTCCCGCCC 3', NI-5: 5' CACACACAAGGCTAC TTCCCT 3') PCR conditions were as follows: 94°C for 12 min followed by 29 cycles of amplication at 95°C for 30 sec, 69°C for 30 sec, and 72°C for 1 min and an additional incubation at 72°C for 10 min. The final product was visualized on a 1% agarose gel. β-actin (β-actin 5': CCTAAGGCCAACCGTGAAAAG, β-actin 3': TCTTCATGGTGCTAGGAGCCA) was also amplified to serve as a loading

control.

Immunoblots. Whole cell extracts were prepared by suspending cells in lysis buffer (10 mM Tris–HCl pH 7.4, 150 mM NaCl, 1.0 mM EDTA [pH 8.0], 2.0 mM sodium vanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 1% Nonidet P-40, 1.0 mM phenylmethylsufonyl fluoride, 1.0 mM pepstatin) at 4°C for 30 min. Samples were mixed with 2× SDS loading buffer containing dithiothreitol and heated at 100°C for 3 min before resolving by SDS-PAGE with 10% polyacrylamide unless otherwise specified. Proteins were transferred to PVDF membrane (Millipore, Billerica, MA), blocked with 5% non-fat dry milk in PBS with 0.02% v/v Tween-20, and detected with primary antibodies against human Lck (Lck (3A5; Santa Cruz Biotechnology, Santa Cruz, CA), c-Src (H12; Santa Cruz Biotechnology), HIV-1 p24 (183-H12-5C, NIH AIDS Research & Reference Reagent Program), or mouse β-actin (Clone AC-15, Sigma-Aldrich) and an HRP conjugated secondary antibody against mouse IgG (Sigma-Aldrich). Blots were developed using an ECL-plus kit (Amersham Biosciences, Piscataway, NJ). For reprobing, blots were stripped with 100 mM 2-mercaptoethanol, 62.5 mM Tris–HCl (pH 6.7), 2% w/v SDS for 45 min at 65°C with intermittent shaking, and reblocked for 1 h prior to reprobing.

Transfection and assessment of virus-like particles. 293T cells were co-transfected with 10 μg of p96ZM651gag-opt (NIH AIDS Research & Reference Reagent Program as contributed by Li, Gao, and Hahn) and 10 μg of either pCI (Promega), pCI Lck, pcDNA3.1, pcDNA3.1 Lck, pMEX, pMEX Lck, LC1, LC2, LC1/2, pCIneo-c-Src, K154,

F505, or R273 using the $CaPO_4$ method as previously described (Cook 03). Virus-like particle production was quantified from cell supernatants 48 h post-transfection by p24 ELISA. Lck, c-Src, and Gag protein expression was confirmed by immunoblot.

Immunofluorescence microscopy. Cells were harvested at 5 d post-infection, washed in cold PBS, and fixed with 2% paraformaldehyde in staining media (ice cold 1% FCS/PBS) for 30 min at 22^{O}C. Fixed cells were washed twice in staining media and permeabilized with 0.1% Triton X-100 for 3 min at 22^{O}C. Following two washes in staining media, permeabilized cells were resuspended in 0.1M glycine in PBS for 10 min at room 22^{O}C. Cells were blocked for 30 min at 22^{O}C with 1% BSA/PBS prior to staining for 1 h on ice with mouse anti-HIV-1 capsid protein p17 (gag) (Advanced Biotechnologies, Columbia, MD) in 1% BSA/PBS. Two washes in staining media removed unbound primary antibody before the addition of Alexa Fluor 660 goat anti-mouse IgG (H+L) (Molecular Probes, Eugene, OR) in 1% BSA/PBS for 30 min on ice in the dark. Cells were visualized using an Olympus IX70 laser-scanning confocal microscope with a 60X (NA1.4) oil objective.

Electron microscopy. Electron microscopy was performed by Tom Doman and Douglas Key of the General Virology Laboratory at the Animal Diagnostic Laboratory at Penn State University. Cells were fixed with 2.5 % glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.4, (Electron Microscopy Sciences, Fort Washington, PA) for 2 h on ice. Three 10 min washes with cold 0.1 M sodium cacodylate buffer (pH7.4) were performed

prior to fixing cell pellets for 2 h at 22°C with 2% osmium tetroxide, (Electron Microscopy Sciences) in 0.1M sodium cacodylate buffer. After 3 washes, cells were dehydrated in a graded ethanol series, 25%, 50% , 70%, 95%, 2 x 100% for 15 min each. Infiltration of the cell pellets was accomplished with a 1:1 mixture of EMbed-Araldite resin, medium formulation, (Electron Microscopy Sciences) and propylene oxide, overnight. After 2 changes of undiluted resin for 4 h per exchange, pellets were placed into a 70°C oven overnight to cure. Ultra-thin sections were cut to a thickness of 80nm using a MicroStar diamond knife and an RMC MT-7000 ultra microtome. Sections were collected on 300 mesh copper grids and stained with 5% uranyl acetate in methanol and Reynolds lead citrate for 15 and 10 min respectively. Sections were observed and photographed using a Philips EM-400 electron microscope operated at 80KV.

4.3 Results

Lck is required for efficient HIV-1 replication. To examine if Lck regulates HIV-1 replication, Jurkat T cells and JCaM.1 cells, a Jurkat-derived cell line which lacks a functional Lck protein (Straus 92, Goldsmith 87), were infected with HIV-PLAP. HIV-PLAP expresses placental alkaline phosphatase (PLAP) on the surface of infected cells upon viral transcription (Chen 96), thus providing a marker for infected cells that can be detected by flow cytometry. At various times post-infection, HIV-1 replication as assessed by p24 ELISA was found to be reduced 2-7-fold in T cells lacking Lck as compared to those with Lck (Fig. 4.1A and B). We determined the timepoint with the largest p24 difference and the least amount of syncytium formation or cell death to be at 5d post-infection, thus all subsequent experiments were performed at this timepoint.

Flow cytometric analysis for the PLAP surface marker showed that the percentage of JCaM cells expressing HIV-1 at both 3d and 5d post infection was equal or greater than that observed in Jurkat cells, precluding differential infection and expression as explanations for the reduction in virus replication (Fig. 4.2). Furthermore, diminished HIV-1 replication in JCaM was not due to a general decrease in cellular metabolism or inability to signal since no decrease in virus replication was observed in cells lacking Zap70, a protein tyrosine kinase immediately downstream of Lck (Fig. 4.3). To confirm that the decrease in HIV production was due to the absence of Lck in JCaM cells, several clonal and pooled Lck-expressing JCaM cell lines (JCaM-Lck) were generated and tested for their ability to support HIV-1 replication. Re-introducing functional Lck into JCaM restored HIV-1 replication to levels observed in infected Jurkat cells (Fig.4.1A and data not shown).

To determine whether Lck activity was also required for efficient HIV-1 replication in primary T cells, HIV-infected $CD4^+$ T cells were treated with the Src-kinase inhibitor PP1, which inhibits enzyme maturation and kinase activity (Schmidt-Arras 05). Similar to the trend observed in the cell lines, HIV-1 replication was reduced approximately 2.5 fold in infected $CD4^+$ T cells treated with PP1 (Fig. 4.1C). PP1-treatment did not adversely affect infection since the percentage of infected PP1-treated cells was equal or greater than that of control cells as determined by flow cytometry for PLAP expression (data not shown). Although PP1 is not a specific inhibitor of Lck, Lck and Fyn are the only Src kinases expressed in primary T cells (Lowell 04). Therefore, these data, plus the results from our Lck-deficient cell lines, indicate that Lck promotes

efficient HIV-1 replication in T cells.

Fig. **4-1**

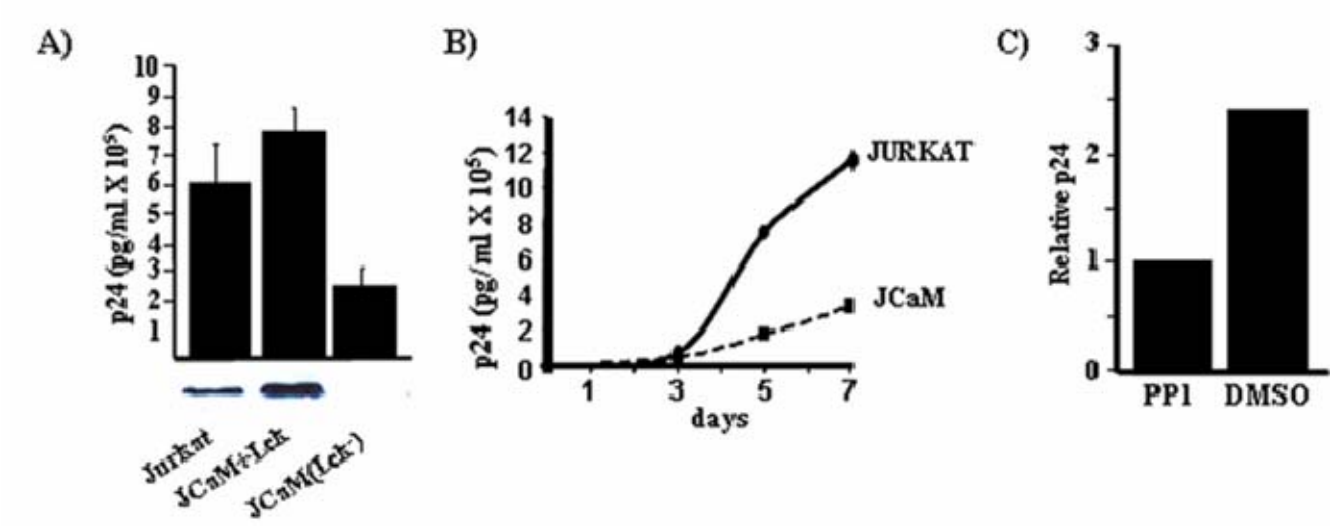


Figure **4-1:** HIV-1 replication is reduced in the absence of Lck**.** A) Jurkat, JCaM, and JCaM that stably express Lck (JCaM + Lck) cells were infected with HIV-PLAP pseudotyped with a VSV-G envelope. At 5 d post-infection, p24 ELISA was performed to assess viral replication. Lck protein expression was confirmed by immunoblot. These data are from a single experiment that includes at least 3 independent infections with error bars showing the standard deviation between infections. These data are representative of 5 independent experiments. B) Jurkat and JCaM cells were infected with HIV-PLAP and p24 was monitored over the course of 7 days. These data are from a single experiment with each data point representing three independent infections. These data are representative of three experiments. C) Positively selected primary $CD4^+$ cells were infected with HIV-PLAP treated with the 10 μm of the Src kinase inhibitor PP1 or a DMSO vehicle control 1d post-infection. 5 d post-infection, viral replication was assessed by p24 ELISA and the percentage of HIV infected cells was quantified using flow cytometric analysis for PLAP expression. Relative p24 units were calculated as p24 values per infected cell and expressed as fold difference over PP1 treated cells. For PP1 treated cells 13.1% of the cells were infected and 3301 pg/ml of p24 was detected, whereas 8.1% of the untreated T cells were PLAP+ and 4864 pg/ml of p24 were detected. These data are from a single experiment performed in triplicate and represent data from 3 experiments.

Fig. **4-2**

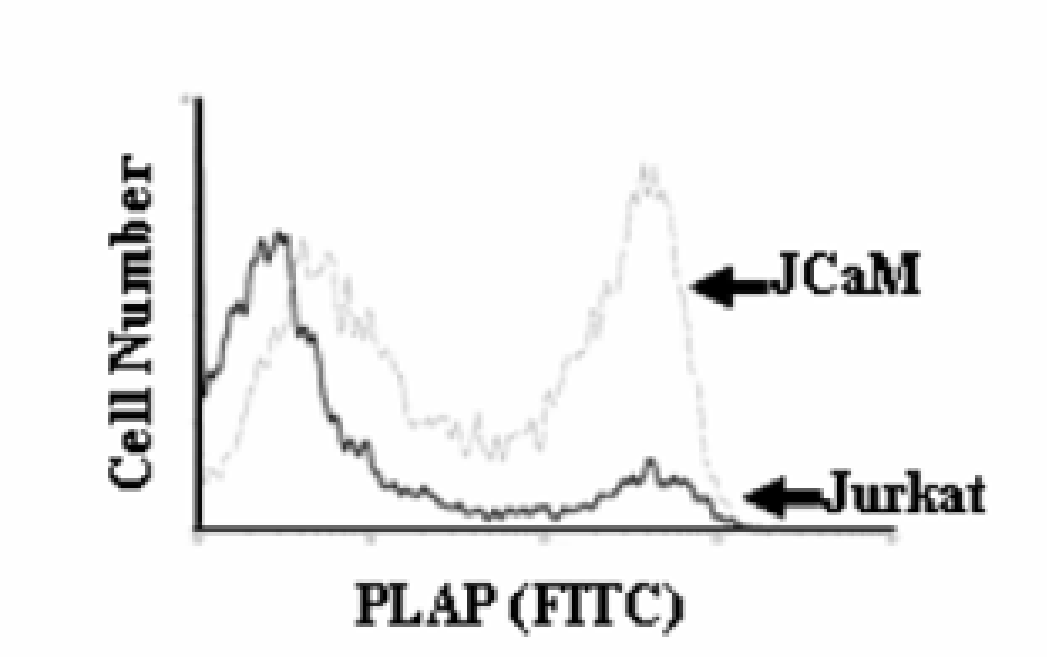


Figure **4-2** Effect of Lck on HIV-1 infection and transcription. Jurkat and JCaM cells were infected with HIV-PLAP and 5 d post-infection flow cytometry was performed using antibodies against PLAP to detect infected cells.

Fig. **4-3**

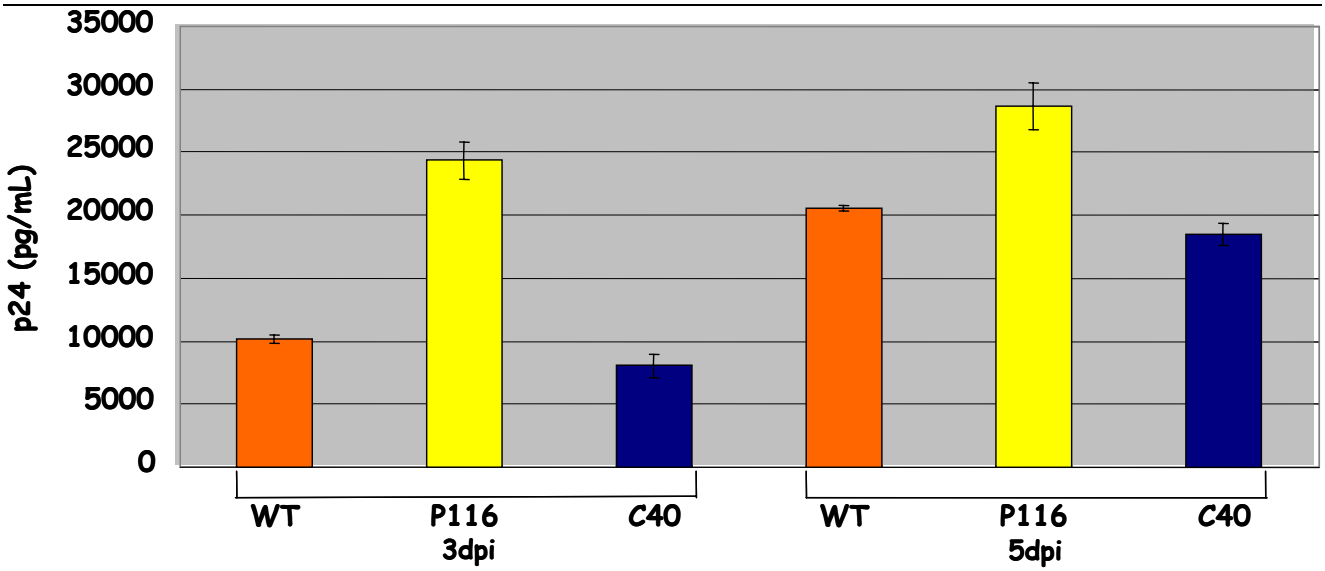


Figure **4-3**: The Role of Zap70 in HIV-1 Replication**.** Jurkat E6.1, P116, a Jurkat-derived Zap70 deficient cell line, and C40, P116 cells reconstituted with Zap70, cells were infected with HIV-PLAP and virus replication assessed at the indicated timepoints via p24 ELISA. Error bars represent the standard deviation of 3 independent infections.

Lck targets HIV-1 Gag to the plasma membrane. In order to identify Lck-dependent processes critical for HIV-1 replication, we examined HIV-1 infection and protein levels in HIV-PLAP-infected Jurkat and JCaM cells. We initially analyzed proviral integration in these cells using a semi-quantitative nested PCR technique (Butler 01). Comparable amounts of integrated provirus were observed in both infected Jurkat and JCaM cells (Fig. 4.4A and B), indicating that Lck is regulating a step after proviral integration. In addition, protein expression of HIV-1 Gag p55 and p24 were similar or greater in

infected JCAM as compared to Jurkat cells as determined by immunoblotting (Fig. 4.4C). These observations verify that Jurkat and JCAM cells are equally susceptible to HIV-1 infection and suggest that Lck is participating in late events of the virus life cycle such as virus assembly or release. To confirm that Lck is involved in HIV-1 packaging or egress, we cotransfected Lck and HIV-1 Gag into 293T cells and measured virus-like particle production by p24 ELISA. Virus-like particle production in the presence of Lck was approximately three fold that of control cells (Fig. 4.4D), signifying that Lck enhances HIV-1 Gag assembly and release. In contrast, another Src family kinase member, c-Src, did not increase virus-like particle release, suggesting that this is an Lck specific activity (Fig. 4.4E).

Fig. **4-4**

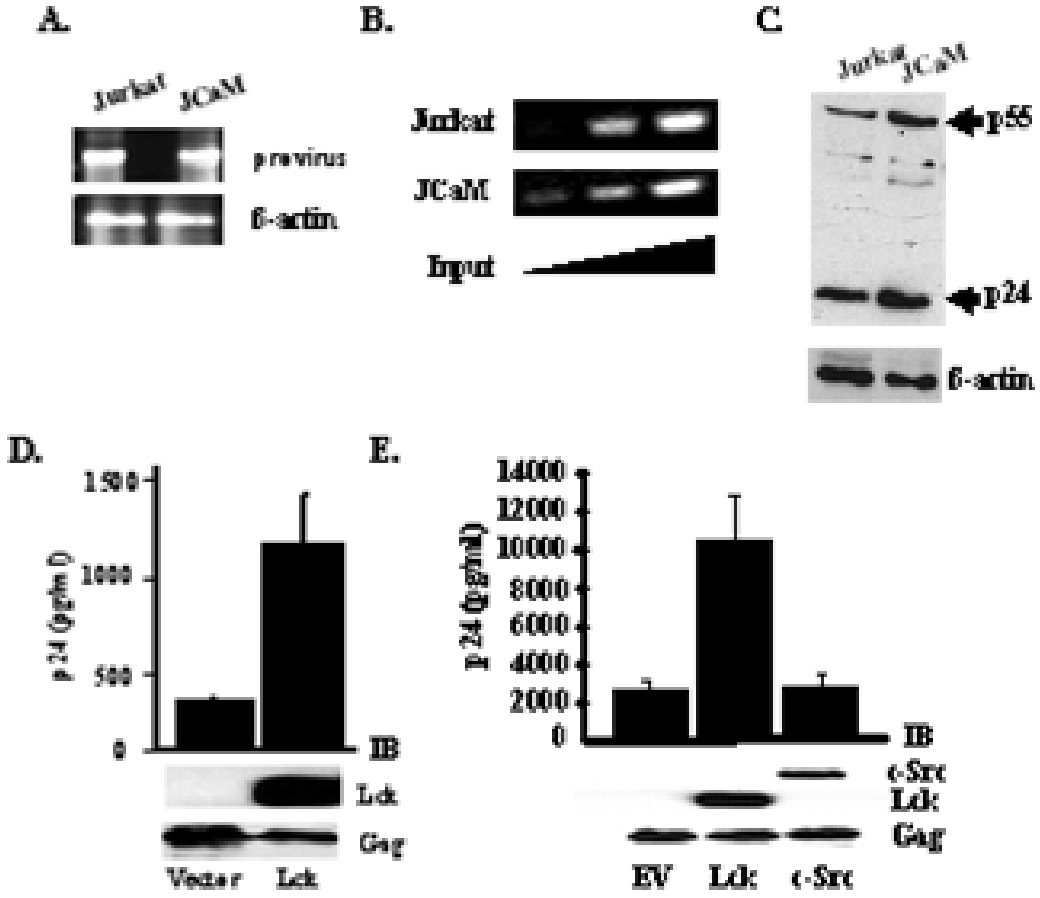


Figure **4-4** Lck is required for HIV-1 Gag assembly. A) Jurkat and JCaM cells were infected with HIV-PLAP for 5 d. Genomic DNA was isolated to measure proviral integration using a nested PCR as described in the methods section. B) A repeat of the PCR shown in A) except that the first round product was diluted by 250 fold, 50 fold or 10 fold prior to the second round of PCR to demonstrate that PCR reactions were semi-quantitative. PCR products were visualized on an ethidium bromide stained agarose gel. C) Jurkat and JCaM cells were infected with HIV-PLAP and at 5 d post-infection, cells were lysed and Gag protein expression determined by immunoblotting. D) HIV-Gagp55 and an Lck expression vector or empty vector were cotransfected in 293T cells and virus-like particle release measured by p24 ELISA. These transfections were performed in triplicate and error bars represent standard deviation between samples. Immunoblots below p24 data show protein expression in cells used in this experiment. The data are from a single experiment that is representative of 5 experiments. E) HIV-Gagp55 and a c-Src expression vector or empty vector were cotransfected in 293T cells and virus-like particle release measured by p24 ELISA. These transfections were performed in triplicate and error bars represent standard deviation between samples. Immunoblots below p24 data show protein expression in cells used in this experiment. The data are from a single experiment that is representative of 3 experiments.

Virus assembly and release are driven by Gag and preferentially occur at the plasma membrane in T cells. Because our experiments implicated Lck in these processes, we examined Gagp17 localization in HIV-1-infected Jurkat, JCaM, and JCaM-Lck cells using confocal microscopy. As expected, Gagp17 was detected exclusively at the plasma membrane in Jurkat (Fig. 4.5B) and JCaM-Lck cells (Fig. 4.5F). In contrast, Gagp17 was detected at the plasma membrane as well as intracellularly in approximately 58% of the HIV-infected JCaM cells (Fig. 4.3D). The intracellular compartment containing Gagp17 colocalized with the late endosomal marker CD63 (Fig. 4.5). Electron microscopy confirmed these results showing that viral particles were associated only with the plasma membrane in Jurkat cells but were detected both at the plasma membrane and in membrane-bound intracellular vesicles in JCaM cells (Fig. 4.6). Furthermore, Gagp17 distribution in the intracellular membranes was more extensive in Jurkat as compared to JCaM cells, in which Gagp17 was primarily associated with the late endosomal marker, CD63 (Fig. 4.7). These data suggest that Lck regulates Gag trafficking through intracellular compartments and targeting to the plasma membrane during HIV-1 assembly in T cells.

Fig. **4-5**

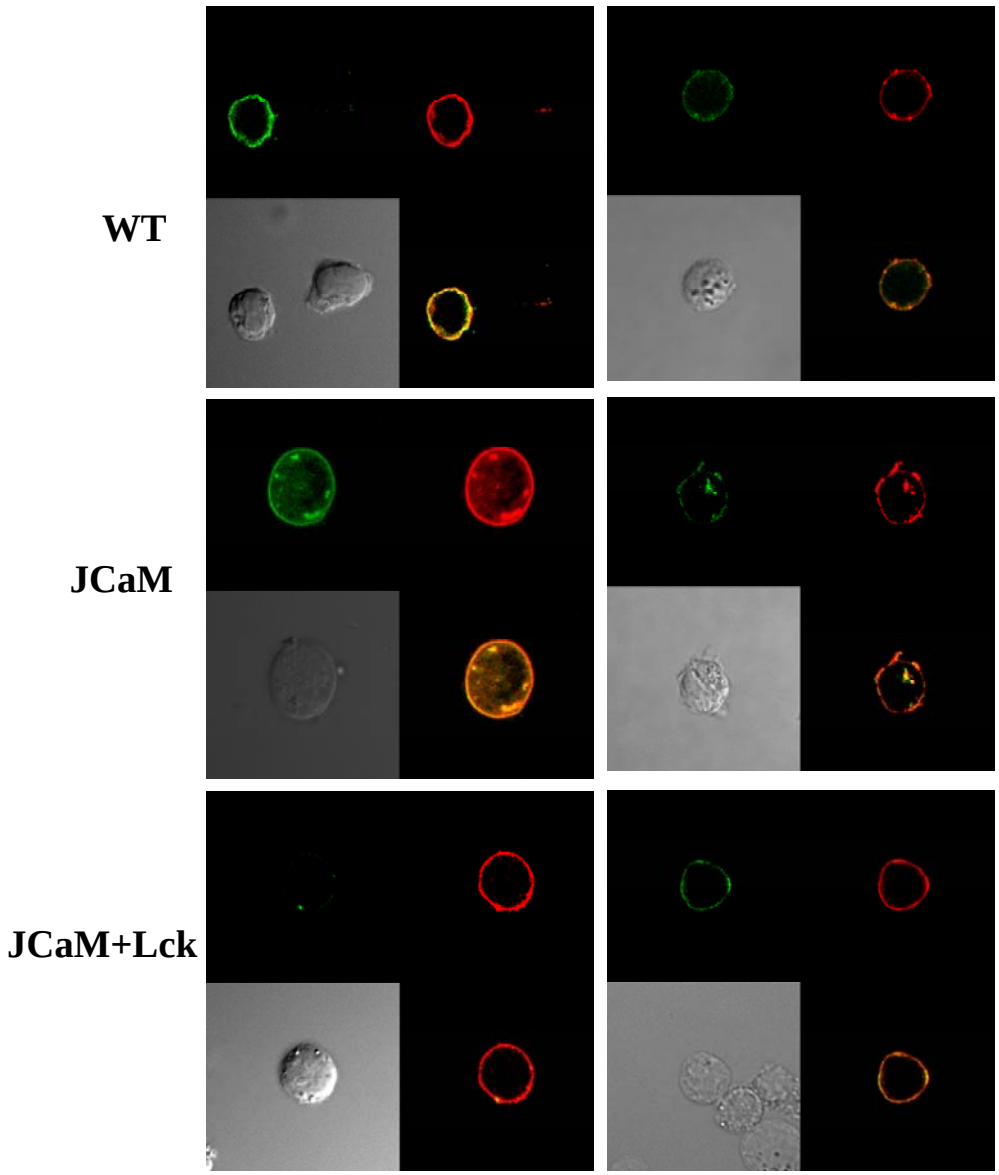


Figure **4-5**: Intracellular accumulation of Gag in the absence of Lck**.** Jurkat (A-B), JCaM (C-D) and JCaM-Lck (E-F) cells were infected with HIV-PLAP. Five days after infection, cells were stained for HIV-1 Gag p17 (red)(upper right quadrant) and CD63 (green)(upper left quadrant). The lower left and right quadrants of each panel represent phase contrast and merged images, respectively. Cells were visualized using an Olympus IX70 confocal laser-scanning microscope with a 60X (NA1.4) oil objective.

Fig. 4-6

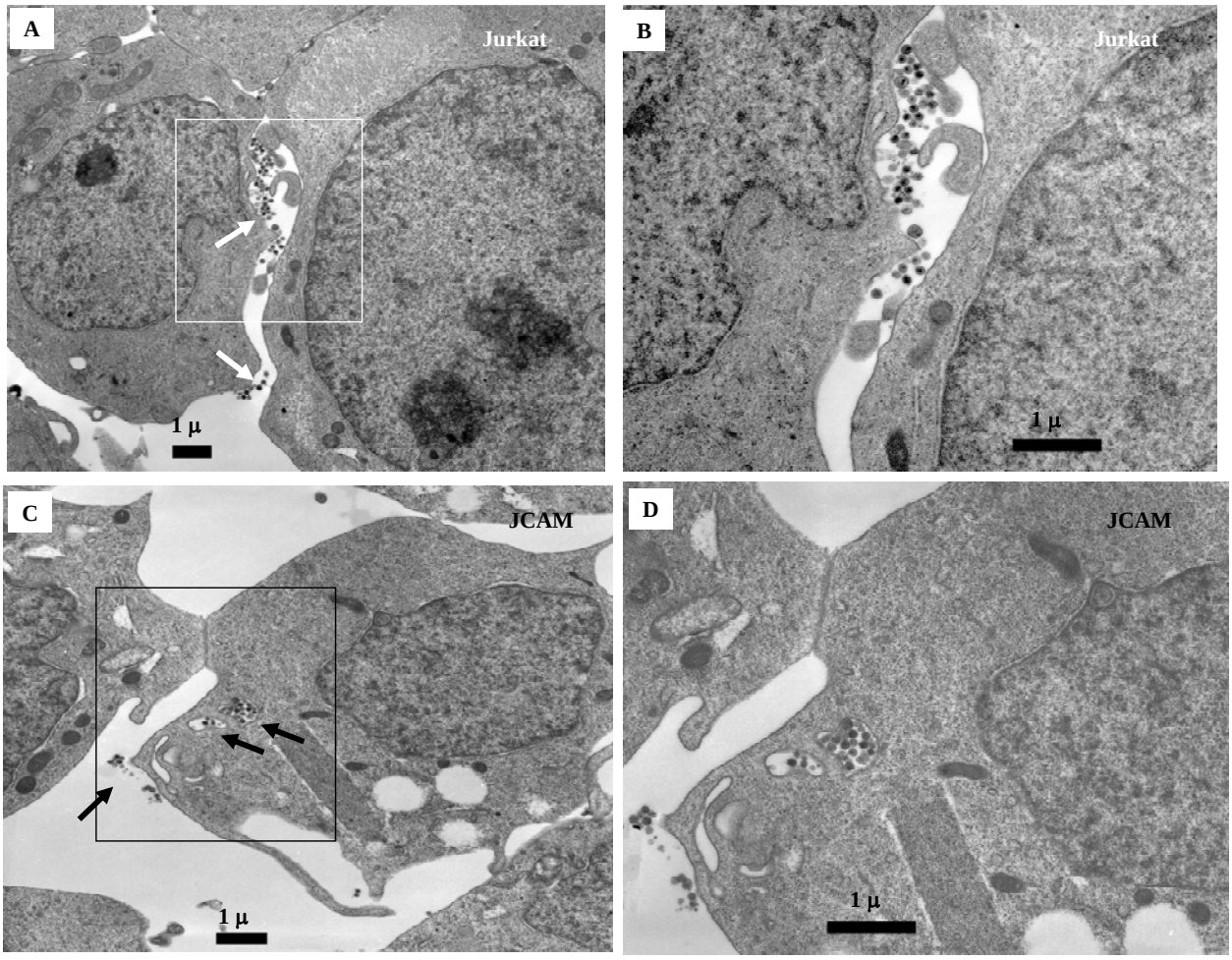


Figure **4-6**: HIV is present in intracellular vesicles in the absence of Lck. Electron micrographs of HIV-PLAP-infected Jurkat T cells (panels A and B) and JCAM cells (panels C and D). Magnification of A) and C) is 6,000X, whereas panels B) and D) are 13,000X. Boxes in panels A) and C) represent areas enlarged in panels B) and D). Arrows highlight location of virus particles. Bars on the figures represent 1 micron.

Fig. **4-7**

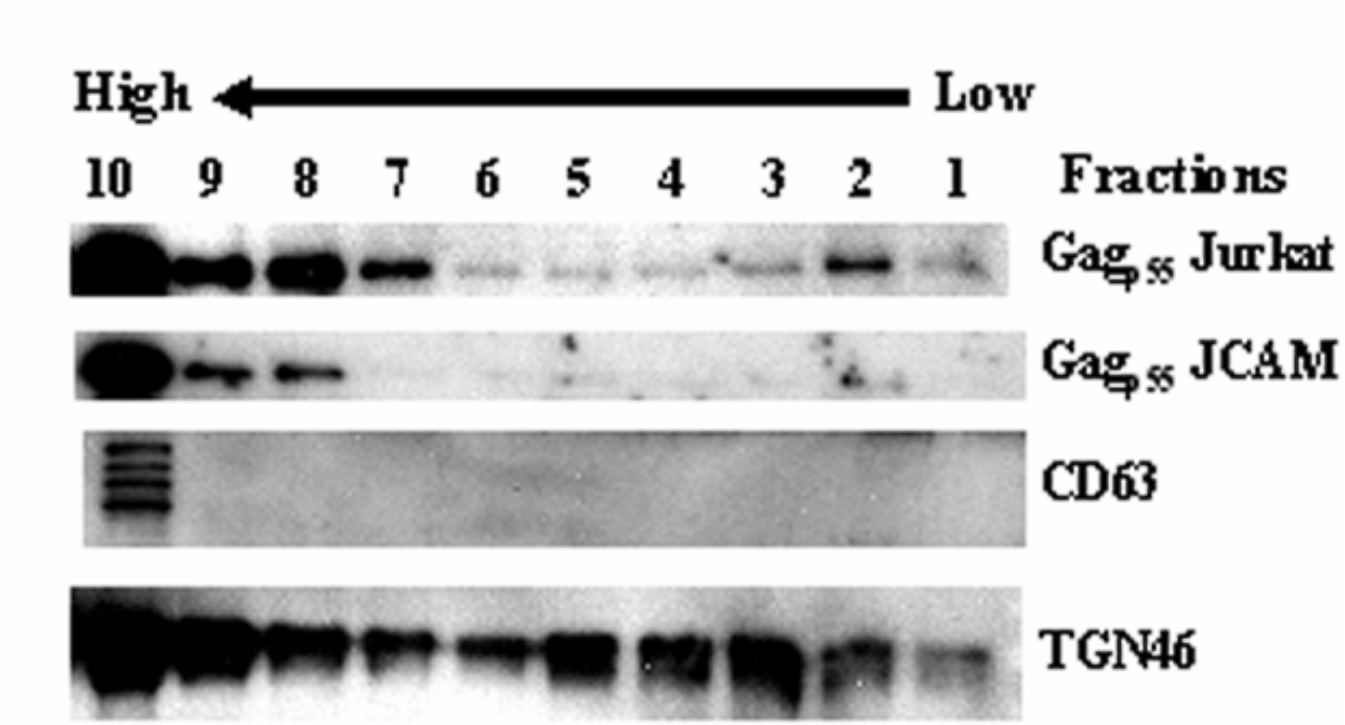


Figure **4-7**: Intracellular accumulation of Gag in the absence of Lck. Intracellular membranes from homogenized HIV-PLAP infected Jurkat and JCaM cells were isolated and separated into 10 equal fractions with an iodixanol gradient and centrifugation. Fractions from Jurkats and JCaM were probed for HIV-1 Gagp55. Jurkat cell extracts were probed for CD63 (late endosomal marker) and TGN46 (Golgi marker) expression by immunoblotting. Multiple CD63 bands represent different glycosylation isoforms. Protein content in each fraction was determined to be similar by Bradford reagent (data not shown).

Lck palmitoylation is required for efficient virus production. In order to gain insight into how Lck influences HIV-1 assembly, we cotransfected HIV-1 Gag with Lck expression constructs that harbored mutations in various functional domains. Both the SH2 (K154) and SH3 (W97ALckGFP) Lck mutants produced virus-like particles at equal or higher levels than that of wild-type Lck (Fig. 4.5B), implying that neither of these

domains are required, and may even be inhibitory, for the Lck effect on HIV-1 assembly. A kinase dead (R273) Lck supported similar or elevated levels and a constitutive active (F505) Lck provided no further enhancement in virus-like particle release compared to wild-type Lck (Fig. 4.8B), suggesting that kinase activity is dispensable for or possibly suppresses HIV-1 packaging and replication.

Palmitoylation of Lck is essential for its localization in plasma membrane lipid rafts and HIV-1 is packaged and released from these lipid rafts in T cells. Thus, we were interested in assessing the importance of Lck palmitoylation in HIV-1 replication. Lck expression constructs that included mutations in individual (LC1 and LC2) as well as both critical residues required for palmitoylation (LC1/2) were reduced by greater than 80% compared to wild-type Lck in their ability to mediate virus-like particle release. It should be noted, that previous studies have reported the kinase activity of this mutation to be comparable to its palmitoylated counterpart in unstimulated cells (Goldsmith 87). In support of this data, the Lck unique domain fused to GFP (UD GFP) was sufficient to enhance virus-like particle release to levels comparable to that of wild-type Lck. (Fig. 4.8D) These data indicate that the palmitoylation sites, but not SH2, SH3, or the kinase activity of Lck are critical for its effect on HIV-1 Gag (Fig. 4.8B, D).

Fig. **4-8**

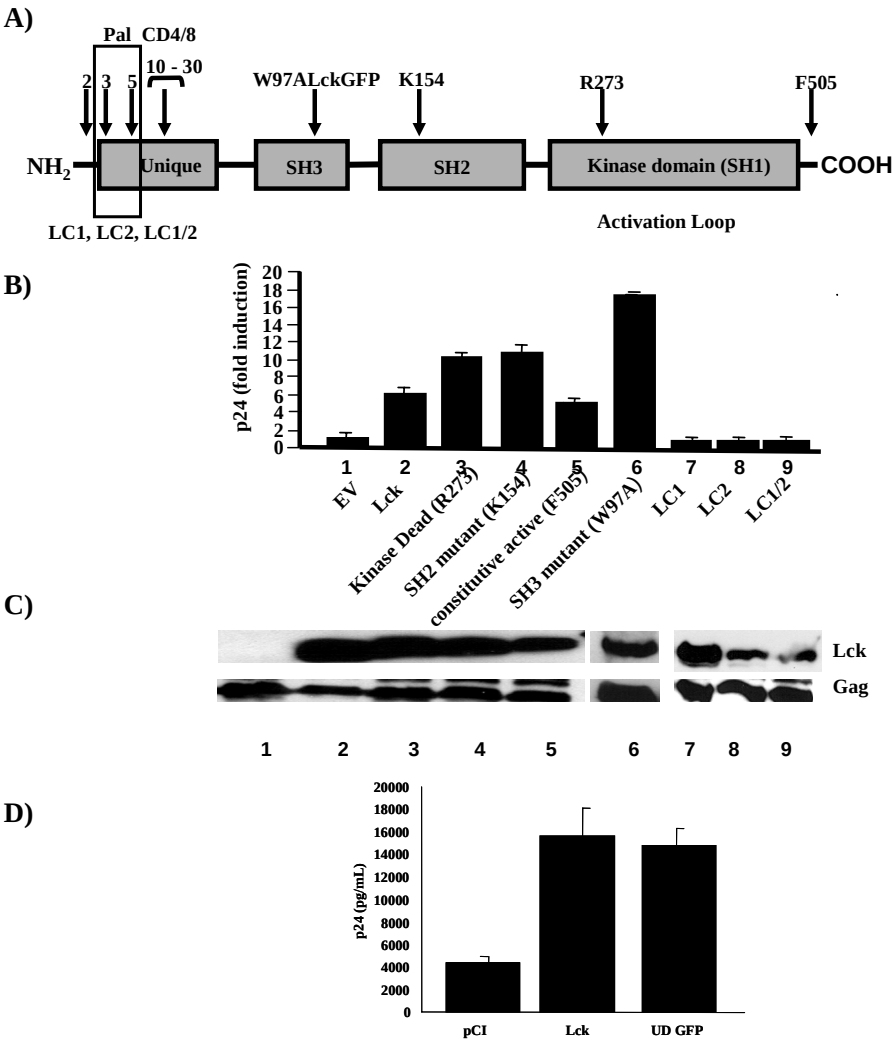


Figure **4-8**: Lck palmitoylation is required for efficient HIV-1 replication**.** A) Schematic of Lck structure and point mutations. B) 293T cells were cotransfected with HIV-1 Gag_{p55} and pCI, pCI Lck, Lck R273, Lck F505, Lck K154,W97A Lck GFP, LC1, LC2, LC1/2 and virus-like particle release measured by p24 ELISA**.** These transfections were performed in triplicate and error bars represent standard deviation between samples. C) Immunoblots below p24 data show protein expression in cells used in this experiment. This is a composite figure. These data represent three independent transfection experiments. D) 293T cells were cotransfected with HIV-1 Gag_{p55} and pCI, pCI Lck, or UD GFP and virus-like particle release measured by p24 ELISA**.** These transfections were performed in triplicate and error bars represent standard deviation between samples

4.4 Discussion

Previous studies have suggested that Lck plays a role in HIV-1 transcription, replication and pathogenesis (Briand 97, Yoshida 92, Coudronniere 98, Tremblay 94, Yousefi 03). In this report, we have identified a novel function for Lck in the later stages of the HIV-1 life cycle, specifically viral packaging. The ability of Lck to directly influence HIV-1 Gag in 293T cells implies that this activity of Lck is specific, CD4-independent, and distinct from its role in T cell signaling. The observation that HIV-1 replication occurs in the absence of Lck indicates that Lck is not necessary for but does increase the efficiency of HIV-1 Gag assembly. In the absence of Lck, HIV-1 Gagp17 accumulated intracellularly and colocalized with CD63 in addition to being detected at the plasma membrane. These data imply that Lck facilitates the progression of HIV-1 Gag from the intracellular compartments to the plasma membrane.

HIV-1 assembly and budding occur at the plasma membrane in T cells (Barre-Sinoussi 83, Gelderblom 87, Levy 84). In contrast, HIV-1 is both packaged and released into the multivesicular bodies (MVB) in macrophages. Recent evidence indicates that ubiquitin and components of the vacuolar protein sorting (Vps) pathway are required for HIV-1 assembly and budding in both cell types (von Schwelder 03, Nydegger 03). However, the pathways and mechanisms by which HIV-1 Gag couples to this machinery and is targeted to the site of virus assembly are not well defined. The distinct locations for HIV packaging and egress in different cell types suggest that cell-specific factors partially determine the site of virus assembly. Lck, a T-cell specific Src kinase, is located at both the plasma membrane (Bijlmakers 99) and in microvesicles (Blanchard 02), and

binds the ubiquitin binding proteins p62 (Vadlamudi 96) and c-Cbl (Rao 02). In fact, a recent report demonstrated an accumulation of Lck in the endosomal compartment of HIV-1 infected cells as compared to uninfected cells (Thoulouze 06). It is possible that Lck and c-Cbl play a role in targeting proteins into these intracellular vesicles (Blanchard 02). This possibility is currently under investigation. Furthermore, Lck may indirectly interact with several components of the cellular protein sorting pathway, including the adapter complexes AP-1 and AP-3, TGN38/41, Rab6, and atypical PKCs. Thus, Lck may regulate HIV-1 assembly by acting as an adapter protein for these or other cellular and viral proteins, including HIV-1 Gag (Fig 4.9).

Fig. **4-9**

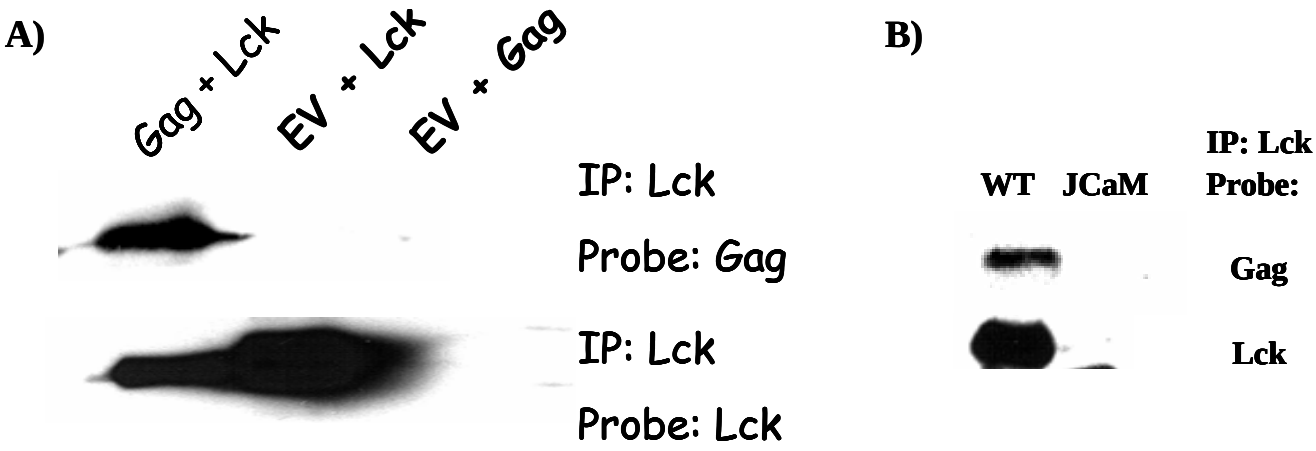


Figure **4-9**: Lck and HIV-1 physically interact**.** A) Lck was immunoprecipitated from whole cell lysates of 293T cells cotransfected with the indicated combinations of empty vector (EV), HIV-1 Gagp55, and Lck expression constructs and probed with either anti- HIV-1 Gag p24 antibody or anti-Lck antibody. Lck and HIV-1 Gag expression were confirmed by immunoblotting whole cell extracts (data not shown). B) Lck was immunoprecipitated from HIV-1 infected whole cell WT Jurkat or JCaM1.6 lysates and probed with either an anti-HIV-1 Gag p24 or Lck antibody.

This is supported by data demonstrating that both single and double Lck palmitoylation mutants were unable to rescue efficient HIV-1 replication. These results imply that Lck palmitoylation and plasma membrane localization are critical for the ability of Lck to impact HIV-1 replication. Interestingly, the Lck SH3 mutant (W97ALckGFP) enhanced virus-like particle production over that of wild-type Lck. Based on the contribution of Lck palmitoylation to its role in HIV-1 assembly, we speculate that the higher activity observed with this mutant may be explained by its hyper-palmitoylated state (Stumptner-Cuvelette 03). Lck is palmitoylated on the intracellular membranes of the early exocytic pathway, which allows for its subsequent transport to the plasma membrane (Bijlmakers 99). Thus, HIV-1 Gag may be usurping

this activity for HIV-1 assembly. Studies to further evaluate adapter function as a mechanism for Lck-dependent HIV-1 assembly are currently underway.

The primary function of Lck is as a tyrosine kinase and it is possible that Lck enhances HIV-1 assembly via phosphorylation of adapter complex proteins. However, our data indicates that the Lck kinase domain is dispensable for its effect on HIV-1 Gag assembly. We are aware that these findings seem to contradict our data demonstrating a reduction in HIV-1 replication in primary $CD4^+$ cells treated with PP1. This discrepancy may be explained by the effect of PP1 on enzyme maturation in addition to its influence on Lck kinase activity (Schmidt-Arras 05). Interestingly, Yousefi et.al. (Yousefi 03) reported an inverse relationship between Lck kinase activity and HIV-1 replication in T cell lines. Thus, it is likely that Lck adapter activity is mediated through direct binding rather than phosphorylation of proteins. In addition, we show that neither the SH2 or SH3 domain is required for the ability of Lck to promote HIV-1 virus-like particle release. Therefore, the unique domain of Lck is likely to be mediating the Lck adapter activity necessary for the enhancement of HIV-1 assembly. One potential mediator of the effect of Lck on HIV-1 assembly and release is the viral protein Nef. Lck physically interacts with Nef (Cheng 99), and Nef has been shown to induce functional and structural changes in the endosomal compartment (Stumptner-Cuvelette 03). However, although Nef may be influencing Lck activity, it is not likely to be critical for the Lck-induced enhancement of HIV-1 assembly for several reasons. First, it was not present in the cotransfection experiments where this effect was observed. In addition, Nef binds the SH2 and SH3 domains of Lck which are dispensible for mediating virus-like particle release.

It is interesting to note that Src family kinases have been implicated in influencing the replication of a number of viruses, including hepatitis B virus (Klein 97,99), vaccinia virus (Newsome 04) and mouse polyoma virus (Messerschmitt 97). Furthermore, herpesvirus saimiri encodes a protein, Tip, which recruits Lck into endosomal vesicles (Park 02). In addition, HIV-1 Vif has been shown to interact with the Src kinase Hck in macrophages (Hassaine 01). Finally, it has been suggested that c-Yes contributes to RSV budding and release (Garnier 96). Similar to our findings for Lck and HIV-1, c-Yes plays a role in the transit of the assembled West Nile virion from the ER through the cellular secretory pathway (Hirsch 05). These data suggest that Lck or other Src kinases may have more general roles in virus replication, including virus assembly and release.

Chapter 5

Conclusions

HIV-1 is highly dependent on T cell activation and has evolved to manipulate the cellular environment to promote its own replication and survival. However, surprisingly little is known regarding the interplay between the virus and intermediates of the T cell signaling network during the course of HIV-1 infection. Thus, the purpose of this thesis was to examine the influence of host cellular proteins in the TcR/CD3/CD28 network on HIV-1 at various stages of its replication cycle. We demonstrated that T cell signals both positively and negatively regulated HIV-1 gene expression, and that the outcome was dependent on the quality of the applied stimulus. Complete T cell activation resulted in optimal HIV-1 transcription that was associated with the binding of a specific factor with a potential CD28 response element (HIV-CD28RE) between -182 and -205 in the HIV-LTR. In contrast, a novel *in vitro* experimental approach revealed that induction of the calcium pathway in the absence of additional signals suppressed HIV-1 expression and promoted a reversible, transcriptionally silent state of the provirus that resembles HIV-1 latency. In addition to transcription, proteins in the T cell signaling pathway promoted HIV-1 assembly and release. Specifically, the Src family kinase Lck facilitated the progression of HIV-1 Gag through the intracellular compartments to the plasma membrane for efficient virus release. Thus, HIV-1 exploits T cell signaling proteins at several stages of the viral life-cycle to ensure its propagation and survival.

5.1 Calcium signaling represses HIV-1 provirus transcription:

Using a novel, *in vitro* system to monitor HIV-1 transcriptional suppression, we determined that the exclusive activation of calcium signaling via ionophore stimulation extinguished HIV-1 gene expression and resulted in a dormant proviral state. Similar to these findings, ionophore-induced calcium release has been shown to promote T cell anergy via an imbalance of NF-AT activation relative to its binding partner, AP-1 and the subsequent up-regulation of anergy-associated genes (Macian 02, Mueller 04, Heissmeyer 04). Calcium signaling has also been shown to inhibit IL-4 transcription by increasing nuclear entry of NF-ATp with respect to NF-ATc (Brogdon 02). Furthermore, the binding of NF-AT to its putative motif in the modulatory region of the HIV-1 LTR attenuated HIV-1 gene expression (Macian 99). Thus, it is possible that HIV-1 transcriptional silencing in response to calcium signaling is mediated by NF-ATp binding the NRE of the HIV-LTR and directly inhibiting transcription.

Alternatively, it has been proposed that HIV-1 latency is regulated via chromatin reorganization into a repressive environment. Acetylation of histone tails has been associated with an open chromatin structure and active transcription. In contrast, histone deacetylases (HDACs) are correlated with a condensed chromatin structure and have been detected at the latent HIV-LTR. In addition, NF-AT is known to repress gene expression via recruitment of HDACs (Baksh 02). Thus, it is possible that calcium signaling following ionophore stimulation results in NF-ATp association with the tandem NF-κB sites and the subsequent recruitment of HDACs to the HIV-LTR enhancer, which ultimately promotes chromatin reorganization and a latent proviral state. As HIV-1 latency is characterized by a closed chromatin structure, it will be important to determine

whether calcium signaling induces stable epigenetic alterations of the HIV-LTR and whether this effect is mediated by NF-ATp. This can be addressed through the assessment of nucleosome remodeling and changes in DNA accessibility in response to ionomycin stimulation via restriction enzyme analysis. The role of NFATp and potential chromatin reorganization in suppressing HIV-1 gene expression can be investigated using ChIP technology to evaluate NFATp binding and histone 3 acetylation at the HIV-LTR in ionomycin-treated cells. Furthermore, histone deacetylase (HDAC) involvement in calcium-induced HIV-1 transcriptional silencing can be evaluated by measuring PLAP re-expression in cells treated with ionmycin in the presence and absence of TsA, an HDAC inhibitor.

Finally, it is plausible that the HIV-1 transcriptional response to calcium activation is independent of NF-AT. Thus, additional molecular targets of calcium signaling should be evaluated as mediators of HIV-1 transcriptional suppression. Similarly, the complexity in the regulation of HIV-1 latency suggests that multiple mechanisms are operative in the establishment and maintenance of a transcriptionally silent provirus. We have developed a novel *in vitro* model which can be adapted to study many different signaling environments and should be utilized for the identification of various cellular intermediates and mechanisms involved in the generation of HIV-1 latency. An additional benefit of this system is the inclusion of cell surface PLAP as an indicator of infected cells. This allowed for the use of an antibody directed against PLAP to quantify and isolate HIV-1 infected cells for the study of the biochemical events occurring specifically in this population. An example of how this strategy can be employed is the recent paper by Yang and Henderson (Yang 05) in which the use of

PLAP to enrich for infected cells led to the identification of c-Cbl as a target for the viral protein, Nef. Alternatively, alkaline phosphatase staining with NBT was employed to detect PLAP infected cells (Appendix A). This technique will allow for the development of high-throughput screen to identify inhibitors and activators of HIV-1 transcription.

5.2 The Role of Lck in HIV-1 assembly:

Lck is a Src family kinase member best known for its integral role in T cell activation and signal transduction in response to antigen presentation. In addition, Lck has been implicated in HIV-1 transcription, replication, and pathogenesis. We have uncovered a unique role for Lck in the late stages of the HIV-1 life cycle, namely the assembly and release of the virus. In the absence of Lck, virus particles accumulated in intracellular compartments containing the late endosomal marker, CD63, and virus replication was attenuated. This finding implied that Lck facilitates the progression of Gag from the MVB's to the plasma membrane for efficient virus release. The role of Lck in HIV-1 assembly was confirmed by the ability of Lck to directly amplify Gag-induced virus-like particle release from 293T cells. Unexpectedly, the kinase activity of Lck was dispensible for this effect, suggesting that Lck adaptor activity was regulating HIV-1 assembly. This function of Lck was also not mediated by its SH2 or SH3 domain. Thus, the most likely possibility was that the Lck effect was mediated by the unique domain (UD). In addition to an N-terminal myristoylation site, the UD of Lck contains two palmitoylation sites, located at cysteine 3 and 5, which are responsible for targeting Lck to the plasma membrane. Indeed, we found that the palmitoylation of Lck was essential for its enhancement of virus- like particle production, suggesting that the Lck UD was

mediating HIV-1 assembly. In support of these findings, expression of the Lck UD was sufficient for the enhancement of virus-like particle release from 293T cells. Furthermore, the UD of Fyn, which is also palmitoylated, fused to Gag promoted the association of this chimeric protein with membrane lipid "barges" and increased virus-like particle production (Lindwasser 01).

Lck is detected in the endosomal compartment (Blanchard 02, Marie-Cardine 96), and Lck palmitoylation occurs at the exocytic membranes, allowing for its subsequent transport to the plasma membrane. Together, these data suggest that Lck binds Gag, either directly or indirectly, via its unique domain and is subsequently palmitoylated at the MVB membrane. Upon palmitoylation of Lck, both Gag and Lck may localize to the plasma membrane for virus assembly and release (Fig. 5.1). Preliminary data indicated that Lck and Gag physically interact (Fig 4.9) and that the UD of Lck is sufficient for this association (data not shown). The Gag domain that mediates this interaction remains unknown. Several domains of HIV-1 Gag have been associated with virus assembly (Fig. 5.2). First, the M domain, comprised of the N-terminal myristoylation site and the basic residue cluster in MA are critical for Gag membrane targeting (Resh 05). The I domain contains several important assembly regions, including the major homology region (MHR) in the N-terminus of CA (amino acids 153 to 172), which is essential for particle formation, the C terminal domain of CA, which contributes to assembly, p2, which is required for higher order multimerization (Morikawa 00), and the N- terminus of NC which has been implicated in assembly based on its interaction with viral RNA (Morikawa 03). Furthermore, basic residues in NC bind host proteins, such as HP68 (Lingappa 06), which are involved in virus assembly, and a dileucine-like internalization

motif within CA mediates Gag association with MVBs (Resh 05). Thus, it would be reasonable to assume that Lck binds to the M, CA, p2, or NC domain. It is interesting to note that the interaction between Lck and CD4 covers a dileucine internalization signal that is required for AP-2-mediated endocytosis of CD4 (Batonik 05). This motif is similar to that found in CA, making this domain an intriguing possibility for the Gag-Lck interaction.

Fig. **5-1**

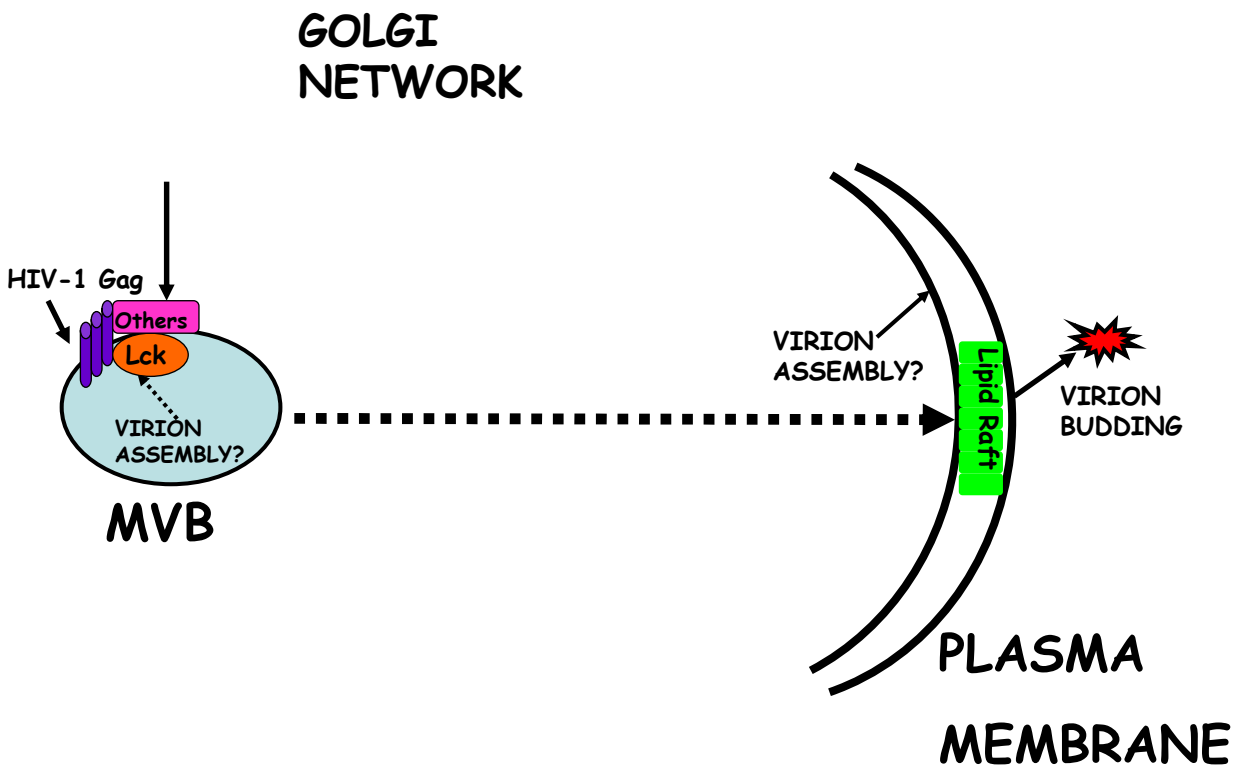
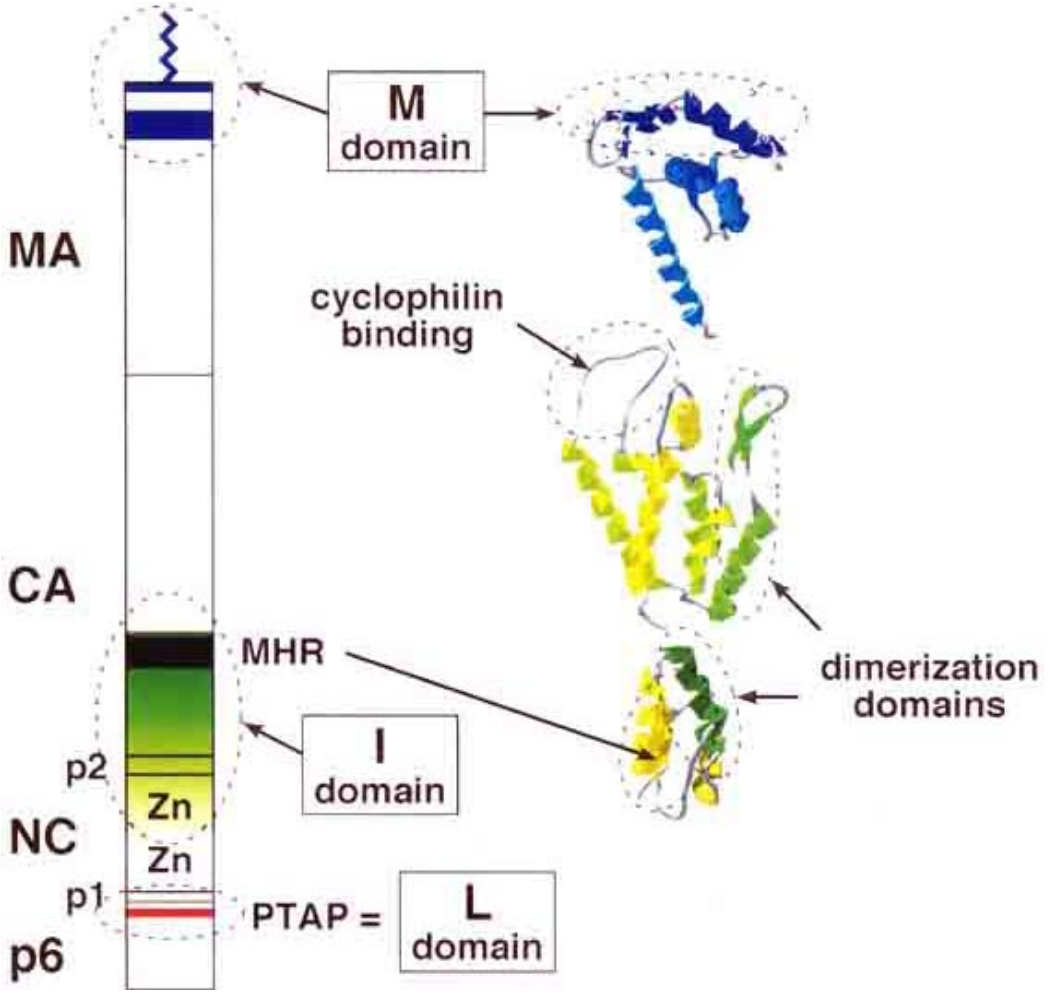


Figure **5-1**: **Proposed model for the role of Lck in HIV-1 Assembly**. Lck binds to HIV-1 Gag and likely other unidentified proteins in the membrane of the multivesicular body and targets HIV-1 Gag to the lipid rafts in the plasma membrane for efficient viral release.

Fig. **5-2**



http://www.bentham.org/chivr/sample/chivr1-1/Morikawa.pdf

Figure **5-2 Schematic of HIV-1 Gag and its domains**

Currently, the molecular mechanisms involved in the Lck mediated enhancement of HIV-1 assembly and egress are unknown. It is likely that Lck and Gag are part of a multiprotein complex that mediates HIV-1 packaging and release. Host factors involved in ubiquitination or the ESCRT protein sorting pathway are known to bind Gag and contribute to virus packaging and egress. Thus, direct or indirect interactions of Lck with

numerous endosomal and ubiquitin-binding factors (Fig. 5.2) represent potential regulatory mechanisms of HIV-1 assembly and release. For example, one attractive candidate is the viral protein Nef. In addition to binding Lck, the Nef-induced dissociation of the Lck/CD4 complex is required for CD4 endocytosis (Kim 99). Furthermore, Nef has been implicated in altering the structure and function of the endosomal compartment (Cheng 99, Stumptner-Cuvelette 03). These functions of Nef involve its interaction with and stabilization of adaptor complexes, such as AP-3 and AP-1, and may contribute to the trafficking of intracellular proteins (Madrid 05, Piguet 99, Coleman 06). Lck has also been shown to interact with the ubiquitin ligase c-Cbl, and Nef mediates the phosphorylation of c-Cbl in an Lck-dependent manner (Yang 05). Additionally, the association of Lck with Hsp90 is essential for the proper synthesis and subsequent membrane binding of Lck (Bijlmakers 00). Interestingly, TGN38/41, a trans-golgi network (TGN) integral membrane protein, has been shown to bind the SH2 domain of the tyrosine kinase Syk (Stephens 97), the ubiquitin-binding protein p62, and Rab6 (Jones 93). Lck interacts with p62, (Vadlamudi 96), thus TGN 38/41, p62, Rab 6, and Lck may form a multiprotein complex. TGN38/41 cycles between the plasma membrane and the TGN (Reaves 93), and the interaction between p62, Rab6, and TGN38/41 is vital in the budding of exocytic vesicles from the TGN (Jones 93). In theory, the association of TGN38/41, p62, Rab6, and Lck could mediate Gag exocytosis from the intracellular compartment to the plasma membrane. In addition, atypical PKCs are anchored by p62 in the lysosome-targeted endosomal compartment, which is essential for growth factor receptor trafficking (Sanchez 98). Therefore, any combination of the above interactions, in addition to potential associations with the more traditional protein

sorting machinery, could mediate the Lck-induced enhancement of HIV-1 assembly and release.

Fig. **5-3**

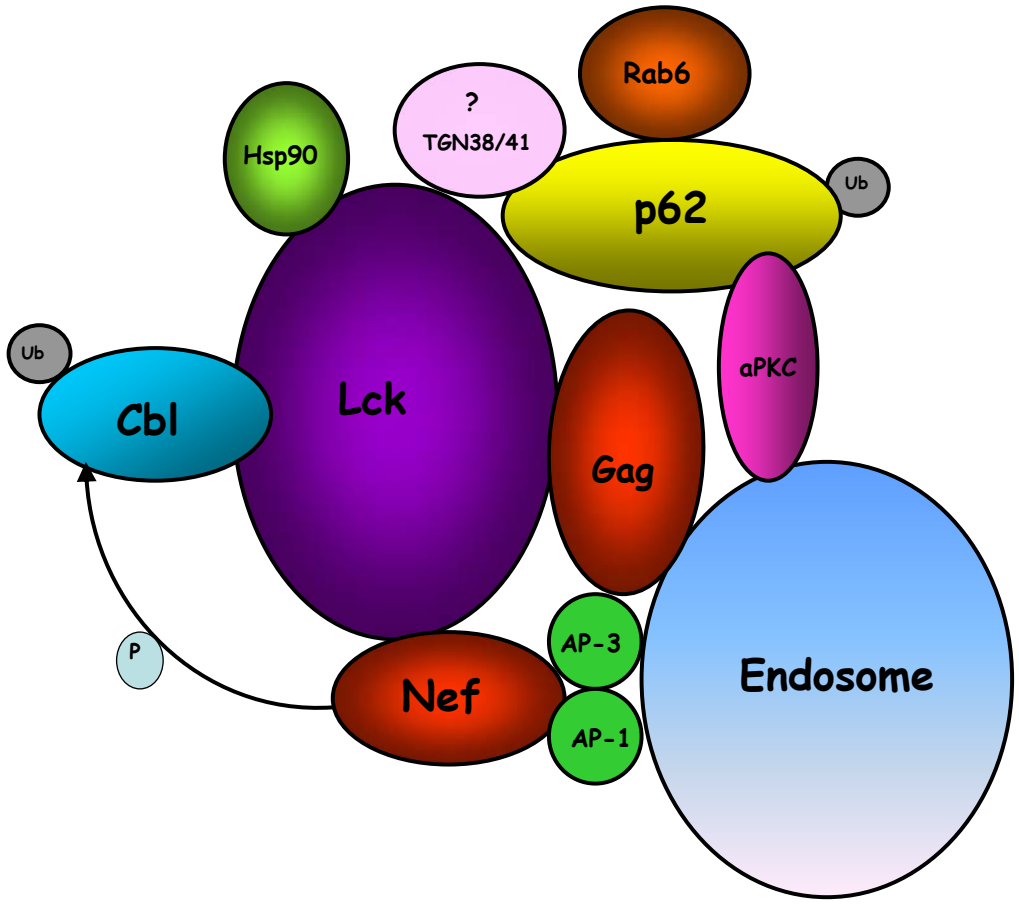


Figure **5-3**: Schematic of Lck-interacting proteins

Previous investigations regarding the role of Lck in HIV-1 replication concluded that the initial level of endogenous Lck kinase activity inhibits some aspect of the viral life cycle, but that eventually, the virus, or a viral protein such as Nef, may target Lck to facilitate

HIV-1 replication (Yousefi 03). In light of this finding and the previous information contained in this thesis, the following overall model for the Lck-mediated enhancement of HIV-1 assembly and release is proposed: HIV-1 infection activates Lck kinase activity, which in turn, inhibits HIV-1 transcription. By three days post infection, Nef targets Lck, possibly via the dissociation of the Lck/CD4 complex during CD4 endocytosis. In conjunction with adaptor protein complexes such as AP-3, Nef initiates the formation of the endosomal compartment and simultaneously transports Lck to these vesicles. Once in the intracellular compartment, Lck binds HIV-1 Gag and other host proteins and is subsequently palmitoylated on the vesicular membrane. In addition, it is possible that Gag is monoubiquitinated by p62 or c-Cbl at some point during this process. Lck palmitoylation allows for the localization of Lck and its interacting proteins to the plasma membrane for efficient release. Of course, this is highly speculative and additional research is required to elucidate the underlying molecular mechanisms of Lck-mediated HIV-1 assembly and egress. Lck is known to interact directly with proteins such as c-Cbl, HIV-1 Nef, and HIV-1 Gag and indirectly with various members of the protein sorting pathway (Fig. 5.3). Investigation into the role of these interactions using cotransfection, siRNA, coimmunoprecipitation, or mutational strategies may provide insight into the means by which Lck influences HIV-1 assembly. The Gag domain(s) required for the Lck-Gag interaction can be identified by performing coimmunoprecipitation experiments using fusion constructs of the various Gag domains and GST. Unidentified proteins interacting with Lck and Gag can be elucidated using mass spectrometry. Determination of the requirement for processes such as ubiquitination and exocytosis may further enhance our understanding of the mechanism

underlying Lck-mediated HIV-1 assembly. Finally, live-cell imaging of HIV-1 Gag progression through cells with and without Lck could help to delineate the specific contribution of Lck to Gag targeting and HIV-1 assembly.

Together, this thesis illustrates the ability of HIV-1 to utilize various techniques throughout the course of its life cycle to manipulate T cell signaling proteins and facilitate its own replication and survival.

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Appendix

Alkaline Phosphatase Staining for HIV-PLAP expression

This experiment was performed in order to adapt the PLAP-PLC *in vitro* transcriptional model for potential use in a high throughput screen.

Fig. **A-1**

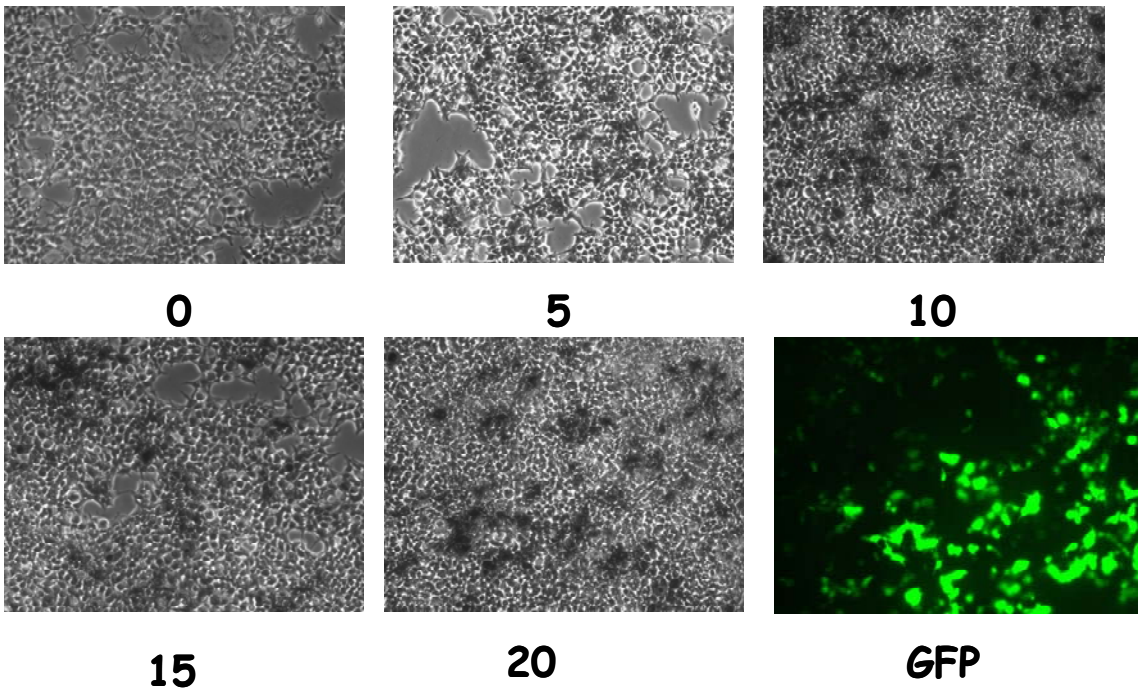

Figure **A-1**:

Appendix. Alkaline Phosphatase staining for HIV-PLAP. 293T cells were transfected with increasing doses of pHXBnPLAPIRESn+ (HIV-PLAP), in a 5:1 ratio with LVSVG and RSV-Rev DNA plasmids via the calcium phosphate method. Numbers

below the figures represent values of HIV-PLAP DNA(μg). Cells transfected with peGFP were included as a positive control for transfection efficiency. Cells were then stained for alkaline phosphatase using the PLAP Reporter Assay (Invivogen, San Diego, CA) per manufacturer's instructions. These results indicate that this is a practical method for the quantification of HIV-PLAP expression that will allow for the development of high-throughput screen technologies to identify inhibitors and activators of HIV-1 transcription.

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