THE ROLE OF RHOA SIGNALING PATHWAYS IN REGULATING HIV-1 REPLICATION

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ABSTRACT

Rebecca Jo Loomis: The Role of RhoA Signaling Pathways in Regulating HIV-1 Replication
(Under the direction of Dr. Lishan Su)

RhoGTPases are involved in regulating important cellular processes including cytoskeletal organization, gene transcription and membrane vesicle transport – poising RhoGTPases to link these pathways to HIV-1 replication. However, RhoGTPase function in HIV-1 replication is essentially unknown.

RhoA activation can be modulated by the cytoplasmic tail of the HIV-1 transmembrane envelope glycoprotein through its interaction with p115RhoGEF, a RhoA specific activator. Furthermore, activation of the RhoA signaling pathway inhibits HIV-1 gene expression.

To investigate how RhoA pathways modulate HIV-1 replication, I tested individual RhoA effectors. Rho-associated kinase (ROCK) specifically inhibits HIV-1 gene expression in T cells. A ROCK specific inhibitor counteracts RhoA inhibition of HIV-1 gene expression, indicating that RhoA signals via ROCK to inhibit HIV-1. HIV-1 LTR mutations in either the NF-κB or Sp1 binding sites abrogate ROCK-mediated inhibition. ROCK may disrupt the cooperative interaction between NF-κB and Sp1 or modulate epigenetic regulation to inhibit HIV-1 gene expression.

Additionally, I identified a disparate role for the RhoA effector, citron kinase. Citron kinase preferentially enhances HIV-1 virion production by promoting exocytosis. This

activity depends on the leucine zipper, Rho-binding and zinc-finger domains, but not the kinase activity, of citron kinase. Although citron kinase-mediated enhancement of HIV-1 replication is late domain independent, an intact late endosomal sorting pathway is required. Citron kinase may act as a scaffold, linking host proteins to the endosomal sorting and exosomal pathways. I also found a functional interaction between citron kinase and the ESCRT-I component, Tsg101. Ectopic expression of either citron kinase or Tsg101 enhances HIV-1 virion production, however, coexpression of citron kinase and Tsg101 inhibits HIV-1 replication. This functional interaction is mediated by the kinase activity of citron and the PTAP-binding region of Tsg101. The two activities of citron kinase involved in HIV-1 regulation are distinct and independent of one another.

In summary, I identified two RhoA effectors, ROCK and citron kinase to regulate HIV-1 replication. ROCK specifically inhibits HIV-1 gene expression while citron kinase demonstrates a disparate role. These RhoA effectors may provide novel targets for developing antiviral therapeutics and further our understanding of the role of RhoA signaling pathways in HIV-1 replication.

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INTRODUCTION

Human immunodeficiency virus (HIV-1) is the causative agent of AIDS. The AIDS pandemic, with over 40 million HIV-1 infected individuals, is one of the worst tragedies of modern times. As of December 2005, UNAIDS and WHO estimated that 37-45 million people live with HIV-1 (Figure 1A), 4.9-6.6 million people were newly infected in 2005 and 2.8-3.6 million people died with AIDS (Figure 1B). Sub-Saharan Africa is the worse-affected area with 23.8-28.9 million people currently living with HIV-1. Two thirds (64%) of all HIV-1 infected people are living in Sub-Saharan Africa [1]. South and South East Asia are the second most affected regions with 18% of all HIV-1 infected people [1]. In 2005, the steepest increases in HIV-1 infection (25% increase) occurred in Eastern Europe, Central Asia and East Asia [1].

HIV-1 was first reported in North America, Western Europe and parts of Sub-Saharan Africa. The pandemic is now worldwide and has become primarily a heterosexually transmitted disease, disproportionately affecting socially and economically underprivileged populations. The spread of HIV-1 in developed countries appears to be slowing [1], but in developing nations, particularly Africa, Latin America and Asia, is rapid and has been characterized as “out of control”.

HIV-1 is transmitted in blood, semen and vaginal secretions and occurs most commonly via sexual intercourse, sharing of HIV-1 contaminated needles and vertical

transmission from mother-to-child during pregnancy and childbirth [2]. Individual susceptibility to HIV-1 is variable, may change over time and is influenced by genetics, relative levels of different cell types, patterns of cytokine secretion and cellular activation and the expression of cell surface cytokine receptors which act as coreceptors required by the virus for cellular entry [3-7].

Progression of HIV-1 Infection

The major cause for AIDS progression in HIV-1 infected individuals is the decline of $CD4^+$ T cells which are the primary target for HIV-1. $CD4^+$ T cells elicit specific humoral and cell-mediated immune responses necessary to fight infections. Approximately 50% of cases in primary HIV-1 infection remain asymptomatic, whereas the other half of patients develop flu-like symptoms within the first few weeks following infection. During primary infection, viral titers are extremely high in the peripheral blood and the number of $CD4^+$ T cells decreases significantly [8].

The pathogenesis of HIV-1/AIDS is best described as the result of two sequentially distinct phases with different pathogenic mechanisms (Figure 2). The first phase; acute viral infection is characterized by a rapid and massive, directly HIV-1 mediated, loss of memory $CD4^+$ T cells found in the mucosal tissues and have a profound effect on immune system function [9-12]. The second phase; chronic infection, typically lasts for several years and is characterized by the immune system's struggle to recover from the earlier assault. Chronic HIV-1 infection results in a slow decline of $CD4^+$ T cells in the peripheral blood, a low fraction of infected $CD4^+$ T cells and increased death rates of both $CD4^+$ and $CD8^+$ T cells.

Once there is sufficient depletion of $CD4^+$ and $CD8^+$ T cells, the host is unable to adequately fight opportunistic infections and viral titers increase, leading to death.

HIV-1 infection requires the engagement of both a receptor and coreceptor on the cell's surface by the HIV-1 envelope glycoprotein for entry of the viral genome into the cytoplasm to occur (Figure 3, 4). CCR5 is the coreceptor most commonly used by primary isolates at the onset of HIV-1 infection and is present on macrophages and a subset of $CD4^+$ T cells [13]. Individuals with a 32-base pair deletion in the CCR5 gene (Δ32) are highly resistant to infection by primary HIV-1 isolates [14]. As the virus continues to replicate, evolutionary pressures result in mutations in gp120 allowing the virus to utilize CXCR4, a receptor present on the majority of $CD4^+$ T cells.

One of the most striking observations about HIV-1 is the extensive genetic variation that the virus can exhibit within a single individual, most notably the hypervariable region of the *env* gene [15]. HIV-1 is one of the fastest evolving organisms. The rapid evolution of HIV-1 is the result of a myriad of factors. First, the virus experiences a high rate of mutation with the viral reverse transcriptase making ~0.2 errors per genome during each replication cycle [16] with further errors occurring during transcription from DNA by RNA Pol II polymerase [17]. Second, HIV-1 has remarkable replication dynamics with its viral generation time being ~2.5 days and producing ~10^{10}-10^{12} new virions per day [18].

Many factors contribute to the detrimental effect of HIV-1 on an individual. Understanding how the virus has evolved to usurp necessary cellular mechanisms to facilitate its replicative capacity and how it evades the immune system are key factors in developing effective control strategies.

HIV-1 Life Cycle

In addition to the four major proteins, Gag, Pro, Pol and Env, encoded by all retroviruses, HIV-1 is a retrovirus that also encodes six accessory proteins that help regulate and control viral replication (Figure 5) [19, 20]. Gag encodes the major structural proteins, Pro and Pol encode the major enzymatic activities such as the viral protease, reverse transcriptase and integrase, and Env encodes the envelope glycoproteins required for recognition and binding of the cellular receptors and viral entry. HIV-1 also encodes Tat which is critical for HIV-1 LTR transcription, Rev which plays a role in transport of viral RNAs from the nucleus to the cytoplasm. Additionally, HIV-1 contains the accessory proteins, Vpu, Vif, Vpr and Nef which are not uniformly required for viral replication. The HIV-1 life cycle can be considered as a sequence of steps which are regulated by both viral and cellular proteins, although many steps are likely to occur in concert (Figure 6).

The HIV-1 glycoprotein consists of two noncovalently associated subunits, gp120 and gp41, that are generated by proteolytic cleavage of a precursor polypeptide, gp160 [21, 22]. Gp120 directs target-cell recognition and viral tropism through interaction with the cell-surface receptor, CD4 and one of several coreceptors, most commonly CCR5 or CXCR4 [23-28]. The CD4 glycoprotein is expressed on the surface of T lymphocytes, monocytes and dendritic cells, the main target cells for HIV-1 infection *in vivo*. The primary function of CD4 binding is to induce conformational changes in the V1/V2 hypervariable region of gp120 glycoprotein, allowing exposure of the secondary chemokine receptor binding site in the V3 hypervariable region (Figure 3, 4).

The membrane-spanning gp41 subunit promotes fusion of the viral and cellular membranes, a process resulting in the release of the viral contents into the host cell. In

addition, gp41c has been implicated in replication and cytopathogenicity of HIV-1. Gp41c has been found to have a role in maintaining stability of the envelope glycoprotein in its oligomerized form [29, 30] and mediating envelope viral protein incorporation into mature virion in a cell-type dependent manner [31-34]. SIV gp41c isolated from human cells contained a premature stop codon, truncating the cytoplasmic domain of gp41, and this truncation was found to be an adaptation of a non-human primate lentivirus to growth in human cell lines because when the SIV-isolated from human cells was put back into simian primary cells, the gp41c reverted to full-length [35, 36]. HIV-1 or SIV mutants with defective gp41c have cell-type and species-dependent phenotypes suggesting host factors potentially mediate functions of gp41c.

Upon viral entry into the host cell, viral uncoating occurs, involving cellular factors and the viral proteins Gag matrix, Nef and Vif. The viral RNA genome is reverse transcribed into a full-length double-stranded DNA copy by the viral reverse transcriptase [37, 38]. This preintegration complex [39], directed by HIV-1 Vpr, docks to the nuclear membrane [40], enters the nucleus through the nuclear pore [41, 42] and is inserted into the host chromosome by the viral integrase [43-45]. Once the proviral DNA is integrated, the first rounds of proviral transcription and translation occur [46]. The newly synthesized viral RNAs are transported to the cytoplasm. The *env* gene is translated into the precursor protein gp160, which is glycosylated within the endoplasmic reticulum. After translation, the env proteins migrate and insert into the plasma membrane.

The Gag-Pro-Pol polyprotein precursor is synthesized to produce the Gag and Gag-Pro-Pol polyproteins (Figure 7). Gag polyprotein is a 55 kDa precursor that is proteolytically processed during the maturation of the virus into six structural proteins (matrix, capsid,

nucleocapsid, late domain and 2 spacer proteins, p2 and p1) which rearrange and produce the mature virion. A regularly occurring ribosomal -1 frameshift, which occurs 5% of the time during translation, leads to the expression of the Gag-Pro-Pol polyprotein that additionally encodes the enzymatic proteins; protease, reverse transcriptase and integrase [47]. Gag and Gag-Pro-Pol polyproteins move to the cellular membrane and start to assemble, a process directed by the Gag polyprotein [48-50]. Additionally, viral enzymes, genomic RNA and cellular components associate with the immature core [51-53]. Later on, this complex buds through the plasma membrane producing an immature virion [19, 54-57]. Budding triggers the activation of the viral protease that autocatalytically cleaves the Gag and Gag-Pro-Pol polyprotein precursors releasing the structural proteins and enzymes. The individual proteins undergo further interactions, with capsid and nucleocapsid forming the conic nucleocapsid, matrix remains associated to the viral envelope [48, 58-60] and the late domain mediates the final step of viral release [47, 61, 62] with these interactions resulting in the formation of a mature, infectious virion.

Mature HIV-1 virions are spheres of 100-120 nm in diameter, with a lipid bilayer membrane surrounding a dense truncated cone-shaped nucleocapsid core that contains the genomic RNA molecules, the viral protease, reverse transcriptase, integrase, Vpu, Vif, Vpr and Nef [38, 63] (Figure 8). The HIV-1 genome consists of two identical 9.2 kb single stranded RNA molecules within the virion.

HIV-1 LTR and Transcription

HIV-1 requires specific and fine-tuned levels of transcription and the strength of the LTR promoter represents an evolutionary optimum in terms of viral fitness. HIV-1 gene

expression is controlled by the interplay of viral factors (Tat, Rev, Nef) and host regulatory proteins (NF-κB), that bind the LTR in a cell-specific and cell activation-dependent manner [64-70]. Too high transcription rates may disturb this well balanced process and over-activation of HIV-1 transcription may exhaust one or more factors present in limiting amounts, resulting in a decrease in infectivity [71]. Tat transactivation may be influenced by Sp1 elements in the HIV-1 core promoter regions since loss of Sp1 elements in LTR dramatically reduces Tat-mediated LTR activity [72].

The core viral promoter is comprised of TATAA box and three Sp1 binding sites – this is the region necessary for efficient HIV-1 proviral DNA-mediated basal transcription [73, 74] (Figure 9). Immediately upstream of the three Sp1 sites is an enhancer region with two inducible NF-κB binding sites and although they are not absolutely necessary for viral replication, they respond to cellular activation signals by stimulating LTR activity and increasing the rate of viral production [73, 75]. Further upstream is the modulatory region with numerous transcription factor binding sites that may increase or decrease transcription [73]. The modulatory region contains binding sites for NFAT, C/EBP, LEF-1 and ATF/CREB [76].

In contrast to the genetic variation found in HIV-1 envelope, the enhancer/proximal region of the HIV-1 LTR is relatively invariant [77-80]. The HIV-1 enhancer/proximal region in most clades of HIV-1 is composed of two NF-κB sites [75] and three Sp1 sites [74]. These sites are highly conserved among divergent isolates suggesting an important regulatory function and non-random association [81]. NF-κB and Sp1 sites function well in both macrophages and T cells, the cell-types most commonly infected by HIV-1 [82, 83].

NF-κB/Rel family members are involved in transcriptional regulation of a number of cellular and viral genes. These family members form hetero- and homodimers between five mammalian subunits p50, p52, p65, c-Rel and RelB, binding with different affinities to a group of related NF-κB DNA binding sites with a consensus sequence of GGGTNNYYCC [84, 85]. Dimerized NF-κB factors are blocked from nuclear translocation by cytoplasmic IκBs until signaling processes lead to induced IκB degradation and nuclear translocation of NF-κB [86-90]. NF-κB/Rel dimers exert specific functions through non-identical DNA binding site preferences, as well as through individual interactions with other promoter-bound gene-specific or basal factors [91, 92]. The ubiquitous transcription factor Sp1 contains three zinc finger DNA binding domains and four transactivation domains [93-95] and binds to GC-rich sites [96, 97].

To induce HIV-1 gene expression, a specific interaction between NF-κB and Sp1 bound to adjacent sites occurs, binding DNA cooperatively and activating transcription synergistically [81]. Transcriptional synergy depends on orientation and spacing of the NF-κB/Sp1 binding sites although transcriptional activation domains are required for functional activation, specific interaction between Sp1 and NF-κB requires the zinc finger region of Sp1 and an N-terminal portion of the Rel homology domain of p65, the DNA binding domains of each protein [98]. Sp1 elements are often found adjacently to NF-κB sites in enhancers/promoters, including those for HIV-1 [74] intracellular adhesion molecule 1 [99], vascular adhesion molecule 1 [100] and granulocyte-macrophage colony-stimulating factor [101], suggesting a precedent for transcriptional cooperativity.

Nuclear factor of activated T cells (NFAT) activity has been defined as a complex family of transcriptional regulators distantly related to NF-κB through the Rel homology

domain [102-104]. Resting T cells express inactive NFAT molecules in their cytoplasm which upon T cell activation translocate to the nucleus [104-107]. NFAT is an immediate early activation factor that plays a role in the process of T cell activation through control of IL-2 gene activation [108]. Ironically, T cell activation itself contributes to virus replication and the progressive immune dysfunction that is associated with HIV-1 infection, inducing events within cells to enhance HIV-1 transcription [109, 110]. Initially, it was thought that NFAT played no role in HIV-1 enhancer transactivation [111] because deletion of the putative NFAT binding site (position -255 to -217) in the HIV-1 LTR had no effect on HIV-1 gene expression in Jurkat T cells [112]. However, NFAT serves as a positive activator of HIV-1 transcription and replication when NFAT is bound to the HIV-1 NF-κB enhancer motifs (nucleotides -104 to -81) [113]. Ca^{2+}-dependent signaling can be activated through the NF-κB enhancer regions and is a hallmark of NFAT activation in T cells [102, 108, 114-118].

A multitude of gene-specific transcription factors which can activate or repress transcription of their target genes in a combinatorial fashion through their individual binding sites have been identified in modulating HIV-1 LTR promoter activity. The synergistic protein-protein or protein-DNA interactions lead to a functional cross-coupling that allows a high degree of complexity.

RhoGTPases and Transcription

The small GTPase family of Rho proteins (Rho, Rac, Cdc42, RhoD, RhoG, RhoE and TC10) are members of the Ras superfamily of monomeric GTP-binding proteins. They regulate a variety of important cellular processes by cycling between an inactive GDP-bound

form and an active GTP-bound form [119-121]. Activation of the GTPase, through GDP-exchange is promoted by guanine nucleotide exchange factors (GEFs) [121], whereas inactivation by an intrinsic GTPase activity, is stimulated by GTPase-activating proteins (GAPs). Rho guanine nucleotide dissociation inhibitors (Rho-GDIs) stabilize the inactive, GDP-bound form of the protein (Figure 10).

RhoGTPases are involved in numerous intracellular signaling pathways including actin cytoskeletal reorganization [122-124], activation of transcription factors such as serum-response factor [125] or NF-κB [126] and cell cycle progression [127, 128]. Rho mediates the integrin-dependent cell adhesion of a variety of cells in the substratum, which are found to form focal adhesions and stress fibers in fibroblasts [120]. Rho is also required for cytokinesis [129-131], inducing and maintaining the contractile ring [130, 132], the actinomyosin-based contractility as it increases calcium sensitivity in smooth muscles [133] and mediates stimulus-evoked neurite retraction in N1E-115 neuroblastoma cells [134]. Rac induces the assembly of small focal complexes at the periphery of cells and controls the production of lamellipodia and membrane ruffles [123, 124, 135, 136]. Cdc42 triggers actin polymerization to form filopodia or microspikes [122-124, 137, 138].

The JNK/stress-activated protein kinase and p38 MAP kinase cascades control gene transcription in response to cellular stresses [139] and these two MAP kinase pathways can be activated by Rac and Cdc42 [140, 141]. Overexpression of constitutively active Rac or Cdc42 lead to a modest activation of JNK reporter plasmids, making the exact role of GTPases in MAP kinase activation unclear [142]. However, in the *S. cerevisiae* pheromone response pathway, where both Cdc42 and a JNK-like MAP kinase pathway are required, the

GTPase is not required for activation of the kinase cascade, but is required for the correct cellular localization of the MAP kinase containing signaling complex [143].

Additionally, Rho, Rac and Cdc42 have each been reported to activate serum response factor (SRF)-dependent transcription and the transcription factor, NF-κB [125, 126, 144]. The c-*fos* serum response element (SRE) forms a ternary complex with the transcription factors SRF and TCF (ternary complex factor). By itself, SRF can mediate transcriptional activation induced by serum, LPA or intracellular activation of heterotrimeric G proteins. Activated forms of the GTPases RhoA, Rac1 and Cdc42Hs also activate transcription via SRF and act synergistically at the SRE with signals that activate TCF [125]. RhoA, Cdc42, and Rac-1 proteins efficiently induce the transcriptional activity of NF-κB by a mechanism involving phosphorylation of IκBα and translocation of p50/p50 and p50/p65 dimers to the nucleus in a variety of cell-types [145], but independently of the Ras GTPase and the Raf-1 kinase [126]. Overexpression of either normal or constitutively active forms of RhoA, Rac1, and Cdc42Hs induced the transactivation of the HIV-1 promoter, which is partly mediated by the presence of two NF-κB binding sites in its enhancer region [75, 145].

Rho, Rac and Cdc42 activities are required during G_1 cell cycle progression, but it is unclear if this is because of their effects on actin cytoskeleton and integrin adhesion complexes or if it is because of more direct effects on gene transcription [128, 146-151]. RhoA has been suggested to regulate cell cycle progression by modulating the protein stability of cell cycle regulators such as $p27^{KIP1}$ [152] and transcription of specific genes such as cyclin D1 [148], c-*fos* [125], or $p21^{WAF}$ [153].

Activation of GTPases is temporally and spatially controlled with GEFs playing an essential role. A large family of GEFs (>40) have been identified, each of which shares two

common motifs. The Dbl homology domain has been shown to encode the catalytic nucleotide exchange activity [154, 155] and the pleckstrin homology domain whose function is less clear but may determine subcellular localization [121, 156-158]. Experimentally, it appears that some GEFs are specific for an individual GTPase, whereas others are more promiscuous [159-161].

G proteins transduce signals from a large number of cell surface heptahelical receptors to various intracellular effectors including adenylyl cyclases, phospholipases and ion channels. Each heterotrimeric G protein is composed of a guanine nucleotide-binding α-subunit and a high affinity dimer of β and γ subunits. Gα subunits are commonly grouped into four subfamilies (G_s, G_i, G_q and G_{12}) on the basis of their amino acid sequence and function [162, 163]. The G_{12} subfamily only has two members, $G\alpha_{12}$ and $G\alpha_{13}$ [164]. Members of the RGS family of proteins, which includes at least 19 members, negatively regulate G protein signaling [165-168].

A RhoA-specific exchange factor, p115RhoGEF, directly links $G\alpha_{13}$ to regulation of RhoA (Figure 11). p115RhoGEF was initially isolated as a protein that bound tightly to the nucleotide-free form of RhoA and increased the nucleotide exchange rate of RhoA [169]. p115RhoGEF contains both a regulator of G protein signaling (RGS) domain which allows specific binding to either $G\alpha_{12}$ or $G\alpha_{13}$, resulting in the activation of their GTPase activity (negative regulator) and a Dbl homology (DH) domain which acts as a guanine exchange factor to specifically activate the small GTPase RhoA (positive regulator) [170-172]. Furthermore, the activated form of $G\alpha_{13}$, *in vitro*, stimulates the ability of p115RhoGEF to catalyze dissociation of GDP from RhoA by 3-4 fold [173]. Overexpression of either $G\alpha_{13}$ or p115RhoGEF potentiated transcription of a reporter gene regulated by the modified SRE

which drives transcription of SRF when activated by Rho-dependent mechanisms [125, 174], demonstrating a link between $G\alpha_{13}$, p115RhoGEF and RhoA in mediating cellular processes. Once GEFs have catalyzed the replacement of bound GDP for GTP, Rho proteins can specifically interact with their effectors or targets and transmit signals to downstream molecules.

The small GTP-binding protein Rho functions as a molecular switch in the formation of focal adhesions and stress fibers, cytokinesis and transcriptional activation. RhoA's diverse functions are mediated by its association with downstream RhoA effector proteins when in its active GTP-bound form [175]. These include three families of Ser/Thr kinases; (1) Rho kinase (ROCK) and other ROCK family kinases, (2) protein kinase N and its related kinases and (3) citron kinase. The conformational changes between the GTP and GDP-bound forms of RhoA are restricted primarily to two surface loops, named switch regions I and II [176, 177]. Effector proteins must utilize these differences to discriminate between GTP- and GDP-bound forms, though they can also interact with other regions of the GTPase. Using RhoA effector domain mutants, with point mutations introduced into the switch I region, it was demonstrated that distinct effectors are involved in RhoA-mediated transformation of NIH3T3 cells, SRF activation and actin stress fiber formation [178, 179].

RhoGTPases, T cell Activation and HIV-1

T cell development within the thymus involves an ordered sequence of differentiation and proliferation events. Hematopoietic stem cells from the bone marrow enter the thymus undergoing phases of proliferative expansion, T cell antigen receptor (TCR) rearrangements and positive and negative selection processes to mature into peripheral T cells. In a normal

thymus, double positive (DP, $CD4^+CD8^+$) thymocytes are the most abundant. DP thymocytes undergo positive selection where the TCRα chain rearranges and only T cells producing a TCR capable of recognizing either MHC I or MHC II and transducing a signal are able to survive. The surviving thymocytes undergo negative selection to eliminate cells responding too strongly to self-antigen presentation [180, 181]. These thymic developmental events ensure only cells with the appropriate immune repertoire exit to the periphery [182]. Lymphocyte development in the thymus is dependent on signaling pathways generated by a complex array of extracellular stimuli involving antigen receptor- and cytokine receptor-derived signals [183].

G protein coupled receptors (GPCRs) play critical roles in facilitating T cells to react to external signals by controlling thymocyte survival, differentiation and proliferation [184-186]. Both p115RhoGEF and RhoA have been shown to affect T cell function. $G\alpha_{13}$ knockout embryos die at day nine due to defects in angiogenesis [187, 188] and in G2A knockout mice, a GPCR known to work specifically through the $G\alpha_{13}$ pathway, develop a late onset autoimmune disorder [189, 190]. Additionally, blocking $G\alpha_{13}$ signaling by using a dominant-negative mutant of p115RhoGEF (p115ΔDH) blocked $G\alpha_{12}$ and $G\alpha_{13}$ signaling in T cell progenitors leading to impaired thymopoiesis, reduced proliferation and increased cell death [191]. Peripheral T cells in p115RhoGEF knockout mice hyperproliferate even in the absence of antigenic stimulation [192]. A transgenic lck C3 transferase mouse, which expresses the RhoA inhibitor C3 transferase under the control of the lck promoter, showed a specific requirement for RhoA at the $CD4^-CD8^-$ stage for survival [193]. Inactivation of Rho function in the thymus demonstrates that differentiation of progenitor cells to mature T cells can occur with both positive and negative selection of T cells remaining intact [182].

However, these transgenic mice show maturational, proliferative and cell survival defects during T cell development that severely impair the generation of normal numbers of thymocytes and mature peripheral T cells [182]. An additional study demonstrated that processes of proliferation and survival are independently regulated during thymopoiesis and establish two different functions for Rho in the development of early thymic progenitors. In pre-T cells, Rho function was required for survival but not G_1/S phase cell cycle progression and in late pre-T cells, Rho regulates cell cycle progression but not survival [193]. Collectively, these studies indicate that the function of the GTPase Rho is essential for normal thymic development and define Rho as a critical intracellular signaling molecule in T lymphocytes.

Although RhoGTPases have been implicated in various steps of T cell activation [182, 191, 193], little is known about how RhoGTPases affect HIV-1 replication. The transmembrane envelope glycoprotein (gp41c) of HIV-1 contains a highly conserved long cytoplasmic domain that has been implicated in regulating HIV-1 replication and cytopathogenicity [194]. We have previously shown that gp41c interacts directly with the carboxy-terminal regulatory domain of p115RhoGEF [195], the upstream activator of RhoA. Ectopic expression of p115RhoGEF or $G\alpha_{13}$, which activates p115RhoGEF activity, leads to inhibition of HIV-1 replication via a RhoA-dependent mechanism (Figure 11). The RhoA effector activity inhibiting HIV-1 replication is genetically separable from its activities in activation of SRF and actin stress fiber formation, suggesting RhoA inhibits HIV-1 regulation via a novel effector activity (Figure 11) [196].

To investigate how RhoA signaling pathways modulate HIV-1 replication, we tested several RhoA effectors in human T cells. We discovered that Rho-associated kinase

(ROCK), a RhoA effector, specifically inhibits HIV-1 gene expression in T cells. Ectopic expression of wild-type ROCK inhibited viral gene expression in a dose-dependent manner. At very low levels of ROCK overexpression, we saw a significant inhibitive effect on viral production (i.e. assembly/release) with a minimal effect on viral gene expression, whereas high levels of ROCK efficiently inhibited HIV-1 gene expression. A ROCK specific inhibitor, Y27632, counteracted RhoA inhibition of HIV-1 gene expression. Coexpression of ROCK with constitutively active RhoA further inhibited viral gene expression than when either ROCK or constitutively active RhoA were expressed alone. Mutations in the second NF-κB and/or the first two Sp1 binding sites of the HIV-1 LTR abrogated ROCK-mediated inhibition of HIV-1 gene expression. Further experiments need to be completed to elucidate the mechanism used by ROCK to mediate inhibition of HIV-1 transcription.

HIV-1 Viral Assembly and Release

HIV-1 assembly and release occur in a series of essential steps driven by the viral Gag precursor protein, $Pr55^{Gag}$ [20, 47]. In the absence of all other viral proteins, HIV-1 Gag can still promote the release of virus-like particles [197]. HIV-1 Gag is organized into four distinct regions; matrix, capsid, nucleocapsid and late domain (Figure 7). The N-terminal matrix domain harbors a myristoylation signal essential for transport of Gag from within the cell to the plasma membrane [198, 199]. Additionally, a basic patch on the surface of the globular core of matrix contributes to the selective association of $Pr55^{Gag}$ with the plasma membrane by interacting with acidic phospholipids [200]. Capsid, which follows matrix in the context of $Pr55^{Gag}$, can by itself assemble into hollow cylindrical particles reminiscent of viral cores *in vitro* but requires relatively high protein concentrations [201-203]. Cleavage at

the matrix/capsid junction results in the conformational rearrangement of capsid into a conical structure during virus maturation [201, 203, 204]. The nucleocapsid domain contains two copies of a conserved zinc finger-like motif which are required for the encapsidation of the genomic viral RNA [47]. The late (L) domain catalyzes the pinching off and detachment of virus particles from the cell surface and from each other [47]. In the case of HIV-1, the L domain is encoded by PTAP in the C-terminal, p6 domain of $Pr55^{Gag}$. HIV-1 Gag^{p6} is also required for the incorporation of the accessory protein, Vpr [205-207], the viral Pol and envelope proteins [208, 209] and to control particle size [210, 211]. The L domain has two highly conserved domains; one located near the C terminus which is essential for Vpr incorporation [205, 206, 212] and the second, located at the N terminus of p6, is critical for late domain's role in viral release [61, 62]. Point mutations in the PTAP motif of p6 or deletion of the entire p6 domain produces a striking defect in the production of virus particles [61, 62]. Mutant virus remains attached to the cell surface via a thin tether rather than budding off from the plasma membrane, identifying the p6 domain as crucial for HIV-1 budding (Figure 12).

The late domain is a highly conserved Pro-rich motif found in most enveloped viruses, including rhabdoviruses [213, 214], filoviruses [215] and Epstein-Barr virus [216], in addition to retroviruses. L domains map to three classes of tetrapeptide motifs with sequence PT/SAP [61, 62], PPXY [217-219] or YPXL [220]. PPXY motifs appear to be the most common sequence associated with L domain function, stimulating budding of Rous sarcoma virus [217, 221], Mason-Pfizer monkey virus [218], murine leukemia virus [219, 222], human T-cell leukemia virus type I [223], bovine leukemia virus [224], the rhabdoviruses [213, 214, 225] and the filoviruses [215]. Equine infectious anemia virus

(EIAV) L domain activity is encoded by a YPDL motif [220]. In addition, a number of retroviruses and the Ebola filovirus, contain adjacent or overlapping PTAP and PPXY sequences [218, 223, 226]. The presence of both PTAP and PPXY motifs may provide functional redundancy or enable sequential association of L domains with multiple host factors (Table 1).

L domains interact with the cellular ubiquitination and endosomal sorting machinery [197, 227], but the role of ubiquitination is unclear. Evidence suggests there is a positive correlation between late domain function and ubiquitination; (1) L domain containing proteins of several retroviruses (i.e. HIV-1, HIV-2, MLV and EIAV) are ubiquitinated [228-230], (2) proteasome inhibitors disrupt budding of rhabdoviruses and some but not all retroviruses [230-236], (3) L domains of Rous sarcoma virus [237], Mason-Pfizer monkey virus [238], the rhabdoviruses [231] and Ebola virus [215] appear to functionally interact with proteins related to Nedd4, a ubiquitin E3 ligase, (4) the EIAV L domain bind and colocalize with the AP-50 subunit of the AP-2 complex which is involved in endocytosis [239] and (5) the host protein Tsg101, a ubiquitin enzyme 2 variant protein, was identified in *S. cerevisiae* yeast two-hybrid screens as a HIV-1 Gag^{p6}-interacting protein [204, 240, 241]. In HIV-1, Gag^{p6} ubiquitination enhances the binding of Tsg101 and Gag^{p6} but is not required for that binding to occur [240]. In HIV-1, the PPEY motif from Ebola's VP40 late domain was unable to replace the L domain function normally provided by the PTAP motif and all PPXY motifs examined stimulated Gag ubiquitination. These findings suggest that ubiquitination of HIV-1 Gag is not sufficient to promote viral egress and that active L domains may recruit deubiquitinating enzymes as a consequence of class E Vps factor recruitment [242].

Despite retroviral L domains differing in amino acid sequence and location within their respective viral structural proteins, they tend to be functionally exchangeable, suggesting commonality of function, perhaps as docking sites for host proteins [222, 234, 243]. The YPXL-type L domain of EIAV recruits the AP-2 clathrin adaptor complex [244] and has been shown to interact with host protein, AIP1, a central component of the multivesicular body (MVB) pathway [245-247]. The PPXY-type L domains of rhabdoviruses, retroviruses and Ebola virus have been shown to bind to WW domain-containing proteins [197, 215, 231]. Among these, Nedd4 family ubiquitin E3 ligases are of particular interest because cellular ubiquitin pools are critical for the release of several retroviruses [227]. Additionally, PTAP-type L domain viruses, like HIV-1, bind the UEV-like protein, Tsg101 [204, 240, 248], a component of the endosomal sorting complex required for transport-I (ESCRT-I).

Host factor requirements can be altered by exchanging L-domain usage, yet all retroviruses are dependent on the VPS pathway for efficient release [240, 249, 250]. Retroviruses use different host factors to complete viral release suggesting that retroviruses utilize different sites in cellular processing pathways to accomplish a common assembly step in viral replication in their respective host cells. EIAV YPXL L domain has been shown to be critical for the release of assembling virions and to interact *in vitro* with AP-50 subunit of the AP-2 complex [244]. However, it was found that the functionally interchangeable late domains of RSV Gag^{p2b} protein and HIV-1 Gag^{p6} protein, which utilize PPPY and PTAP L domains, respectively, do not bind AP-50 *in vitro,* but are still able to facilitate EIAV release. A role for the AP-2 complex in viral assembly is supported by specific colocalization of the α-adaptin subunit of AP-2 with the EIAV Gag^{p9} protein at sites of viral budding on the

plasma membrane [244]. EIAV Gag release is blocked by inhibition of the VPS pathway using dominant-negative Vps4, an ESCRT recycling factor, but is insensitive to Tsg101 depletion [250]. An N terminal truncation of Tsg101, TSG3' impairs budding of PPPY- and PTAP-containing viruses [251] but not YPDL-dependent EIAV [252]. In addition, fusing a mutant EIAV Gag directly to another cellular component of the ESCRT-I complex, Vps28, restored efficient viral release of the EIAV Gag late domain mutant suggesting that EIAV normally enters the Vps pathway downstream of ESCRT-I [250]. AIP1/Alix recognizes proteins bearing the YPXL sequence [252] directly interacts with ESCRT-III component CHMP4 [253] suggesting EIAV Gag may be recruited to ESCRT-III/Vps pathway by interaction with AIP1/Alix [246, 252].

The L domain of RSV is located near the N-terminus of Gag in the p2b region and has the core sequence PPPPY [217, 221] and the deltaretroviruses, which include bovine leukemia virus (BLV) and human T-cell leukemia virus type 1 (HTLV-1) and HTLV-2, have a conserved PPPY motif in the C-terminal region of the matrix domain of Gag, while HTLV-1 also encodes a PTAP motif in the matrix domain [254]. The PY motifs, like those found in RSV Gag, resemble ligands for WW domains, which are approximately 38 amino acid modules containing two widely spaced, conserved tryptophans that are found in a wide variety of signaling, regulatory and cytoskeletal proteins [255, 256]. For RSV, vesicular stomatitis virus and Ebola virus, the L domains have been shown to interact *in vitro* with WW domains from Yes-associated protein (Yap), a signal-transducing molecule and Nedd4, an E3 ubiquitin ligase [214, 215, 237, 257]. A dominant-negative form of Vps4, an AAA ATPase interferes with RSV Gag release, indicating that RSV uses the endocytic trafficking

machinery [249]. Retroviruses can interact with the Vps machinery in several different ways to accomplish particle release.

Several viruses, including HTLV-1, MPMV and Ebola, carry closely spaced PPPY and PT/SAP motifs. In HTLV-1, mutation of either motif reduced budding efficiencies, switching the locations of the two late domains eliminated particle release, replacement of the PTAP motif with either PPPY or YPDL motifs had no effect on particle release but replacement of the PPPY motif with either PTAP or YPDL eliminated particle release indicating that the PPPY motif plays an essential role in HTLV-1 viral budding [254]. The retrovirus, MPMV, had complete loss of virus release with mutation of the PPPY motif. The PSAP motif acted as an additional L domain to promote efficient viral release but required an intact PPPY motif to perform its function implying that in the context of MPMV, the PPPY and PSAP motifs are not redundant [258]. Ebola virus matrix protein VP40 is the major structural protein and plays a key role in viral assembly and release. Efficient viral release is dependent on the presence of the intact N-terminal of VP40 which contains a PPXY and a PSAP motif [259]. The PPXY motif mediates interaction with proteins that contain WW domains [260] and Tsg101 interacts with monomeric and oligomeric VP40 through the PSAP motif [226]. The overlapping motifs of PPXY and PSAP of Ebola virus VP40 suggest that Tsg101 and Nedd4 may interact with VP40 at two different steps during budding and may involve different cellular pathways [226].

The PTAP motif of HIV-1 Gag^{p6} was found to bind to Tsg101 (tumor susceptibility gene 101) in the UEV domain at its N-terminus [204, 240, 248, 261]. Tsg101 is an ESCRT-I component, a 350 kDa cellular complex essential in the VPS pathway. Point mutations in HIV-1 PTAP motif block virus release at late stages [61, 62] and disrupt binding to Tsg101

[240]. Small inhibitory RNA-mediated Tsg101 depletion potently blocks HIV-1 release [240]. Furthermore, overexpression of a dominant-negative form of VPS4 inhibits particle release of HIV-1 and other enveloped viruses [240] indicating that the VPS machinery is involved in the budding of PTAP-encoding retroviruses [240, 261] (Figure 13).

A second region in HIV-1 Gag^{p6} has been defined to contribute to viral release and interacts with AIP1, a host protein [246, 247]. AIP1 interacts with Tsg101 and CHMP proteins of ESCRT-III complex, coupling HIV-1 Gag^{p6} to the early and late-acting endosomal sorting complexes by providing an interaction scaffold. AIP1 also binds the HIV-1 Gag via the LRSL Gag^{p6} sequence and EIAV Gag^{p9} proteins, indicating it can function directly in virus budding [246, 247]. HIV-1 release can be arrested at late stage by deletion or mutation of at least 8 different human class E proteins, which function both early (Tsg101, Vps28) and late (CHMP2A, CHMP3, CHMP4B, CHMP4C, VPS4A, VPS4B) in the MVB pathway [246]. HIV-1 particles package multiple copies of proteins that act in the MVB pathway (i.e. Tsg101, Vps28 and AIP1) [246, 247]. The viral release of HIV-1 is dependent on a protein network that connects early and late components of MVB biogenesis (Figure 14).

Additionally, HIV-1 Gag has been shown to interact directly with the δ subunit of the AP-3 complex at an early step in viral particle assembly, in an interaction that is mediated by the N-terminal α-helical region of the Gag matrix [262]. AP-3 is an adaptor protein complex that mediates sorting of cargo proteins to specific membrane compartments (trans-Golgi network and peripheral endosomal compartments) within the cell [263-267]. Disruption of this interaction prevents Gag from reaching the MVB compartment and inhibits viral particle formation, suggesting that trafficking of Gag to this compartment is part of the normal

productive pathway in HIV-1 assembly, even in cell types where particle release takes place predominantly at the plasma membrane [262].

Collectively, retroviruses and other enveloped viruses seem to interact with a diverse set of host proteins, all linked to the endosomal sorting pathway in some manner, to mediate viral assembly and release at sites of budding. These interactions can be complicated and may have redundancy, suggesting the overall functional importance these host proteins perform in terms of viral assembly/release.

Endosomal Sorting Pathway

The endosomal sorting pathway controls a variety of cellular processes and is involved in the sorting of mono-ubiquitinated proteins into the lumen of the MVB [54, 268]. Ubiquitinated proteins are recognized by endosomal sorting complexes, taken to the limiting endosomal membrane and sorted, resulting in either MVB fusion with the lysosome to degrade contents or release of material into the extracellular environment via exosomal vesicles [269-271]. Ubiquitination is the covalent attachment of ubiquitin to specific lysine residues in target proteins and occurs in three steps mediated sequentially by ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin-protein ligase (E32) enzymes.

ESCRT-I, composed of Tsg101, Vps28 and Vps37, is a conserved component of the endosomal sorting pathway which recognizes the mono-ubiquitinated tag on proteins and recruits two other class E protein complexes (ESCRT-II/III) to participate in protein sorting and vesicle formation [268, 272, 273]. ESCRT-I recognizes ubiquitinated cargo and requires the presumed ubiquitin enzyme 2 variant region (UEV) in Tsg101, implying that MVB sorting machinery is regulated by ubiquitin or binding to ubiquitin membrane proteins to

direct them into the MVB [274]. Mutation of the Tsg101 UEV-like domain not only prevents sorting of ubiquitinated cargo into internal MVB vesicles but also prevents the formation of the vesicles themselves. The recognition of cargo by ESCRT-I activates downstream components of the vesicles budding machinery (ESCRT-II/III) [273]. ESCRT-II is a soluble, stable, heterotrimeric 155 kDa complex and the four structurally related components of ESCRT-III are monomeric in the cytosol, associating only when they are recruited to membranes. High levels of ESCRT-II can partially overcome the need for ESCRT-I suggesting a sequential assembly of the three ESCRT complexes. ESCRT-III components also recruit another class E protein, Vps4p, an AAA-ATPase, which catalyzes the dissociation of the ESCRT machinery at the endosomal membrane [269] (Figure 15).

More than 50 gene products are involved in vacuolar protein sorting with Vps proteins functioning at distinct steps of protein transport [275]. All 17 class E Vps proteins are required for the sorting of MVB cargo proteins. Functional loss of any individual class E Vps protein results in a malformed late endosome/MVB known as the “class E compartment”, an enlarged aberrant endosome [269]. In vps4 mutant cells, ESCRT-I/II/III all accumulate on the class E compartment membranes without dissociating.

Sorting into MVB vesicles is a highly coordinated event. The Vps pathway is regulated as follows: the binding of ubiquitinated cargo by ESCRT-I leads to the activation of ESCRT-II, ESCRT-II interacts directly with the ESCRT-III Vps20/Snf7 subcomplex to promote the assembly of multiple copies of ESCRT-III on the membrane and in the final step, the AAA-ATPase Vps4p interacts with the ESCRT-III Vps2/24 subcomplex to catalyze the release of the entire machinery from the membrane [272, 275]. Fusion of the limiting membrane of the MVB with the lysosomal membrane results in delivery of the luminal MVB

vesicles and their contents to the hydrolytic interior of the lysosome, where they are degraded [276]. Proteins that are spared this fate are excluded from these inner MVB vesicles and remain in the limiting MVB membrane where they can be recycled to the plasma membrane or transported to other sites within the cell (Figure 16).

The sorting of transmembrane proteins into topologically distinct limiting and intralumenal membranes may serve two functions; (1) transmembrane proteins in the intralumenal membrane are susceptible to degradation by lysosomal hydrolases whereas proteins in the limiting membrane are resistant and (2) intralumenal vesicles might represent storage vehicles for transmembrane proteins that are to be released from the cell in a regulated manner. In specialized cell types (melanocytes and hematopoietic cells), MVBs serve as intermediates in the formation of secretory lysosomes, such as melanosomes, MHC II compartments and lytic granules [277]. The formation of these specialized organelles requires sorting of specific proteins at the level of MVBs, where upon appropriate stimulus, secretory lysosomes fuse with the plasma membrane and all intralumenal vesicles will be shed into the extracellular space (as exosomes) or transferred to neighboring cells (Figure 17).

Recent studies have shown that several enveloped RNA viruses reprogram the endosomal sorting machinery for their budding processes [278]. Viruses, like HIV-1, do not encode their own machinery for viral budding and require a cellular protein function that is activated by their late domain membrane-associated structural proteins. The PTAP motif of HIV-1 binds Tsg101 and when Tsg101 is depleted, HIV-1 is unable to bud from the plasma membrane, forming stalks of unbudded virions [240]. HIV-1 budding is inhibited by dominant-negative Vps4, indicating that the ESCRT machinery is required for completion of

viral release [279] (Figure 14). In macrophages, HIV-1 buds into MVBs indicating the virus is capable of using the ESCRT machinery at its normal location [280], not solely through recruitment of the endosomal machinery to the plasma membrane (Figure 16).

Viruses not only parasitize the machinery for MVB formation and viral release, they also usurp the MVB sorting machinery to subvert immune surveillance. The Kaposi's sarcoma-associated herpes virus gene product, K3 (KK3) prevents MHC class I antigen presentation by downregulating MHC class I from the plasma membrane. KK3 associates with MHC class I molecules, promotes their ubiquitination and once internalized, directs them to the late endocytic pathway for degradation by the lysosome in a Tsg101-dependent manner [281]. In the case of antigen-presenting cells, exosomes act as vehicles of immunomodulation [282, 283] and are important players in intracellular communication during the immune response. B-cell derived exosomes carrying peptide-loaded MHC class II can stimulate $CD4^+$ T cells *in vitro* [284]. Exosomes, from cultured DCs, loaded with tumor-derived peptides stimulated CTL-mediated anti-tumor responses *in vivo* [285] and tumor cells can secrete exosomes with tumor antigens, which after transfer to the DC, can mediate $CD8^+$ T cell dependent anti-tumor effects [286].

MVBs are also important for the activation of the immune response. Immature dendritic cells (DCs) package and store MHC class II molecules in the luminal vesicles of the MVB-like compartments that are known as MHC class II compartments (MIIC). Upon stimulation of the cell by antigen, the luminal MIIC vesicles fuse with the limiting membrane, the MHC class II molecules are loaded with antigenic peptide and the MHC class II peptide complexes are transported to the plasma membrane for presentation to naïve T cells [287]. The antigenic stimulation of DCs can also cause the limiting membranes of

MIICs to dock and fuse with the plasma membrane, releasing the luminal vesicles of MIICs from the cell. These secreted vesicles known as exosomes, contain MHC molecules and CD86, a co-stimulatory factor for T cells [285]. Exosomes are also secreted by other hematopoietic cell types which include immature reticulocytes, B cells and platelets, but their exact physiological targets are not yet known (Figure 17).

The endosomal sorting pathway operates in most cell types and is used to sort and transport a wide variety of proteins within the cell. It makes sense that viruses would usurp such a key pathway to facilitate in its replication and to subvert immune recognition.

Role of RhoGTPases in Membrane Trafficking Events

RhoGTPases play a pivotal role in the dynamic regulation of the actin cytoskeleton and, through this, modulate cell morphology, motility and adhesion [120, 288]. In recent years, studies in mammalian cells and other organisms have uncovered multiple links between RhoGTPases and membrane trafficking events. Not only can RhoGTPases act locally on individual trafficking events, they can act globally to control the spatial organization of membrane traffic in response to cues from the extracellular environment. Vesicle trafficking events require an active process of either actin disassembly to mediate their movement through cortical actin or actin-based motor proteins to drive budding and fusion.

The most clear-cut interface of RhoGTPases with the endocytic trafficking is in the process of clathrin-independent internalization from the cell surface. All clathrin-independent routes into the cell are driven by actin polymerization and therefore, have obligatory requirements for RhoGTPases (Figure 18). For example, Type II phagocytosis,

phagocytes displaying the CR3 complement receptor, recognize particles opsonized with the complement iC3b [289]. This morphologically distinct process is dependent on RhoA, but not Cdc42 or Rac [290]. The RhoA effector, Rho-associated kinase (ROCK) is required for Type II phagocytosis and may contribute to the process of engulfment by regulating the actinomyosin contractility [291, 292] and recruitment of the actin nucleating Arp2/3 complex [293]. Inhibition of the RhoA-ROCK- myosin-II pathway via inhibitors specifically targeting ROCK and myosin-II, caused a decreased accumulation of Arp2/3 complex and F-actin around bound particles thereby reducing phagocytic engulfment [292]. These finding suggest an essential role for RhoA and ROCK in mediating Type II phagocytosis.

Additionally, there are two distinct classes of clathrin-independent receptor endocytosis; one involving GPI-coupled receptor internalization and requiring Cdc42 [294] and the second, involving interleukin-2 (IL-2) receptor internalization and requiring RhoA [295]. The IL-2 receptor is internalized from lipid rafts [296] in a dynamin-dependent fashion [295]. This process is inhibited by expression of dominant-negative RhoA or Rac, but is not affected by the constitutively active versions of these GTPases, suggesting roles for both Rho and Rac in IL-2 receptor endocytosis.

Internalized receptors travel through a number of distinct endocytic sub-compartments where sorting decisions are made to determine whether the receptor is recycled to the plasma membrane or targeted to lysosomes for degradation. Several Rho-GTPases have been localized to these compartments indicating the actions of Rho-GTPases are not confined to the plasma membrane. RhoD GTPases have been localized to both the plasma membrane and early endocytic vesicles [297]. Constitutively active RhoD promotes the alignment of early endocytic vesicles with actin filaments by activation of an isoform of

Diaphanous (Dia) which leads to the loss of early endocytic motility through a mechanism requiring tyrosine kinase c-Src activity [298].

Receptors following the lysosomal degradation pathway are sorted into MVBs with the final step involving fusion of these MVBs with lysosomes, resulting in transfer and degradation of cargo. RhoB and RhoA share about 85% amino acid identity and differ primarily in their C-terminal hypervariable region, a crucial determinant of membrane targeting [299]. RhoB, unlike RhoA, can target to both the plasma membrane and internal membranes [299-302]. RhoB is activated by protein kinase C related kinase 1(PRK1) which delays intracellular trafficking of the EGF receptor to lysosomes [301, 303, 304] and blocks the transfer of the EGF receptor from the MVB to the lysosome. However, overexpression of wild-type RhoB had no effect on endosome transport suggesting that endogenous RhoB only transiently inhibits endocytosis [305]. The RhoB effector, PRK1 may play a key role in membrane trafficking. RhoB targets PRK1 to endosomal membranes and overexpression of kinase-inactive PRK1 releases the inhibitory effect of RhoB on the trafficking of internalized receptors [306].

These findings collectively suggest that RhoGTPases link the actin cytoskeleton with endocytic trafficking events at multiple levels as a means to control and regulate these processes. RhoGTPases, because of their roles in actin cytoskeleton and endocytic trafficking, are ideally poised to be involved in mediating signaling pathways necessary for HIV-1 replication.

RhoGTPases and HIV-1

Rac, Cdc42 and Rho are three members of the Rho family of small GTPases which act as molecular switches cycling between an active GTP-bound and inactive GDP-bound state. Activation, in response to extracellular stimuli, is mediated by the Dbl family of GEFs while downregulation involves poorly characterized family of GAPs and RhoGDIs [156, 307]. In their active states, Rho, Rac and Cdc42 interact with a variety of effector proteins to elicit cellular responses [121]. Once activated, Rac induces polymerization of monomeric actin at the cell periphery to produce a dense meshwork of actin filaments forming extending lamellipodia and membrane ruffles [124], Cdc42 stimulates actin polymerization to form filopodia [122-124, 137, 138] and Rho mediates integrin-dependent cell adhesion found to form focal adhesions and stress fibers [120].

Members of the RhoGTPase family also regulate gene transcription. All three GTPases have been reported to stimulate the JNK/SAPK and p38 MAP kinase cascades, the transcription factor NF-κB and the transcription factor SRF [125, 126, 128, 140, 141, 144, 308, 309]. Ectopic expression of Rac effector, hPOSH, leads to activation of the JNK pathway and to nuclear translocation of NF-κB, independent of either actin reorganization or JNK activation [310] suggesting hPOSH acts as a scaffold protein linking signaling events with transcriptional changes. Additionally, Rho, Rac and Cdc42 can trigger G_1 progression when introduced into quiescent fibroblasts and are required for serum-induced cell cycle progression and Ras-induced cell transformation [128, 311-313]. Stimulation of G_1 progression and cell transformation correlate with the ability of the GTPases to induce

cytoskeletal changes and signals may be induced in response to actin polymerization or integrin complex assembly [147, 148, 314].

Despite RhoGTPases being implicated in actin cytoskeletal events, T cell activation, vesicle trafficking and a myriad of other cellular processes, little is known about the role RhoGTPases may play in HIV-1 replication. There is increasing evidence supporting key roles for RhoGTPases in regulating various steps of HIV-1 replication. In T cells and monocyte-derived myeloid cells, inhibition of myosin light chain kinase (MLCK), through the use of wortmannin, an inhibitor of MLCK and MLC phosphorylation, suppressed HIV-1 release [315]. Wortmannin did not disrupt transport of viral proteins to the plasma membrane but rather inhibited budding. These findings suggest that after localization to sites of viral budding, the subsequent actinomyosin interactions participate in the release of viral particles from host cells [315]. However, cytochalasin D, an inhibitor that disrupts the equilibrium between monomeric and polymeric actin, only partially inhibited HIV-1 viral release most likely due to cytochalasin D's partial depolymerization of actin filaments [315].

In most HIV-1 infected cells, release occurs as virus buds and pinches off from the plasma membrane, although even in cell-types where HIV-1 egress occurs primarily at the plasma membrane, Gag assembly can be detected in late endosomes [316]. Analysis of MLV- and MPMV-infected cells showed association of Gag with late endosomal membranes and subsequent movement to the plasma membrane, the latter requiring a functional vesicular transport system [317, 318]. Protein ubiquitination is a major regulator of intracellular protein transport and serves as a signal for sorting of proteins from the cell surface or the TGN into late endosomes and lysosomes [319-324].

A productive HIV-1 infection requires the exploitation of the host protein sorting and trafficking pathways. Ubiquitination is known to be involved in the late steps of the HIV-1 life cycle [232, 234, 325] therefore it is likely that an E3 ubiquitin ligase activity is involved in HIV biogenesis. The Rac effector, hPOSH, a TGN-associated E3 ubiquitin ligase was identified as a critical factor in the sorting of HIV-1 Gag to the plasma membrane [326]. In HIV-1 infection, hPOSH functions independently of the viral late domain but acts upstream of virus budding at the cell membrane since depletion of hPOSH ablated accumulation of particles at the cell surface. An E3 ligase mutant of hPOSH was unable to restore production of VLP in hPOSH-inhibited cells implying that targeting of Gag from the TGN to the plasma membrane requires the ubiquitination activity of hPOSH [326].

Previously, we had shown that the cytoplasmic tail of the HIV-1 transmembrane envelope glycoprotein, gp41c, interacts directly with the carboxy-terminal regulatory domain of p115RhoGEF [195], a GEF and activator of RhoA. Ectopic expression of p115RhoGEF or $G\alpha_{13}$, which activates p115RhoGEF activity, leads to inhibition of HIV-1 replication via a RhoA-dependent mechanism (Figure 11). The RhoA effector activity inhibiting HIV-1 replication is genetically separable from its activities in activation of SRF and actin stress fiber formation, suggesting RhoA inhibits HIV-1 regulation via a novel effector activity Figure 11) [196].

To investigate how RhoA signaling pathways modulate HIV-1 replication, we tested several RhoA effectors in 293T and human T cells. We found citron kinase, a Ser/Thr kinase, to preferentially enhance virion production of both HIV-1 and MLV, in a late domain independent manner. Depletion of endogenous citron kinase inhibited HIV-1 virion production. The leucine-zipper, Rho-binding and zinc-finger domains, but not the kinase

domain, were necessary for the enhancement activity. Ectopic expression of citron kinase led to the formation of cytoplasmic structures containing citron kinase and HIV-1 Gag proteins. Citron kinase enhanced secretion of exosomes and microvesicles that copurified with HIV-1 virions. Even though citron kinase-mediated enhancement of HIV-1 replication was HIV-1 late domain independent, an intact late endosomal sorting pathway was necessary.

To more clearly define the role of citron kinase in modulating HIV-1 virion production, we wanted to identify possible component(s) of the endosomal sorting pathway that may facilitate citron kinase's role in HIV-1 replication. We identified a functional interaction between citron kinase and Tsg101, a component of the ESCRT-I complex. The kinase activity of citron kinase was necessary and sufficient for the functional interaction with Tsg101. Additionally, the functional interaction was dependent on maintaining the integrity of Tsg101's PTAP-binding region. Collectively, our findings suggest novel mechanisms for regulation of HIV-1 virion release involving citron kinase-mediated exocytosis and components of the endosomal sorting pathway.

Concluding Remarks

HIV-1 is one of the most serious infectious diseases to plague humans. Despite increased knowledge involving the mechanics of HIV-1 infection, we are still a long way from fully understanding it. The HIV-1 genome encodes only ten open reading frames and therefore requires use of host pathways to facilitate in the completion of its life cycle. Therefore it is necessary to identify host proteins involved in HIV-1 regulation. RhoGTPases are involved in a number of diverse cellular processes, many of which potentially could

influence the outcome of HIV-1 infection. RhoGTPases play key roles in mediating T cell activation, regulating the actin cytoskeleton and vesicle transport and in inhibiting/activating transcription factors – thus RhoGTPases may link these pathways with HIV-1 replication. Our lab previously found that the cytoplasmic tail of the HIV-1 envelope glycoprotein binds directly to p115RhoGEF, a guanine nucleotide exchange factor, to inhibit HIV-1 replication. This thesis further explores the role of RhoA signaling pathways in regulating HIV-1 replication.

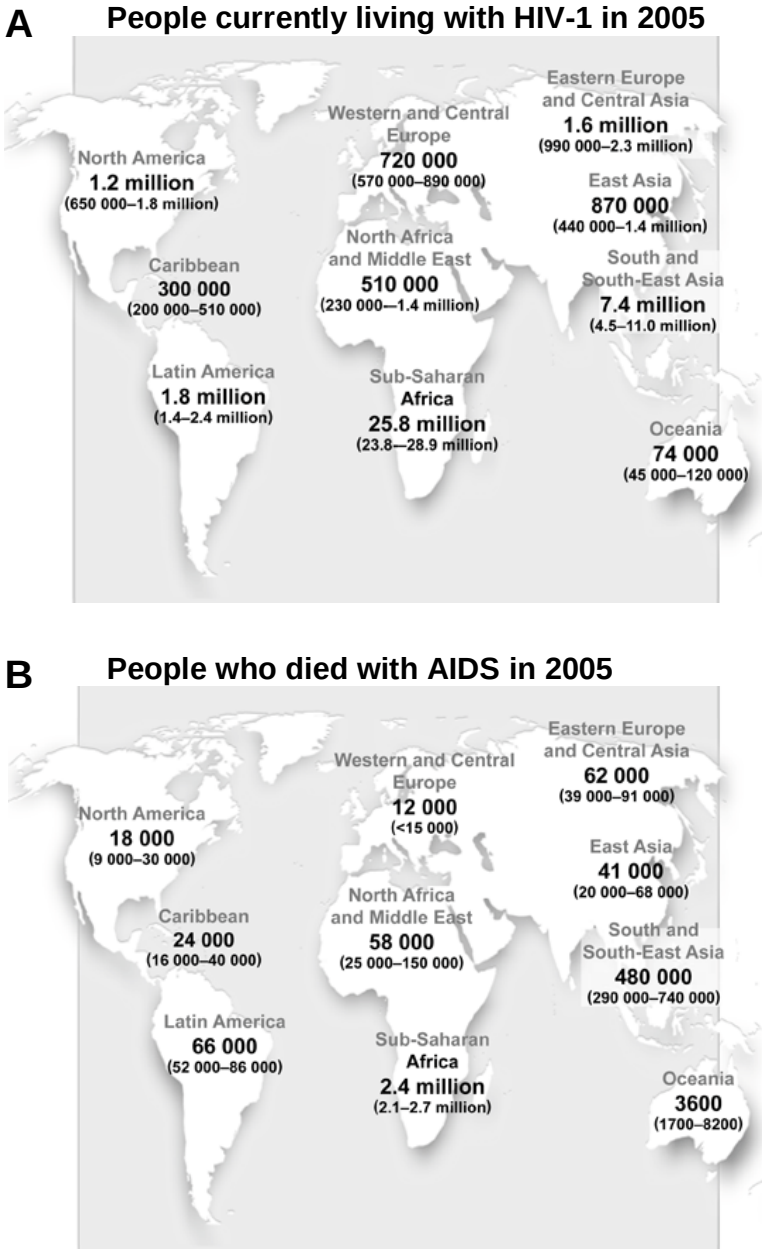

Figure 1: UNAIDS/WHO Epidemic Update 2005. (A) At the end of 2005, UNAIDS and WHO estimated that 37-45 million people live with HIV-1, with the majority of those people living in Sub-Saharan Africa. (B) An estimated 2.8-3.6 million people died with AIDS in 2005 according to the 2005 UNAIDS/WHO Epidemic Update.

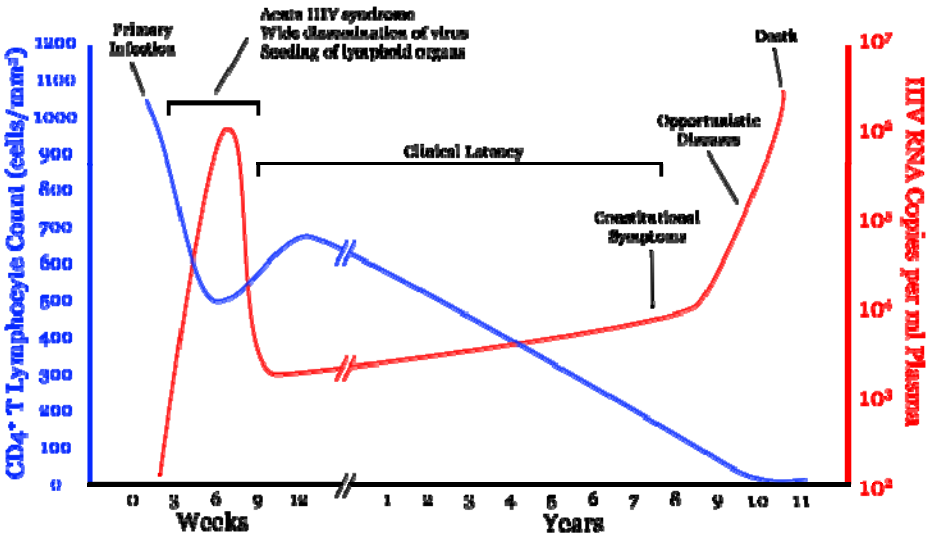

Figure 2: Acute vs. Long-term Infection. HIV-1 is best described as the result of two sequentially distinct phases with different pathogenic mechanisms. The first phase; primary infection is characterized by a rapid and massive, directly HIV-1 mediated, loss of memory $CD4^+$ T cells (blue line) and a high viral load (red line). The second phase; chronic infection (clinical latency) which can last for several years, results in a slow decline in $CD4^+$ T cells in the peripheral blood (blue line), leading to increased death rate of T cells. The loss of $CD4^+$ and $CD8^+$ T cells prevents the host from adequately fighting opportunistic infections and causes viral titers to increase (red line).

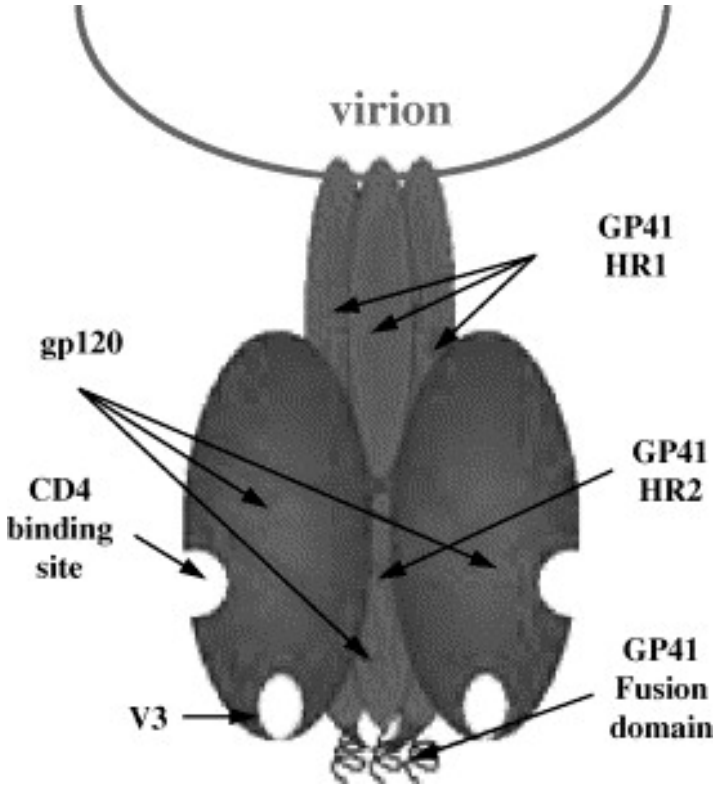

Figure 3: HIV-1 Envelope Glycoprotein. The envelope glycoprotein is a heterodimer composed of the external gp120 noncovalently attached to the transmembrane domain, gp41, organized into a trimeric structure. Gp120 contains both the CD4-receptor binding site and the coreceptor binding site. The coreceptor binding site is not fully exposed until CD4 is bound. Gp41 forms pores in the plasma membrane and anchors the gp120 in both virions and infected cells.
Sierra S, Kupfer B and Kaiser R. Basics of the virology of HIV-1 and its replication. *Journal of Clinical Virology* 2005;34:233-44.

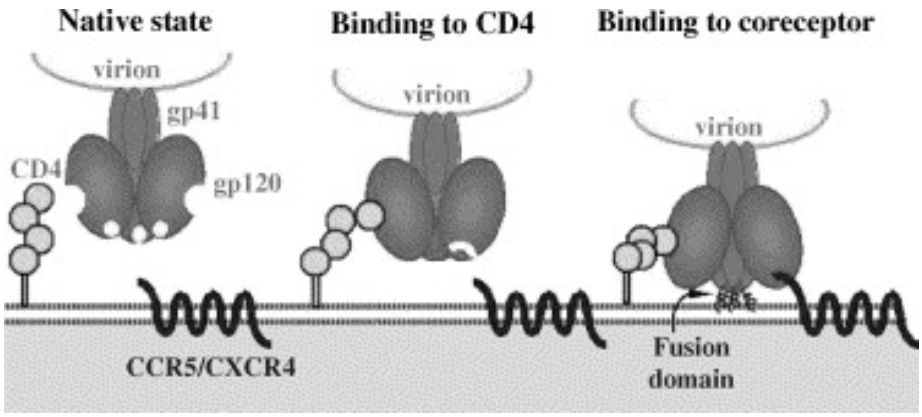

Figure 4: Model for HIV-1 Virion Attachment and Entry. The external gp120 domain interacts with the CD4 receptor on the cellular plasma membrane triggering a conformational change in gp120 which displaces a previously hidden region which acts as the coreceptor binding site. The binding of a cellular coreceptor induces additional conformational changes in the envelope protein allowing the HR1 and HR2 domains of gp41 to interact with each other to form a stable six-helix bundle structure leading to membrane fusion and core entry into the target cell.
Sierra S, Kupfer B and Kaiser R. Basics of the virology of HIV-1 and its replication. *Journal of Clinical Virology* 2005;34:233-44.

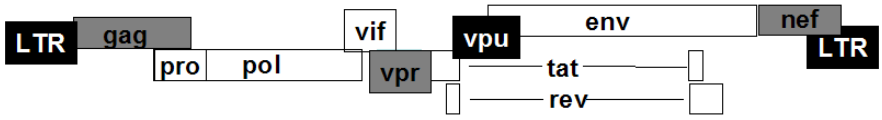

Figure 5: Schematic of the HIV-1 Genome. HIV-1 is a retrovirus with ten open reading frames. In addition to the four major proteins encoded by all retroviruses; Gag, Pro, Pol and Env, HIV-1 also encodes six accessory proteins that help regulate and control viral replication. HIV-1's major structural proteins are encoded by Gag, its major enzymatic activities by Pro and Pol and the envelope glycoproteins required for recognition and binding of cellular receptors for viral entry are encoded by Env. Tat is critical for transcription and Rev plays a major role in the transport of viral RNAs from the nucleus to the cytoplasm. Additionally, Vpu, Vif, Vpr and Nef are accessory proteins that are not uniformly required for virus replication.

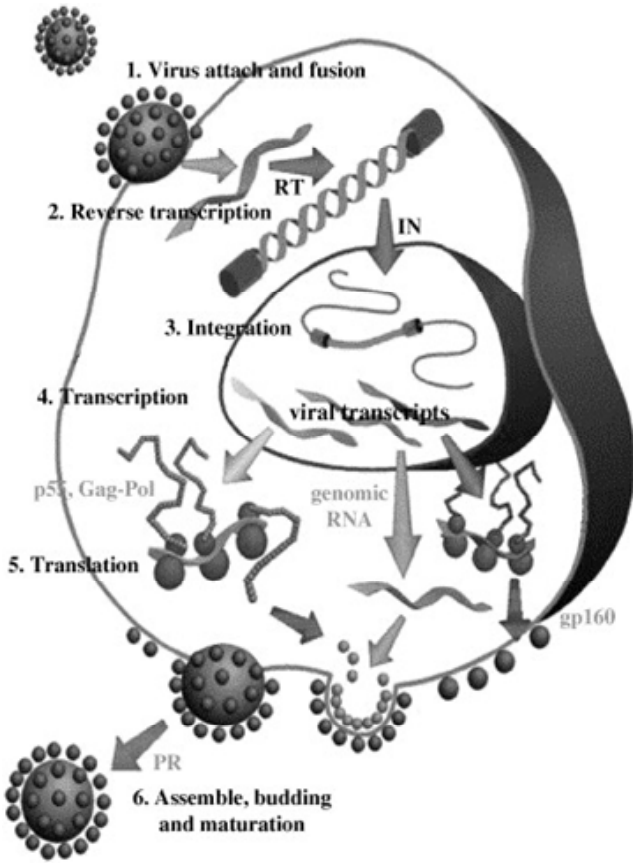

Figure 6: The HIV-1 Life Cycle. The main steps in HIV-1 replication are sequentially numbered 1-6. (1) Virus binds to CD4 and the appropriate coreceptor resulting in fusion of the viral envelope with the cellular membrane and release of the viral nucleocapsid into the cytoplasm. (2) Following uncoating, viral RNA is reverse transcribed by the RT. (3) The resulting dsDNA migrates into the cell nucleus and is integrated into cellular DNA by the integrase. (4) The proviral DNA is transcribed by the cellular RNA polymerase II. (5) The mRNAs are translated by the cellular polysomes. (6) Viral proteins and genomic RNA are transported to the cellular membrane and assemble. Immature virions are released. Gag polypeptide precursors are processed by the viral protease to produce mature infectious viral particles.
Sierra S, Kupfer B and Kaiser R. Basics of the virology of HIV-1 and its replication. *Journal of Clinical Virology* 2005;34:233-44.

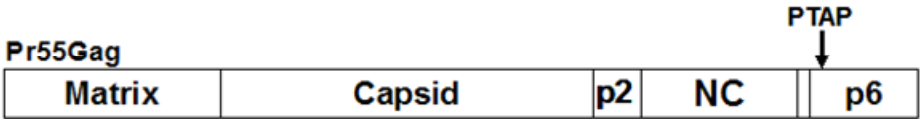

Figure 7: Schematic of HIV-1 Gag Polyprotein. The HIV-1 Gag-Pro-Pol message is primarily translated to produce the Gag and Gag-Pol polyproteins. The Gag polyprotein is a 55 kDa precursor that is proteolytically processed during maturation of the virus into six structural proteins (matrix, capsid, nuclocapsid, p6 late domain and two spacers; p2 and p1) which rearrange and produce the mature virion. A regularly occurring ribosomal -1 frameshift (occurs 5% of the time) during translation, leads to the expression of the Gag-Pro-Pol polyprotein that encodes the major enzymatic proteins; protease, reverse transcriptase and integrase.

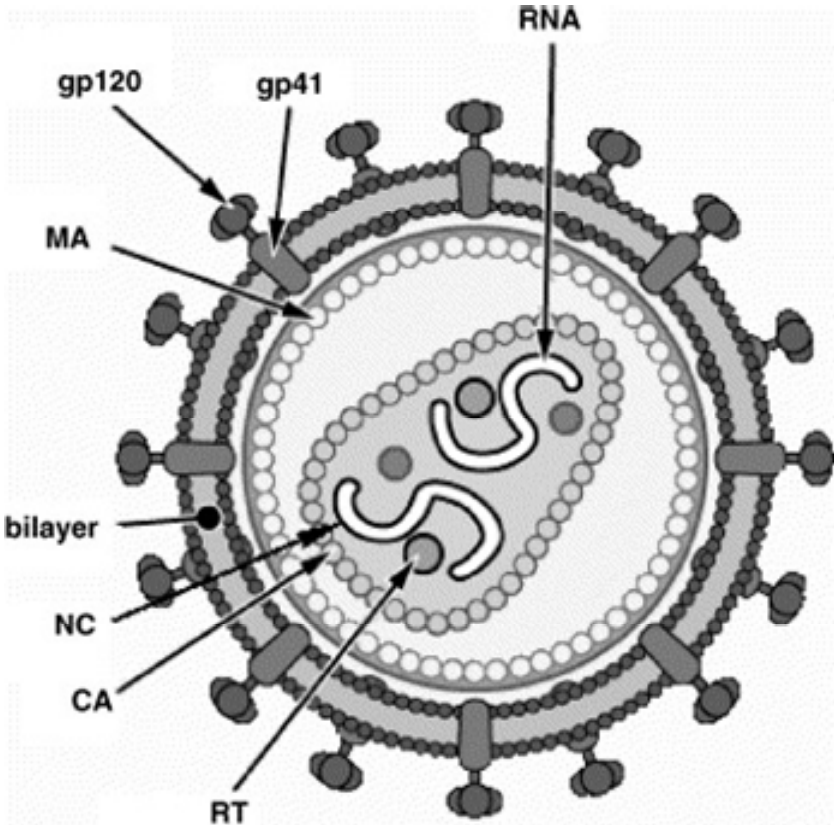

Figure 8: Diagram of HIV-1 Mature Virion. Mature virions are spheres of 100-120 nm in diameter, with a lipid bilayer membrane surrounding the dense truncated cone-shaped core that contains the genomic RNA molecules, the viral protease, reverse transcriptase, integrase, Vpu, Vif, Vpr and Nef. The principal structural proteins forming the core are matrix (MA) which forms the shell associated directly to the inner side of the membrane through the N terminus, capsid (CA) with its N-terminal domain assembling into hexameric rings, nucleocapsid (NC) which has been implicated in the formation and stabilization of the genomic RNA dimers and in NC assembly and p6 which is the domain required for the late stages of viral assembly, efficient release from the cell and internalization of the Vpr protein into the assembled virion. Sierra S, Kupfer B and Kaiser R. Basics of the virology of HIV-1 and its replication. *Journal of Clinical Virology* 2005;34:233-44.

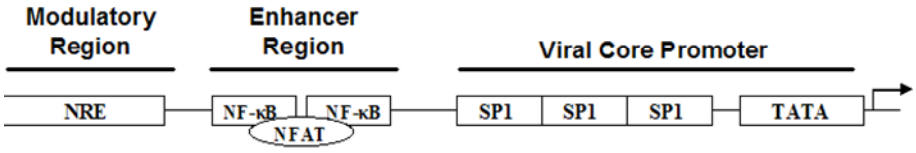

Figure 9: Schematic of HIV-1 Viral Promoter. The viral core promoter is composed of the TATAA box and three Sp1 binding sites. This region is necessary for efficient HIV-1 proviral DNA-mediated basal transcription. Immediately upstream of the three Sp1 sites is an enhancer region with two inducible NF-κB binding sites and an NFAT site, and although these sites are not absolutely necessary for viral replication, they respond to cellular activation signals by stimulating LTR activity and increasing the rate of viral production. Further upstream is the modulatory region with numerous transcription factor binding sites that may increase or decrease transcription. The modulatory region contains binding sites for NFAT, C/EBP, LEF-1 and ATF/CREB.

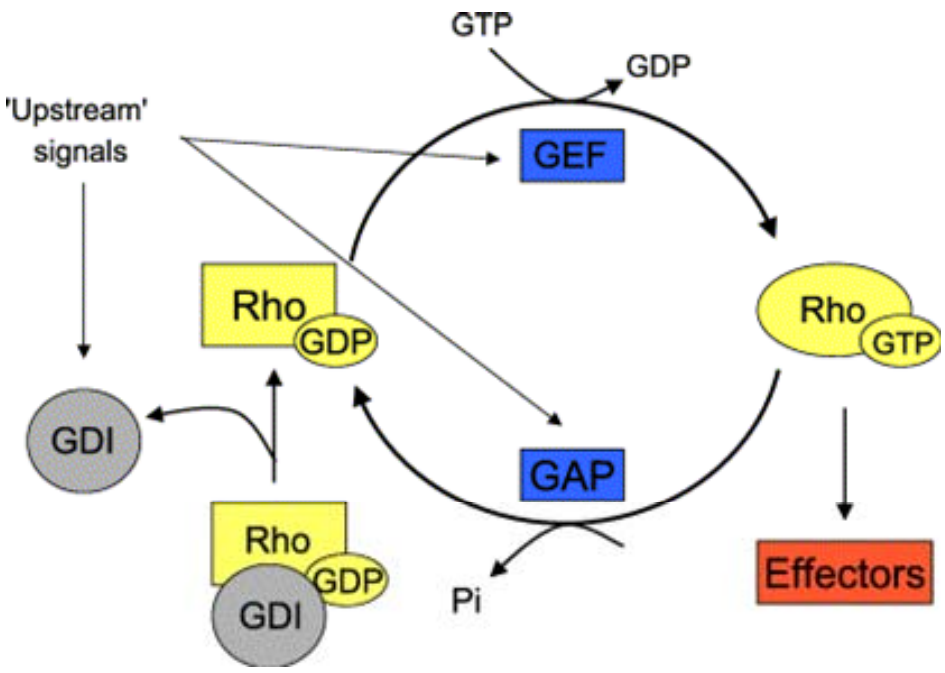

Figure 10: The RhoGTPase Cycle. RhoGTPases cycle between an inactive GDP-bound form and an active GTP-bound form. The cycle is tightly regulated mainly by guanine exchange factors (GEFs), GTPase activating proteins (GAPs) and guanine dissociation inhibitors (GDIs). In their active form, RhoGTPases can bind to effector molecules such as kinases and scaffold proteins.
Raftopoulou M and Hall A. Cell Migration:Rho GTPases lead the way. *Developmental Biology* 2004;265:23-32.

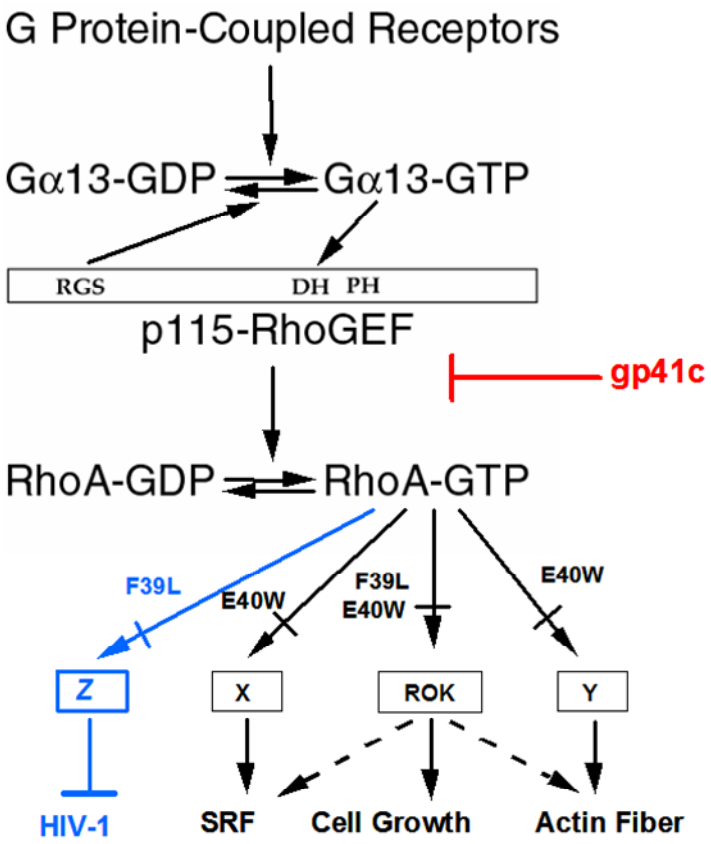

Figure 11: p115RhoGEF Links $G\alpha_{13}$ to Regulation of RhoA and HIV-1 Replication. The putative GPCR linking $G\alpha_{13}$ and the RhoA-signaling pathway trigger a cascade of events to modulate actin cytoskeletal organization (actin fiber), transcriptional activation (SRF), cell survival and growth. p115RhoGEF contains both a regulator of G protein signaling (RGS) domain which allows specific binding to either $G\alpha_{12}$ or $G\alpha_{13}$, resulting in the activation of their GTPase activity (negative regulator) and a Dbl homology (DH) domain which acts as a guanine nucleotide exchange factor (GEF) to specifically activate the small GTPase RhoA (positive regulator). Both p115RhoGEF and RhoA63L reduced the luciferase gene expression (~5-fold) of pNL4.Luc when coexpressed in 293T cells. pNL4-3 was cotransfected with vector or different RhoA mutant derivatives resulting in a distinct profile. The RhoA E40W mutant showed efficient inhibition of HIV-1 replication despite impaired activity in transformation of NIH3T3 cells, SRF activation, and actin stress fiber formation. Furthermore, the RhoA F39L mutant, similar to the RhoA E40W mutant in cell transformation, but still with low or normal activity in SRF activation and actin stress fiber formation, showed significantly reduced inhibition of HIV-1 replication. Therefore, activation of SRF (partially) and actin stress fiber formation by RhoA is neither necessary nor sufficient to inhibit HIV-1 replication. These results suggest that the HIV-1 inhibitory activity of RhoA is genetically separable from its other activities.
Wang L, Zhang H, Solski PA, Hart MJ, Der CJ, Su L. Modulation of HIV-1 Replication by a Novel RhoA Effector Activity. *J Immunol* 2000;164:5369-74.

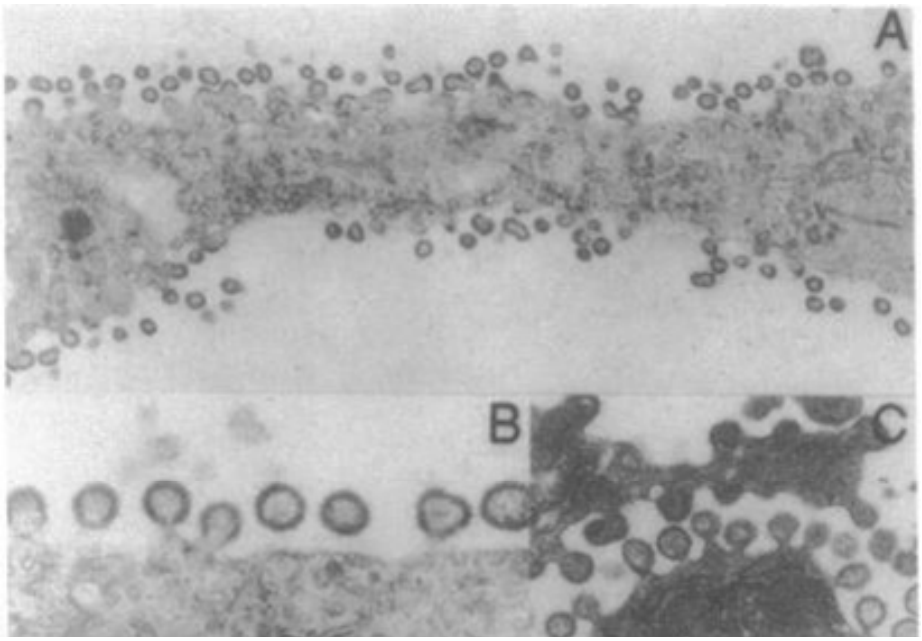

Figure 12: HIV-1 Late Domain Mutants Show a Novel Phenotype. There is a marked reduction in virion release displayed by L1/s and P10, 11/L mutants. The L1/s mutant replaces the N-terminal leucine of p6 and is predicted to create truncated capsid precursors that lack p6 entirely. The P10,11/L mutants replaced the two adjacent proline residues within the 5 amino-acid stretch of PTAPP. (A) Numerous particles were evident on outer surface of the cell membrane but very few infectious particles left the cell surface. (B) Viral particles produced by L1/s mutant had an immature appearance with a thick electron-dense outer shell and an electron-lucent center. (C) Virus remains attached to the cell surface via a thin tether.
Gottlinger HG, Dorfman T, Sodroski JG, Haseltine WA. Effect of mutations affecting the p6 gag protein on human immunodeficiency virus particle release. *PNAS* 1991;88:3195-9.

Virus	L domain	Location	Interactions with Host Proteins
HIV-1	PTAP[60,61], LRSL[247,248]	p6[Gag]	Tsg101[205,241, 249,262] AIP1/Alix[247,248]
MLV	PPPY[220,223]		Nedd4[258]
Ebola	PPPY, PTAP[216]	N-term VP40	Nedd4[216], Yap[261], Tsg101[227]
RSV	PPPY[218,222]	N-term p2b[Gag]	Nedd4[232,238], Yap[258], Tsg101[250]
MPMV	PPPY, PSAP[219]		Nedd4, Tsg101[259]
HTLV-1	PPPY, PTAP[224,255]	C-term of MA	Nedd4, Tsg101[Brouamr 2003]
BLV	PPPY[225]	C-term of MA	
EIAV	YPXL[221]	p9[Gag]	AP-50 of AP-2[245], AIP1/Alix[246-8]

Table 1: Most enveloped viruses have L domains to facilitate in viral release. The summarizes data regarding the specific L domain sequence used by each virus, its location within the virus and known host protein interactions.

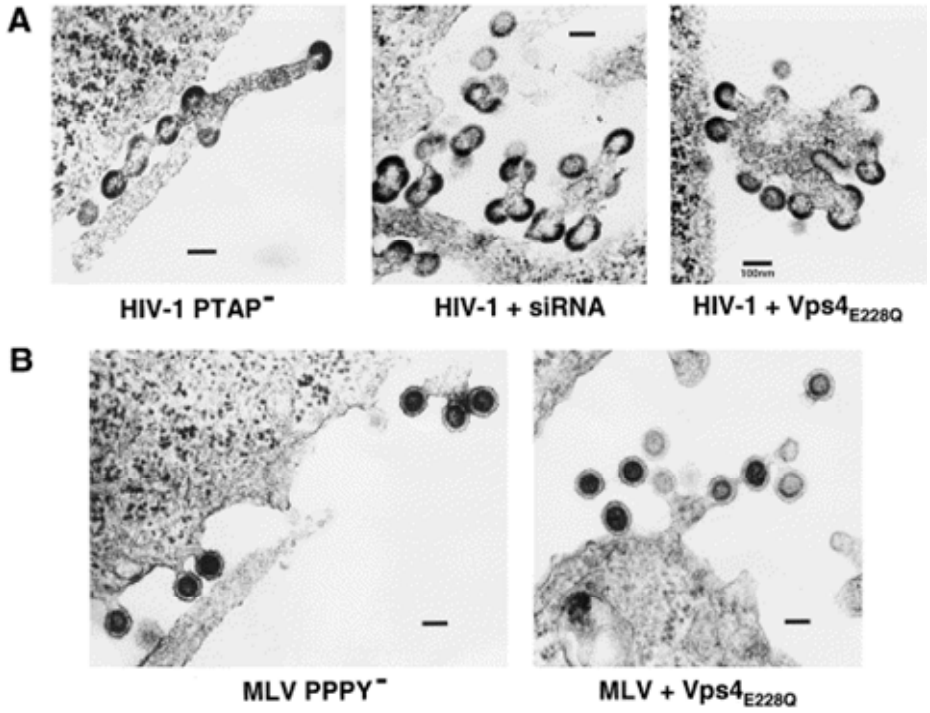

Figure 13: Tsg101 Depletion and Vps4 Dominant-Negative Mutants Block Virus Budding at a Late Stage. (A) TEM showing the effects on HIV-1 budding when mutating the Gag^{p6} PTAP sequence (left), depleting cellular Tsg101 (center) or overexpressing the dominant-negative Vps4E228Q protein (right). In each case, virus budding was arrested at a late stage, with immature particles remaining connected to the plasma membrane via thin membrane tethers or to other budding particles forming clusters of interconnected particles. (B) TEM showing the effects on MLV budding when mutating the p12 PPPY motif (left) or overexpressing the dominant-negative Vps4E228Q protein (right). These MLV late domain phenotypes are similar to those described for HIV-1.

Garrus JE, von Schewedler UK, Pornillos OW, Morham SG, Zavitz KH, Wang HE, Wettstein DA, Stray KM, Cote M, Rich RL, Myszka DG and Sundquist WI. Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. *Cell* 2001;107:55-65.

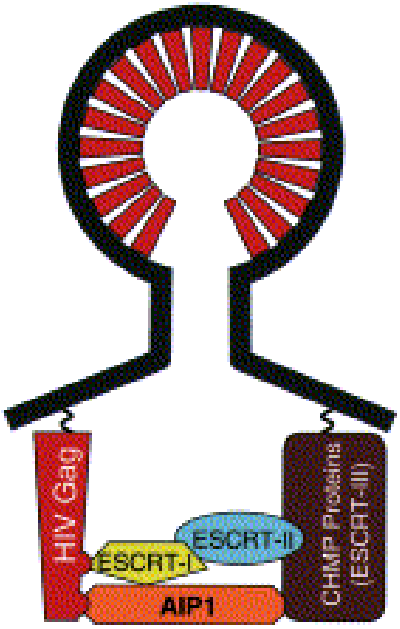

Figure 14: Model for HIV-1 Late Domain Function. The HIV-1 Gag^{p6} domain forms a ternary complex with host proteins; AIP1 and ESCRT-I component, Tsg101, which then recruits CHMP proteins (components of ESCRT-III) directly via AIP1 and indirectly via ESCRT-II to form ESCRT-III.
Strack B, Calistri A, Craig S, Popova E and Gottlinger HG. AIP1/ALIX is a binding partner for HIV-1 p6 and EIAV p9 functioning in virus budding. *Cell* 2003;114:689-99.

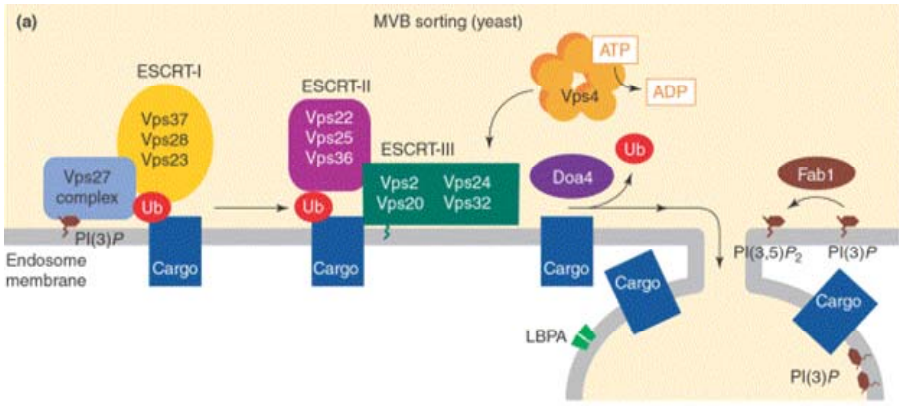

Figure 15: MVB Sorting in Yeast. Yeast Class E Vps protein complexes and auxiliary factors that function in protein sorting and vesicle formation during MVB biogenesis. ESCRT-I recognizes ubiquitinated cargo and requires the UEV domain of Vps23 (yeast homolog of Tsg101). The binding of ubiquitinated cargo by ESCRT-I leads to activation of ESCRT-II. ESCRT-II interacts directly with the ESCRT-III Vps20/Snf7 subcomplex to promote the assembly of multiple copies of ESCRT-III on the membrane. In the final step, the AAA-ATPase Vps4p interacts with the ESCRT-III Vps2/24 subcomplex to catalyze the release of the entire machinery from the membrane.
Pornillos O, Garrus JE and Sundquist WI. Mechanisms of enveloped RNA virus budding. *TRENDS in Cell Biology* 2002:12(12)569-79.

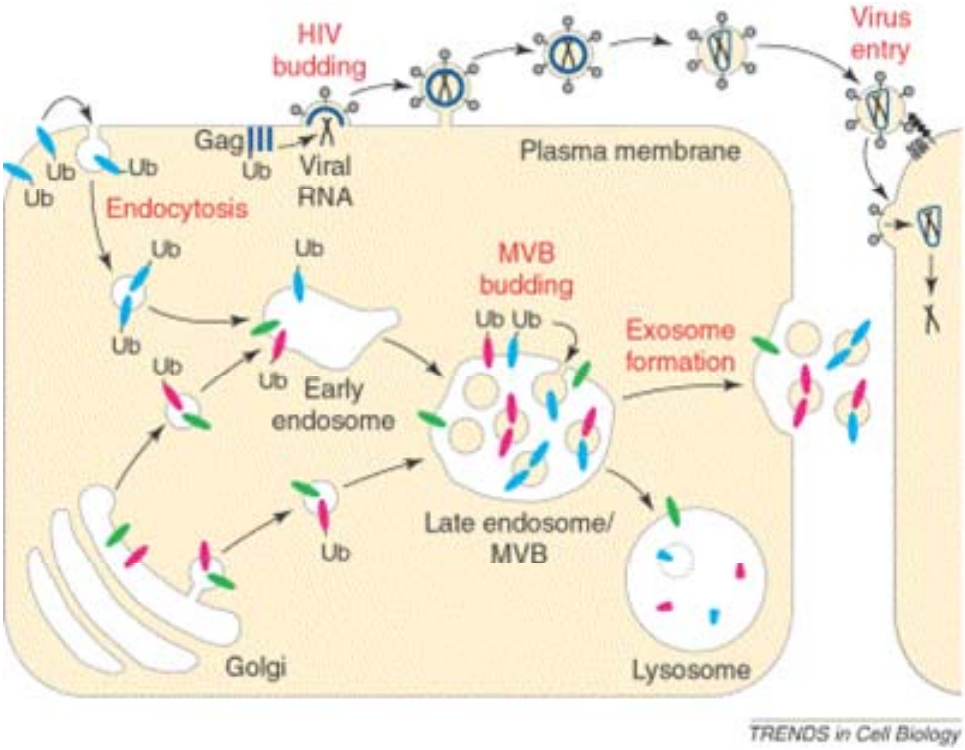

Figure 16: The Endosomal Sorting Pathway, HIV-1 Assembly/Budding and Exosome Formation. HIV-1 Gag proteins are shown in dark blue and protein cargos sorted through the Vps pathway are shown in magenta, green and light blue. The process of HIV-1 viral assembly/budding is analogous to the sorting of ubiquitinated cargo by the endosomal sorting pathway and formation of the multivesicular body. Sorting of proteins by the endosomal sorting pathway can lead to one of three outcomes; (1) the MVB fuses with the lysosome and contents are degraded, (2) the MVB fuses with the plasma membrane and contents are released as exosomes into the extracellular space or (3) the proteins remaining in the intraluminal membrane are recycled to their place of origin.
Pornillos O, Garrus JE and Sundquist WI. Mechanisms of enveloped RNA virus budding. *TRENDS in Cell Biology* 2002:12(12)569-79.

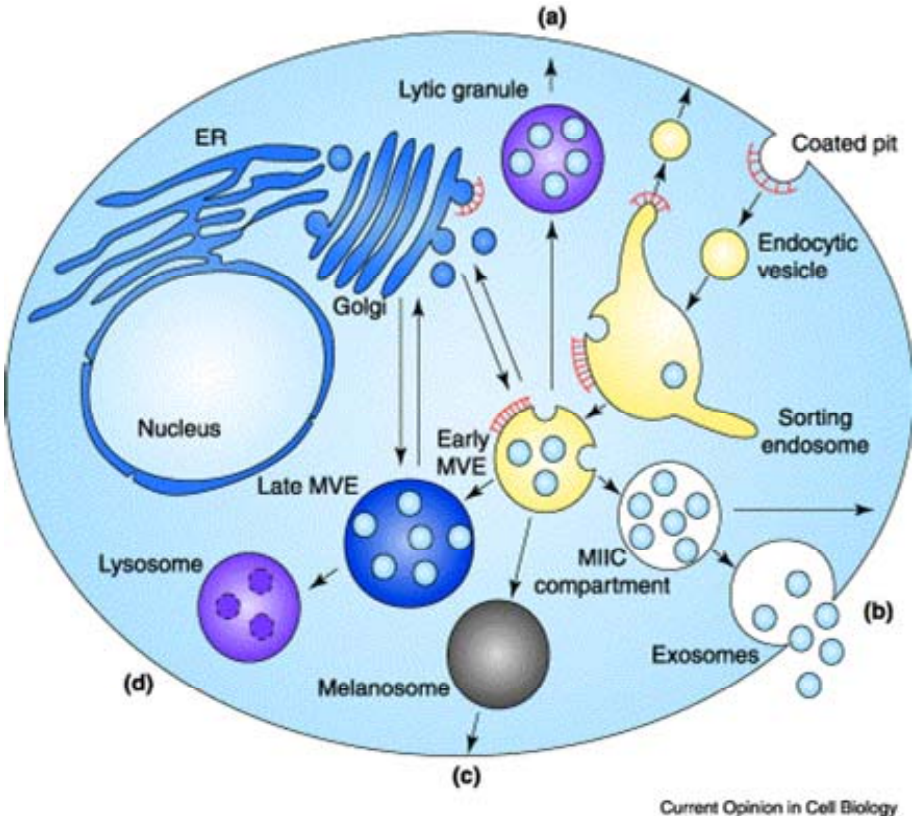

Figure 17: Formation and Function of MVEs. Multivesicular endosomes (aka MVBs) are formed after invagination of the limiting membrane of the sorting endosome. They are versatile and can serve different functions in different cell types, such as being precursors for (a) lytic granules in T lymphocytes, (b) MHC class II compartments and exosomes in antigen-presenting cells, (c) melanosomes in melanocytes, and (d) late MVEs/lysosomes in most nucleated cells. Both endocytic and biosynthetic proteins are sorted in and out of MVEs, indicated by the arrows.
Raiborg C, Rusten TE and Stenmark H. Protein sorting into multivesicular endosomes. *Current Opinion in Cell Biology* 2003;15:446-55.

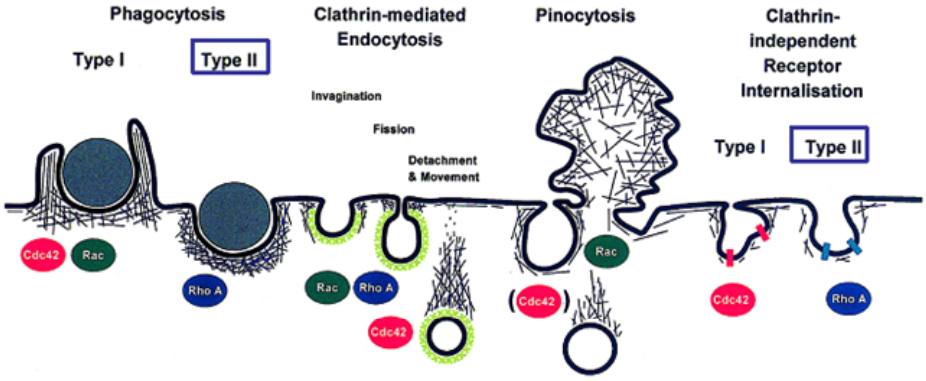

Figure 18: Diagram Summarizing the Known and Proposed Sites of Action of RhoGTPases in Internalization from the Plasma Membrane. The most clear-cut interface of RhoGTPases with the endocytic trafficking is in the process of clathrin-independent internalization from the cell surface. All clathrin-independent routes into the cell are driven by actin polyermization and therefore have obligatory requirements for RhoGTPases. In type II phagocytosis, phagocytes display the CR3 complement receptor, recognizing particles opsonized with the complement iC3b – this process is dependent on RhoA but not Cdc42 or Rac. Additionally, RhoA is involved in type II clathrin-independent receptor internalization of IL-2. The IL-2 receptor is internalized from lipid rafts in a dynamin-dependent manner – this process is inhibited by expression of dominant-negative RhoA or Rac.
Qualmann B and Mellor H. Regulation of endocytic traffic by Rho GTPases. *Biochem J.* 2003;371:233-41.

CHAPTER TWO:
INHIBITION OF HIV-1 REPLICATION BY RHOA IS MEDIATED BY RHO-ASSOCIATED KINASE (ROCK)

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Rebecca J. Loomis performed all experiments.
Patricia A. Solski and Channing J. Der provided the ROCK constructs used in these experiments.

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ABSTRACT:

RhoGTPases play essential roles in modulating the actin cytoskeleton, controlling cell processes and activation of transcription factors. Previously, we reported that the cytoplasmic tail of the HIV-1 transmembrane envelope glycoprotein gp41 (gp41c) interacts directly with the carboxy-terminal regulatory domain of p115RhoGEF, a specific guanine nucleotide exchange factor and activator of RhoA. Binding of gp41c to p115RhoGEF inhibits activation of RhoA by p115RhoGEF [195]. Ectopic expression of p115RhoGEF, $G\alpha_{13}$, which activates p115RhoGEF activity, or constitutively active RhoA leads to inhibition of HIV-1 replication. The RhoA effector activity involved in inhibiting HIV-1 replication is genetically separable from its other known activities suggesting that RhoA inhibits HIV-1 replication via a novel effector activity [196].

We found Rho-associated kinase (ROCK), a RhoA effector, to specifically inhibit HIV-1 gene expression in T cells. ROCK overexpression inhibited viral gene expression in a dose-dependent manner. At very low levels of ROCK overexpression, we saw a preferential inhibitive effect on HIV-1 virion production (i.e. assembly/release) with a minimal effect on viral gene expression, whereas higher levels of ROCK overexpression, significantly inhibited HIV-1 gene expression. A ROCK specific inhibitor, Y27632, counteracted RhoA inhibition of HIV-1 gene expression. Coexpression of ROCK with constitutively active RhoA further inhibited viral gene expression than when either ROCK or constitutively active RhoA were expressed alone. Therefore ROCK is the RhoA effector that contributes to RhoA-mediated inhibition of HIV-1 gene expression. Mutations in the second NF-κB and/or the first two Sp1 binding sites of the HIV-1 LTR abrogated ROCK-mediated inhibition of HIV-1 gene expression. The mechanism of RhoA-mediated inhibition via ROCK has eluded us.

INTRODUCTION:

The small GTPase family of Rho proteins (RhoA, Rac1 and Cdc42) regulate a variety of important cellular processes by cycling between an inactive GDP-bound form and an active GTP-bound form [119-121]. RhoGTPases are involved in numerous intracellular signaling pathways including actin cytoskeletal reorganization [122-124], activation of transcription factors such as serum-response factor [125] or NF-κB [126], and cell cycle progression [127, 128]. RhoA's diverse functions are mediated by its association with RhoA effector proteins when in its active form [175]. These include three families of Ser/Thr kinases; (1) Rho-associated kinase (ROCK) and other ROCK family kinases, (2) protein kinase N and its related kinases and (3) citron kinase. The conformational changes between the GTP and GDP-bound forms of RhoA are restricted primarily to two surface loops, named switch regions I and II [176, 177]. Effector proteins utilize these differences to discriminate between GTP- and GDP-bound forms. Using RhoA effector domain mutants, it was demonstrated that distinct effectors are involved in RhoA-mediated transformation of NIH3T3 cells, SRF activation and actin stress fiber formation [178, 179].

G protein coupled receptors (GPCRs) play critical roles in facilitating T cells to react to external signals ranging from developmental cues to chemotactic molecules. GPCRs transduce signals through their direct interaction with members of the heterotrimeric G protein families: $G\alpha_i$, $G\alpha_s$, $G\alpha_q$ and $G\alpha_{12}$. The p115RhoGEF protein couples $G\alpha_{13}$ signaling to RhoA activation. p115RhoGEF contains both a regulator of G protein signaling (RGS) domain which allows specific binding to either $G\alpha_{12}$ or $G\alpha_{13}$, resulting in the activation of their GTPase activity, and a Dbl homology (DH) domain which acts as a guanine exchange

factor to specifically activate the small GTPase RhoA [170-172]. Both p115RhoGEF and RhoA have been shown to affect T cell function.

Although RhoGTPases have been implicated in various steps of T cell activation [182, 191, 193], little is known about how RhoGTPases affect HIV-1 replication. We have previously shown that the long cytoplasmic tail of HIV-1 gp41c, interacts directly with the carboxy-terminal regulatory domain of p115RhoGEF, a specific guanine nucleotide exchange factor and activator of RhoA [195]. Ectopic expression of p115RhoGEF or $G\alpha_{13}$, which activates p115RhoGEF activity, leads to inhibition of HIV-1 replication via a RhoA-dependent mechanism. The RhoA effector activity involved in inhibiting HIV-1 replication is genetically separable from its activities in activation of serum response factor and actin stress fiber formation [196], suggesting RhoA inhibits HIV-1 regulation via a novel effector activity.

To investigate how RhoA signaling pathways modulate HIV-1 replication, we tested several RhoA effectors in human T cells. We found that Rho-associated kinase (ROCK), a RhoA effector, specifically inhibits HIV-1 gene expression in T cells. At very low levels of ROCK overexpression, HIV-1 virion production (i.e. assembly/release) was significantly inhibited without a similar effect on viral gene expression. Ectopic expression of ROCK inhibited HIV-1 gene expression in a dose-dependent manner, with high levels of ROCK efficiently inhibiting HIV-1 transcription. A ROCK specific inhibitor, Y27632, counteracted RhoA inhibition of HIV-1 gene expression. Coexpression of wild-type ROCK with constitutively active RhoA synergistically inhibited HIV-1 gene expression than when either wild-type ROCK or constitutively active RhoA was expressed alone. These findings suggest RhoA signals through ROCK to repress HIV-1 transcription. Mutations or deletions in the

second NF-κB and/or the first two Sp1 binding sites of the HIV-1 LTR abrogated ROCK's ability to inhibit HIV-1 gene expression. Disruption of either the NF-κB or the Sp1 sites was sufficient to abrogate HIV-1 gene repression by ROCK, suggesting that the cooperative interaction between NF-κB and Sp1 may be disrupted.

Our data suggests that ROCK may inhibit HIV-1 gene expression by affecting binding to the NF-κB and/or Sp1 sites. We demonstrated that ROCK does not disrupt binding at either the NF-κB or Sp1 sites individually. However, we have not fully addressed whether the cooperative interaction between NF-κB and Sp1 is disrupted. Alternatively, ROCK may recruit an HDAC to interact with NF-κB and/or Sp1 to prevent activation of the HIV-1 LTR. However, ROCK-mediated HIV-1 gene expression was further repressed by an HDAC inhibitor. Another potential mechanism for ROCK-mediated HIV-1 transcriptional repression is through phosphorylation, whether direct or indirect, given that the kinase activity of ROCK is necessary for its inhibitive effect on HIV-1 gene expression. Therefore, it is critical to identify possible phosphorylation target(s) of ROCK and any phosphorylation changes that occur during HIV-1 infection with constitutively active ROCK.

Materials and Methods:

Reagents, plasmids and cell lines

The pNL4-3 plasmid encodes the entire HIV-1 genome DNA in pUC18 [327]. The pNL4.LucR-E- plasmid was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program [328]. ROCK constructs were previously described (Figure 19E) [329]. The constitutively active RhoA mutants (RhoA63L) were cloned in the pAX142 mammalian expression vector [330]. Jurkat T cells (kindly provided by G. Crabtree, Stanford, CA) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin and L-glutamine. HeLa-MAGI cells (NIH AIDS Research and Reference Reagent Program, [331]) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, penicillin/streptomycin and L-glutamine.

HIV-1 Production and Replication in Transfected Human Cells

Transient production of HIV-1 was performed by transfecting NL4-3 (0.3 μg) with vector, wild-type ROCK, ROCKΔ4 or ROCK KDIA (0.6 μg) in Jurkat T cells in 48-well plates at $1x10^5$ cells/well with GenePorter (Gene Therapy Systems, San Diego, CA). At 48 hours post-transfection, HIV-1 virions in cell supernatant were measured by p24 ELISA or RT assays [332] and infectious units were determined by titering the supernatant on HeLa-CD4-LTR-lacZ cells (MAGI) as described previously [331].

To analyze HIV-1 gene expression in transfected cells, pNL4.Luc (0.3 μg) was transfected into Jurkat T cells with vector, wild-type ROCK, ROCKΔ4 or ROCK KDIA (0.6 μg) in Jurkat T cells in 48-well plates with GenePorter (Gene Therapy Systems, San Diego,

CA). Cell extracts were analyzed at 48 hours post-transfection for luciferase activity (AutoLumat LB953, EG&G berthold; FluoStar Optima, BMG LabTech).

To define the binding site(s) of the HIV-1 LTR necessary for ROCK-mediated inhibition of HIV-1 gene expression, Jurkat T cells were cotransfected with HIV-1 LTR-luciferase constructs (0.12 μg), HIV-1 tat (0.24 μg) and vector, ROCKΔ4 or ROCK KDIA (0.6 μg) in a 48-well plate at $1x10^5$ cells/well. Cells were harvested 48 hours post-transfection and analyzed for luciferase activity (AutoLumat LB953, EG&G berthold; FluoStar Optima, BMG LabTech).

For transfections involving the ROCK specific inhibitor (Y26732), Jurkat T cells were cotransfected with pNL4.Luc (0.3 μg) and vector, wild-type ROCK or constitutively active RhoA (RhoA63L) (0.6 μg) in a 48-well plate at $1x10^5$ cells/well. At 24 hours post-transfection, the Y26732 inhibitor was added at a final concentration of either 10 μM or 25 μM. Cells were harvested at 48 hours post-transfection and analyzed for luciferase activity (AutoLumat LB953, EG&G berthold; FluoStar Optima, BMG LabTech).

For transfections involving the use of HDAC1 inhibitor, trichostatin A (TSA), TSA was added at 100 ng/mL at 24 hours post-transfection. Cells were harvested at 48 hours post-transfection for analysis.

Western Blotting

Transfected Jurkat T cells were lysed and resolved (50-100 μg of total protein) on 10% SDS-PAGE. Gels were transferred to PVDF membranes (Amersham Biosciences) and blocked with 5% nonfat milk. The membrane was probed with mouse α-p24 (NIH AIDS Research and Reference Reagent Program), a mouse α-Myc (AbCam, Cambridge, MA) and

mouse α-actin (Sigma-Aldrich) specific antibodies and visualized using an ECL Kit (Amersham Biosciences).

Electrophoretic Mobility Shift Assays

To prepare the nuclear extract, the collected cell pellets were resuspended in 90 μL of CE+NP-40 buffer. CE+NP-40 buffer is made of 500 μL 1M Hepes pH 7.6, 3 mL 1M KCl, 100 μL 0.5M EDTA, 50 μL 1M DTT, 45.1 mL dH_2O, 0.2% NP-40 and protease inhibitors. Cell lysates were incubated on ice for 3 minutes and then spun at 2500 rpm for 4 minutes. The supernatant was discarded (cytoplasmic extract). The nuclear pellet was resuspended in 90 uL of CE+NP-40 buffer and spun at 2500 rpm for 4 minutes. The supernatant was again discarded. The pellet is resuspended in 90 μL of NE buffer. NE buffer is composed of 1 mL 1M Tris pH 8, 4.2 mL 5M NaCl, 75 μL 1M $MgCl_2$, 40 μL 0.2M EDTA, 12.5 mL 100% glycerol and 31.54 mL dH_2O. The nuclear pellet was incubated for 10 minutes at room temperature and spun at full speed for 10 minutes. The supernatant was transferred to a new tube (nuclear extract) and protein concentration was determined using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL).

Sp1 and NF-κB consensus sequence probes were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. The Sp1 consensus oligonucleotide sequence was 5'ATTCGATCGGGGCGGGGCGAGC 3', underlined portion represents Sp1 binding site. The NF-κB consensus oligonucleotide sequence was 5'AGTTGAGGGGACTTTCCCAGGC 3', underlined portion represents NF-κB binding site. The labeled probes necessary for this experiment were prepared as follows; 7.4 μL Sp1/NF-κB consensus oligo, 2 μL PNK buffer, 5 μL $\gamma^{32}P$, 1 μL PNK and 4.6 μL dH_2O. Mix everything together and incubate at 37°C for 1

hour. The mixture is spun through a G25 Sephadex Spin column to purify at 1.6 krpm for 5 minutes. The labeled probe was then counted via a scintillation counter.

The gel was prepared as follows; 7 mL acrylamide, 4 mL TGE (10x TGE = 0.25M Tris, 1.9M glycine, 10mM EDTA pH 8.5), 4 mL 50% glycerol, 25 mL dH_2O, 300 μL 10% APS and 40 μL Temed.

Reactions were prepared to contain 20-50 μg protein, 50,000 cpm of labeled probe, 0.05 μg dIdC/reaction, 4 μL 5xbinding buffer (50mM Tris pH 7.6, 50% glycerol, 5mM DTT and 2.5mM EDTA). Cold competitor or non-specific competitor was added at 50 times the amount of labeled probe used. Water, 5xbinding buffer and protein with either cold competitor/non-specific competitor were incubated for 20 minutes at RT. Labeled probe was then added and again the mixture was incubated for 20 minutes at RT.

The gel was run using 1xTGE as running buffer, at 40mA for approximately 2 hours. Once the gel was finished running, it was placed on Watman paper, wrapped in seran wrap and dried before exposure to film.

RESULTS:

RhoA Specifically Inhibits HIV-1 Gene Expression in T cells by ROCK

To identify the novel RhoA effector activity involved in inhibiting HIV-1 replication, we tested various RhoA effectors for their effect on HIV-1 viral replication in 293T and human T cells. Ectopic expression of ROCK, a RhoA effector, was shown to inhibit HIV-1 gene expression (Figure 19A) and HIV-1 viral release (Figure 19B) in Jurkat T cells. Both wild-type ROCK and constitutively active ROCKΔ4 inhibited HIV-1 replication while the kinase-dead ROCK showed no effect on HIV-1 gene expression suggesting that the kinase activity of ROCK is required for its inhibitive effect on HIV-1 gene expression (Figure 19E). Likewise, we demonstrated that cell-associated viral protein expression was inhibited when wild-type ROCK and constitutively active ROCKΔ4 were overexpressed (Figure 19C). We further investigated the effect of ROCK on viral protein expression, specifically looking at processing of Gag, and found no evidence that ROCK altered Gag processing (data not shown). We conclude ROCK inhibits HIV-1 gene expression.

Next, we sought to determine whether ROCK specifically inhibited HIV-1 gene expression by using plasmids with promoters other than HIV-1 LTR. Jurkat T cells were cotransfected with either pGK-GFP (housekeeping promoter) or CMV-GFP (viral promoter) and vector, constitutively active ROCKΔ4 or kinase-dead ROCK. We analyzed promoter activity by measuring GFP expression. ROCKΔ4 did not inhibit GFP expression from either the pGK or CMV promoters (Figure 19D) suggesting that ROCK specifically inhibits HIV-1 LTR gene expression.

Ectopic expression of ROCK had a dose-dependent effect on HIV-1 viral replication. We cotransfected Jurkat T cells with pNL4.Luc or pNL4-3 and increasing amounts of wild-

type ROCK. At very low levels of ROCK overexpression (10 ng), there was a significant inhibition of HIV-1 viral release but no effect on viral gene expression (Figure 19F, G). HIV-1 viral gene expression was inhibited by ROCK overexpression in a dose-dependent manner with higher levels of ROCK expression inhibiting gene expression more significantly (Figure 19G).

To determine if ROCK was the sole RhoA effector involved in repressing HIV-1 gene expression, we used a ROCK-specific inhibitor, Y27632, to block ROCK activity. In Figure 20A, we cotransfected Jurkat T cells with pNL4.Luc and vector or wild-type ROCK and added various concentrations of Y27632 (0 μM, 10 μM or 25 μM), 24 hours post-transfection. At 10 μM concentration of Y27632, wild-type ROCK was unable to inhibit HIV-1 gene expression. In Figure 20B, we cotransfected Jurkat T cells with pNL4.Luc and vector or constitutively active RhoA (RhoA63L) and added various concentrations of Y27632 (0 μM, 10 μM or 25 μM), 24 hours post-transfection. At both 10 μM and 25 μM concentrations of Y27632, RhoA63L was unable to inhibit HIV-1 gene expression to the same degree as when no drug was present. The ROCK inhibitor, Y27632, partially counteracts RhoA inhibition of HIV-1 gene expression.

To further support our finding that RhoA inhibited HIV-1 gene expression through activation of ROCK, we found that coexpression of ROCK and RhoA63L inhibited HIV-1 gene expression more than when expressing either ROCK or RhoA63L alone (Figure 20C). We conclude ROCK is the RhoA effector involved in mediating RhoA's inhibitive effect on HIV-1 gene expression.

ROCK Inhibits HIV-1 Gene Expression via NF-κB and Sp1 Binding Sites

To define the binding site(s) of the HIV-1 LTR necessary for ROCK-mediated inhibition of HIV-1 gene expression, we used various LTR truncation and deletion mutants. First, we cotransfected Jurkat T cells with HIV-1 long-terminal repeat (LTR)-luciferase truncation mutants. We found that the NF-κB binding sites are required for ROCK-mediated HIV-1 gene repression (Figure 21A). Deletion of all sites upstream of the TATA box or deletion of the three Sp1 sites but maintaining the integrity of the NF-κB sites resulted in LTR gene expression at background levels. Raw data is shown in Figure 22. A schematic of the HIV-1 LTR-luciferase truncation mutants used are shown in Figure 21B.

Next, we used LTR-linker scanning mutants to more accurately define the NF-κB/Sp1 binding site region necessary for HIV-1 gene expression by ROCK. The LTR-linker scanning-luciferase mutants were prepared where 18 base pair regions of the wild-type LTR were replaced with an NdeI-XhoI-SalI (NXS) polylinker [333]. These linker-scanning mutants provide a more accurate assessment of a promoter's regulatory sequences than does deletion analysis, since effects of relatively small substitution mutations are analyzed in the spatial context of the remaining wild-type sequences. The linker-scanning mutants used in this analysis are shown and described in Figure 23B, C. ROCKΔ4 was unable to inhibit HIV-1 gene expression when either NF-κB sites or the first two Sp1 sites were altered, but was able to inhibit HIV-1 gene expression when the third Sp1 site was altered (Figure 23A). Raw data is shown in Figure 24. We, therefore, conclude that ROCK inhibits HIV-1 gene expression by affecting interaction or binding at the NF-κB and the first two Sp1 sites.

To more closely analyze the NF-κB/NFAT site(s) involved in ROCK-mediated HIV-1 gene repression, we used HIV-1 LTR constructs with point mutations in the first NF-κB

site, the second NF-κB site, the NFAT site or both NF-κB sites (Figure 25B). When both NF-κB sites were mutated, the LTR promoter activity was negligible. However, when either the first NF-κB site or the NFAT site were mutated, constitutively active ROCKΔ4 was able to repress HIV-1 gene expression but when the second NF-κB site was mutated, ROCKΔ4 was unable to inhibit HIV-1 gene expression (Figure 25A). Raw data is shown in Figure 26.

Taken together, the results of the HIV-1 LTR mutant analysis demonstrate the necessity of the second NF-κB site (Figure 25A) and the first two Sp1 sites (Figure 23A) in ROCK-mediated inhibition of HIV-1 gene expression. Disruption of either NF-κB or Sp1 was sufficient to abrogate ROCK's effect on HIV-1 gene expression suggesting that ROCK disrupts the cooperative interaction of NF-κB/Sp1 needed to activate the HIV-1 LTR.

Mechanism by which ROCK Mediates HIV-1 Repression

Although we have yet to elucidate the mechanism by which ROCK inhibits HIV-1 gene expression, we have several hypotheses. First, ROCK may disrupt the cooperative interaction between NF-κB and Sp1 to inhibit gene activation (Figure 27A) to inhibit HIV-1 gene activation. NF-κB and Sp1 sites are often found adjacently in promoter regions, including those for HIV-1 [74], intracellular adhesion molecule 1 [99], vascular adhesion molecule 1 [100] and granulocyte-macrophage colony-stimulating factor [101]. In the HIV-1 LTR, the actions of NF-κB and Sp1 are highly cooperative, involving the effects on DNA binding of both factors to their adjacent binding sites, leading to increased transcriptional control [81].

If this cooperative interaction is disrupted, most likely indirectly by ROCK, binding to one or both the NF-κB and Sp1 sites would be inhibited. To test this hypothesis, we

performed electrophoretic mobility shift assays (EMSAs) attempting to determine whether binding to the NF-κB or Sp1 sites was altered in the presence of ROCK and if any alteration correlated with effects on HIV-1 transcription. The preliminary EMSAs revealed no change or perhaps an increase in binding to the NF-κB and Sp1 sites, individually, in the presence of constitutively active ROCK (Figure 27B, C). To fully address whether the cooperative interaction between NF-κB and Sp1 is disrupted by ROCK to inhibit HIV-1 gene expression we need to perform EMSAs using a probe that contains both the NF-κB and Sp1 sites adjacent to one another, as seen in the HIV-1 LTR.

Second, ROCK may enhance histone deacetylase1 (HDAC1) recruitment for interaction with Sp1 at the HIV-1 LTR (Figure 28A). Sp1 binds the GC-box and activates a host of viral and cellular genes [95, 334] and it composed of a several modules including N-terminal inhibitory domain, Ser/Thr domain, glutamine rich domains which are important for transcriptional activation and a zinc-finger DNA binding domain in its C-terminus [95, 334, 335]. HDACs have been implicated in repression of gene expression by facilitating chromatin condensation through the removal of an acetyl group of nucleosomal core histones [336-339] and may be involved in cell-cycle regulation, differentiation and development, as well as human cancers [340, 341]. Sp1 can be a target for HDAC1-mediated transcriptional repression with the interaction between Sp1 and HDAC1 being direct and requiring the C-terminal zinc-finger domain of Sp1 [101, 340]. In this case, the transcriptional repression did not involve the deacetylase activity of HDAC1 and was accomplished by the direct protein-protein interaction between Sp1 and HDAC1, which interferes with GC-box Sp1 binding.

We hypothesize that ROCK inhibits HIV-1 gene activation by enhancing recruitment of HDAC1 to interact with Sp1 and/or NF-κB. To test this hypothesis, we added an HDAC1

inhibitor, trichostatin A (TSA), to our transfected cells. Preliminary results revealed that the addition of the HDAC1 inhibitor further repressed HIV-1 gene expression with overexpression of constitutively active ROCK implying that ROCK does not recruit HDAC1 to the Sp1 binding site of the HIV-1 LTR to maintain gene repression (Figure 28B), although we do not yet know why the addition of TSA led to further repression of the HIV-1 LTR.

However, it is also possible that ROCK mediates HIV-1 transcription through some other mechanism such as phosphorylation. ROCK is a Ser/Thr kinase, whose kinase activity is essential for its ability to inhibit gene expression and Sp1 contains a Ser/Thr region with several sites known to be phosphorylated with effects on promoter activity. ROCK may mediate Sp1 phosphorylation thereby affecting Sp1 promoter activity.

DISCUSSION:

To investigate how RhoA signaling pathways modulate HIV-1 replication, we tested several RhoA effectors in human T cells. We found Rho-associated kinase (ROCK), a RhoA effector, to specifically inhibit HIV-1 gene expression in T cells. Overexpression of wild-type ROCK at very low levels had a significant inhibitive effect on virion production (i.e. assembly/release) without a similar effect on viral gene expression. Wild-type ROCK overexpression inhibited viral transcription in a dose-dependent manner, with the greatest transcriptional inhibition at the highest levels of wild-type ROCK overexpression. A ROCK specific inhibitor, Y27632, counteracted RhoA inhibition of HIV-1 gene expression. Coexpression of wild-type ROCK with constitutively active RhoA further inhibited viral gene expression than when either ROCK or RhoA were expressed alone. Together, these findings suggest RhoA signals via ROCK to inhibit HIV-1 transcription. Mutation or deletion of the second NF-κB and/or the first two Sp1 sites in the HIV-1 LTR abrogated transcriptional repression by ROCK.

HIV-1 latency is a natural consequence of the virus replicating in activated $CD4^+$ T cells. $CD4^+$ T cells are capable of undergoing reversible changes in activation state [8, 342]. After responding to an antigenic challenge, some activated $CD4^+$ T cells return to a resting state and persist as memory cells. In this state, the cells are not permissive for viral replication, however, integration of the viral genome into a host-cell chromosome, guarantees viral persistence for the lifespan of the memory T cell [343]. Upon mitogen stimulation, infectious virus can be recovered from these cells.

A major obstacle to HIV-1 eradication is the establishment of a transcriptionally silent viral infection in infected resting memory $CD4^+$ T cells [344, 345]. The extremely

long half-life of these cells, combined with the tight control of HIV-1 expression, make this reservoir ideal to maintain hidden copies of the virus. The exact mechanism by which HIV-1 establishes latency is not understood, but several models exist; (1) transcriptional repression of the proviral DNA by binding of specific inhibitory DNA binding proteins, (2) modification of the chromatin conformation or (3) methylation of the DNA at the enhancer/modulatory region of the HIV-1 promoter [75, 346-350]. ROCK may facilitate in maintaining a state of viral latency by preventing the transcriptional activation of the HIV-1 LTR.

The mechanism by which ROCK mediates HIV-1 transcriptional repression has eluded us. We have several plausible hypotheses. First, we speculated that ROCK may disrupt the cooperative interaction between NF-κB and Sp1 to inhibit gene activation (Figure 27A) because disruption of either the NF-κB or Sp1 sites, alone, was sufficient to abrogate inhibition of HIV-1 gene expression by ROCK. Our data revealed no change or perhaps an increase in binding to the NF-κB and Sp1 sites, individually, in the presence of constitutively active ROCKΔ4 (Figure 27B, C). The role of NF-κB and Sp1 cooperative interaction in ROCK-mediated HIV-1 gene expression has not been fully addressed. Additional binding studies need to be completed to look at binding to the NF-κB and Sp1 sites together, using a probe that contains both sites adjacent to one another as seen in the HIV-1 LTR. It will also be necessary to determine what protein complexes or factors bind to and interact at the NF-κB and Sp1 sites.

Second, ROCK may enhance histone deacetylase1 (HDAC1) recruitment for interaction with Sp1 at the HIV-1 LTR (Figure 28A). HDACs have been implicated in repressing gene expression by facilitating chromatin condensation [336-339] and to directly

interact with Sp1 [101, 340]. Our results revealed that the addition of the HDAC1 inhibitor, TSA, further repressed HIV-1 gene expression with overexpression of constitutively active ROCK. This finding suggests that ROCK does not recruit HDAC1 to the Sp1 binding site of the HIV-1 LTR to maintain gene repression (Figure 28B), although we do not yet know why the addition of TSA led to further repression of the HIV-1 LTR since acetylation is often associated with transcriptional activation. It is possible that a repressor of HIV-1 LTR transcription is activated by acetylation. Again, it is necessary to identify the protein complexes or factors associated with the NF-κB and/or Sp1 sites.

The third and perhaps, most likely, mechanism for ROCK to mediate HIV-1 gene repression is through phosphorylation. ROCK is a Ser/Thr kinase and Sp1 has a Ser/Thr region with several sites known to be phosphorylated with effects on promoter activity. Sp1 phosphorylation in its N-terminus (Ser^{131} and Ser^{59}) [340, 351-353] generally augments transcription whereas phosphorylation of Sp1 at its C-terminus (i.e. Thr^{579}) correlates with Sp1 inactivation [354, 355]. It is possible that transcriptional activation or repression is regulated through changes in the interaction between Sp1 and other regulatory factors as a result of Sp1 phosphorylation, as opposed to changes in Sp1 site occupancy (Figure 29). In support of this model, the kinase activity of ROCK was necessary for HIV-1 mediated repression, suggesting that the ability of ROCK to phosphorylate a currently unknown target plays an important role in HIV-1 gene repression. Additionally, preliminary EMSAs revealed little change in DNA binding to either the Sp1 or NF-κB sites in the LTR promoter. ROCK may phosphorylate Sp1 to prevent its activation of the HIV-1 LTR.

It is necessary to identify possible phosphorylation targets modified, either directly or indirectly by ROCK, in the context of HIV-1 infection. Understanding the role of ROCK

mediated phosphorylation in modulating HIV-1 repression, will hopefully provide, not only an increased understanding of how host proteins interact with HIV-1 to mediate replication, but also new host targets for the development of anti-HIV-1 therapeutics.

Acknowledgments:

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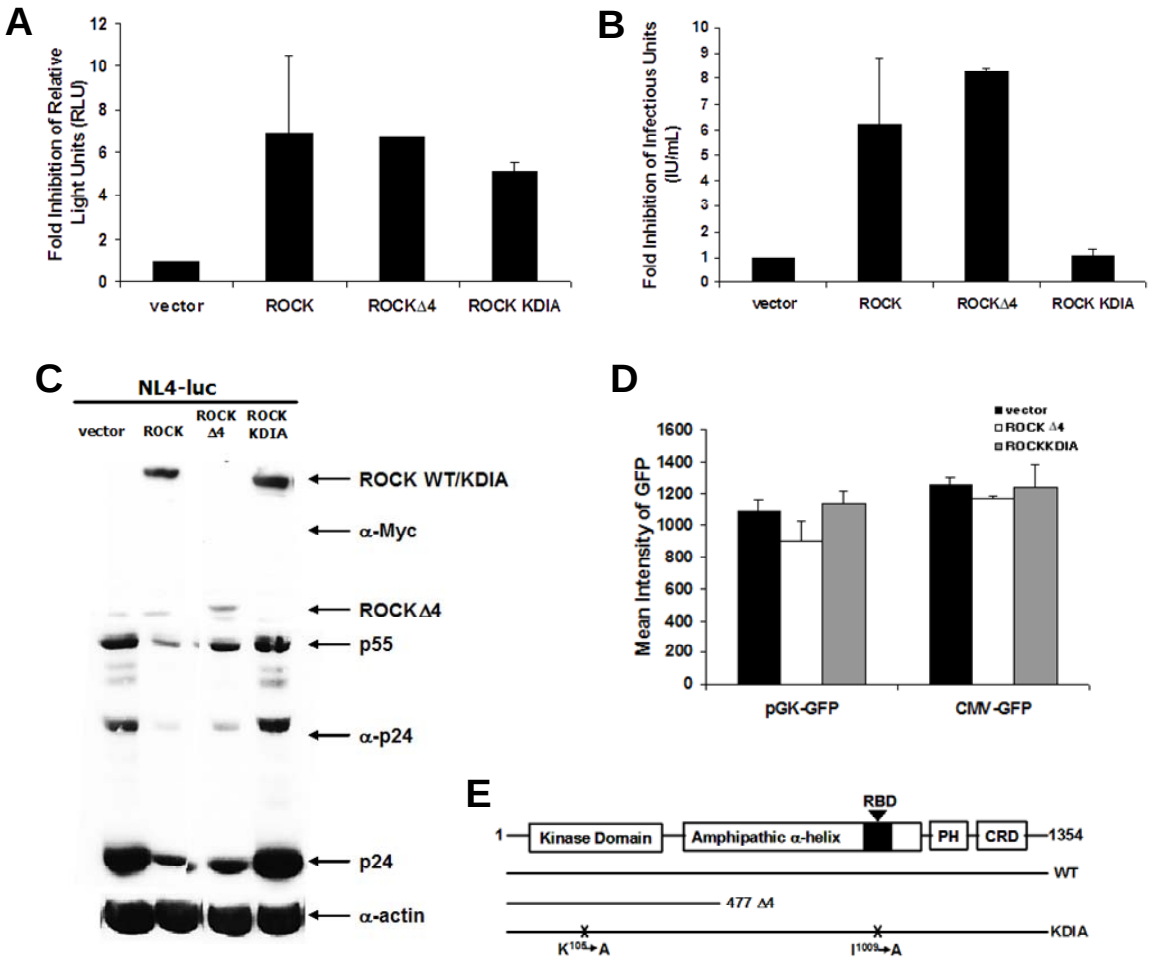

Figure 19: ROCK Specifically Inhibits HIV-1 Gene Expression. (A-C) Jurkat T cells were cotransfected with pNL4.Luc or proviral DNA, NL4-3 and vector, wild-type ROCK, constitutively active ROCK (Δ4) or kinase dead ROCK. Supernatants and cells were collected 48 hours post-transfection. (A) Effect of ROCK on HIV-1 viral gene expression. (B) Effect of ROCK on HIV-1 virion production. (C) Cell-associated viral proteins in Jurkat T cells were analyzed using α-p24, α-Myc and α-actin antibody. (D) ROCK inhibition of HIV-1 gene expression is specific to the HIV-1 LTR. Jurkat T cells were cotransfected with retroviral vectors expressing pGK-driven GFP or CMV-driven GFP and vector, constitutively active ROCK (Δ4) or kinase dead ROCK. GFP expression was analyzed by FACS. The percentage of live cells and GFP positive cells were similar in all samples. (E) ROCK constructs were previously described in Ishizaki *et al.* 1997. ROCK has an N-terminal Ser/Thr kinase domain, a long a-helix region which contains the Rho-binding domain and at its C-terminus, has a pleckstrin homology and cysteine-rich domains.

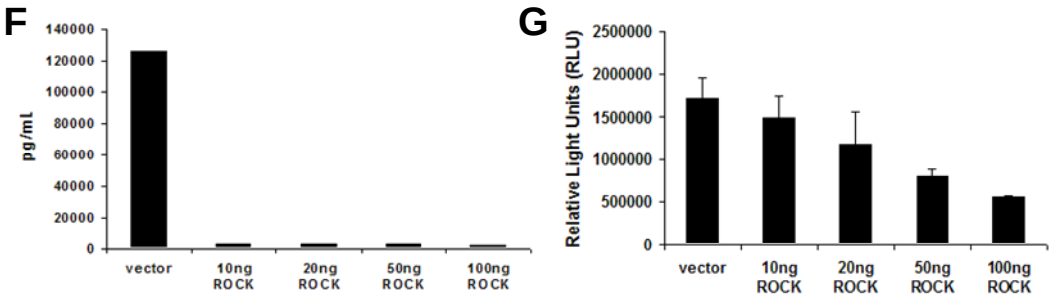

Figure 19: ROCK Specifically Inhibits HIV-1 Gene Expression. (F, G) Dose-dependent response of wild-type ROCK overexpression on HIV-1 virion production (assembly/release) (F) and gene expression (G). (F) At low levels of wild-type ROCK overexpression, ROCK significantly inhibited HIV-1 virion release. Increasing amounts of wild-type ROCK seemed not to have any further effect on virion replication. (G) Dose-dependent response of wild-type ROCK overexpression on HIV-1 viral gene expression. Higher levels of ROCK overexpression were needed to see an effect on viral gene expression.

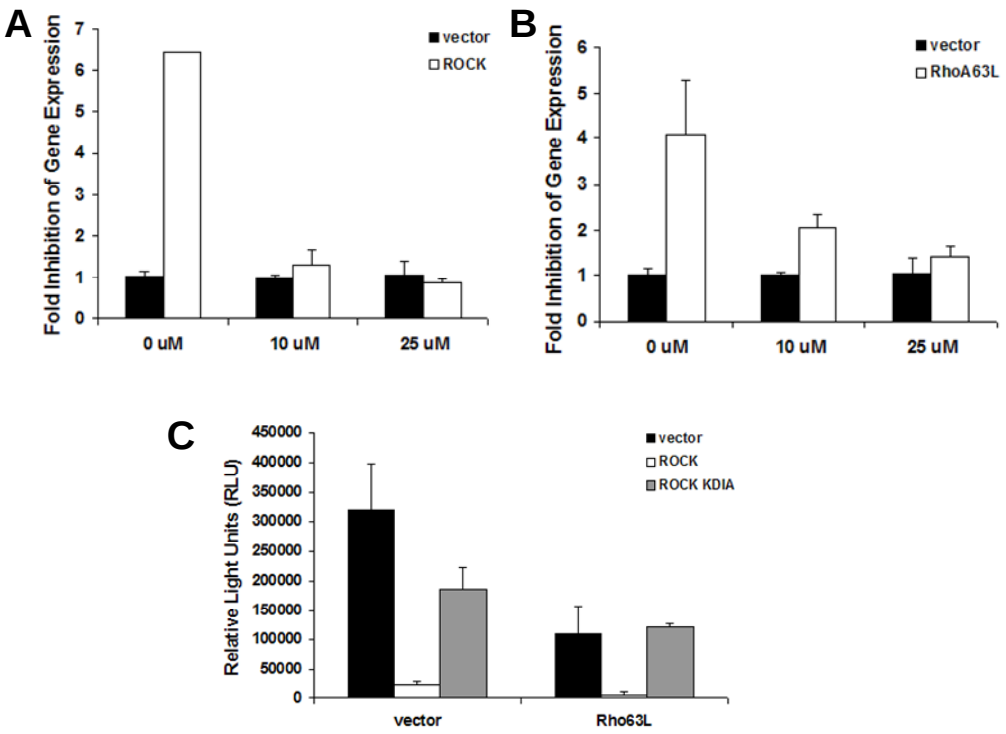

Figure 20: The ROCK Specific Inhibitor, Y27632, Partially Counteracts RhoA Inhibition of HIV-1 Gene Expression. (A, B) Jurkat T cells were cotransfected with pNL4.Luc and vector, wild-type ROCK (A) or constitutively active RhoA (RhoA63L) (B). Inhibitor was added 24 hours post-transfection at 10 μM or 25 μM concentrations. HIV-1 gene expression was analyzed by luciferase assay at 48 hours post-transfection. Y27632 inhibition of ROCK is achieved by competing with ATP for binding to the catalytic site. (C) Co-expression of wild-type ROCK and constitutively active RhoA further inhibited HIV-1 gene expression than when either was expressed alone. Jurkat T cells were cotransfected with pNL4.Luc and vector, wild-type ROCK or ROCK KDIA and/or constitutively active RhoA. Cells were harvested 48 hours post-transfection and analyzed by luciferase assay.

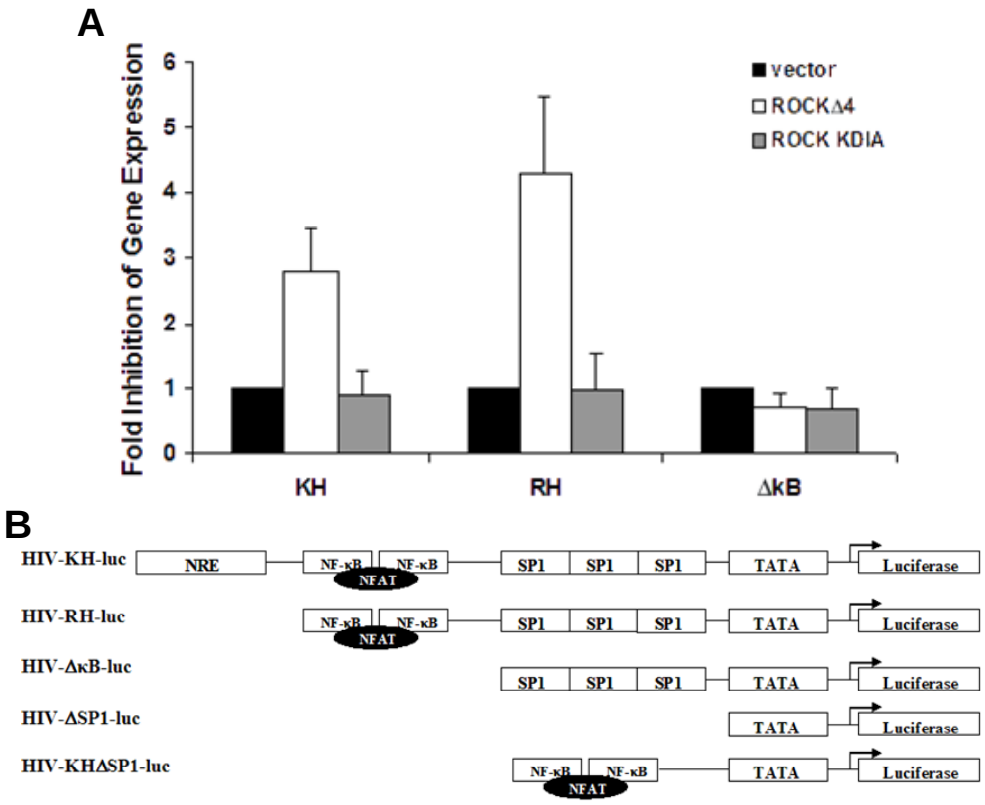

Figure 21: ROCK Inhibition of HIV-1 Gene Expression is Dependent on the NF-κB Sites in the HIV-1 LTR. (A) Jurkat T cells were cotransfected with various LTR-luciferase constructs and vector, constitutively active ROCK (Δ4) or kinase dead ROCK. HIV-1 gene expression was analyzed by luciferase assay at 48 hours post-transfection. Deletion of all sites upstream of TATA box or deletion of the three Sp1 sites but maintaining the integrity of NF-κB sites resulted in gene expression at background levels. (B) Schematic representing LTR deletion constructs used in this experiment.

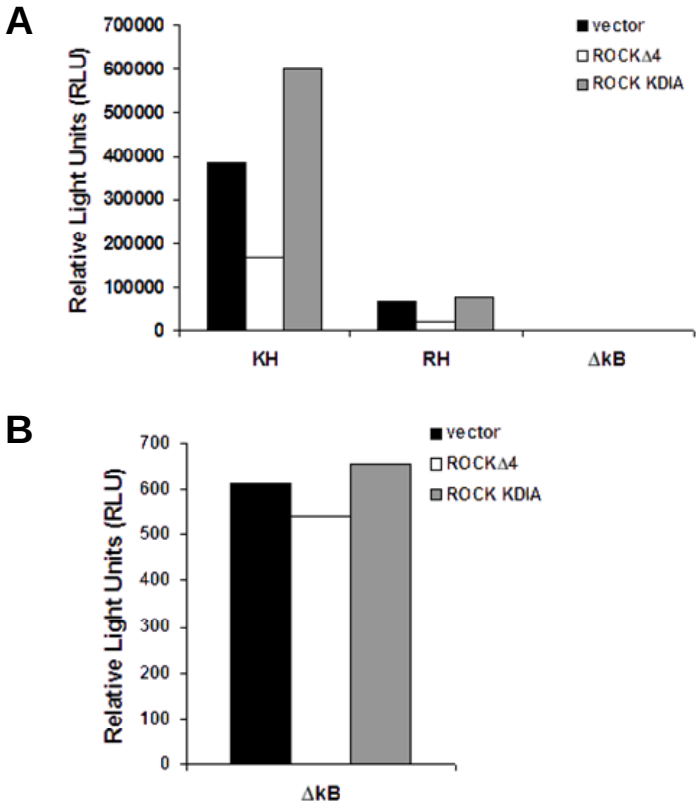

Figure 22: Raw Data Demonstrating that ROCK Inhibition of HIV-1 Gene Expression is Dependent on the NF-κB Sites in the HIV-1 LTR. Jurkat T cells were cotransfected with various LTR-luciferase constructs and vector, constitutively active ROCK **(**Δ4) or kinase dead ROCK. HIV-1 gene expression was analyzed by luciferase assay at 48 hours post-transfection. Deletion of all sites upstream of TATA box or deletion of the three Sp1 sites but maintaining the integrity of the NF-κB sites resulted in gene expression at background levels. All other LTR-deletion constructs had levels of expression, that were above background levels (500 or greater).

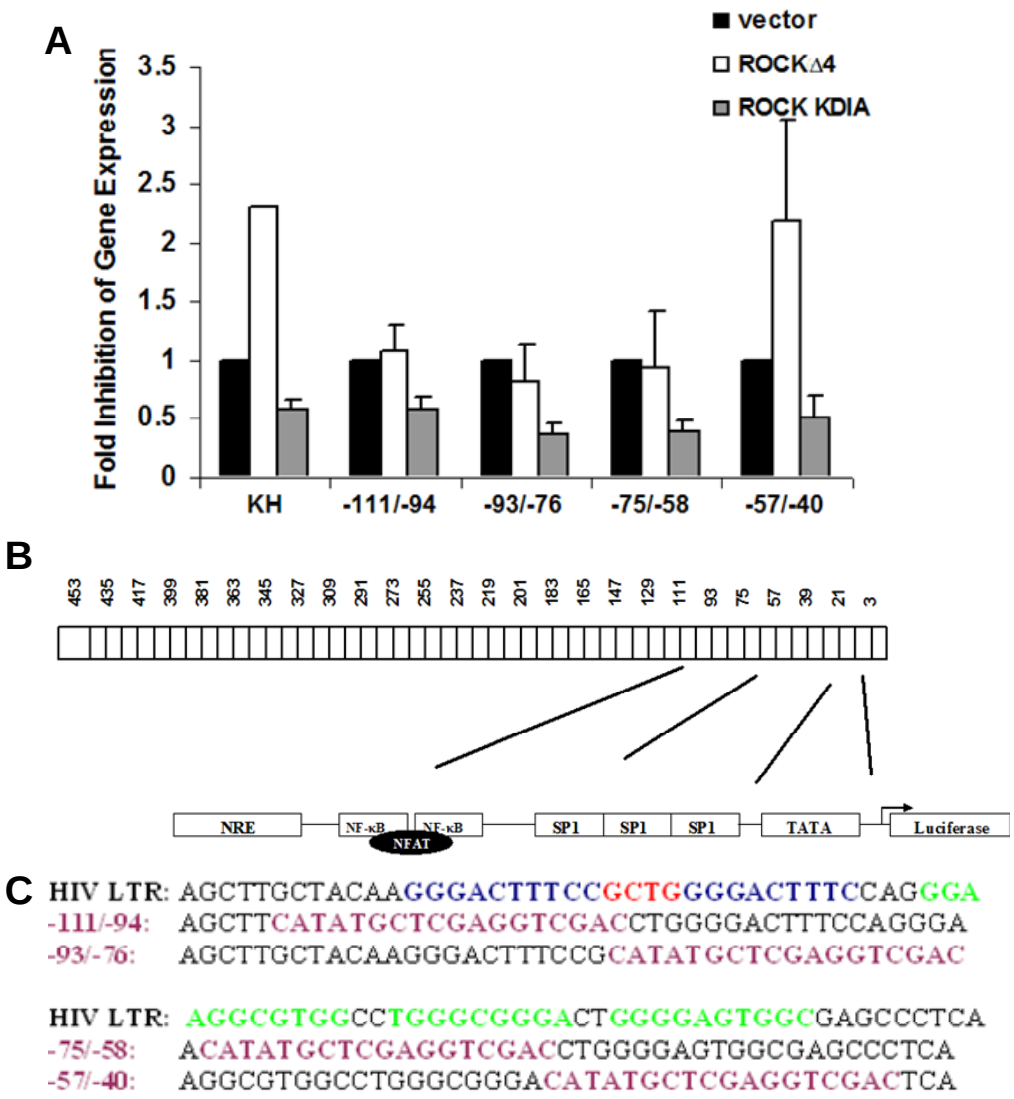

Figure 23: ROCK Inhibits HIV-1 Gene Expression by Affecting Interactions at the NF-κB and the First Two Sp1 Sites. (A) ROCK still inhibited HIV-1 gene expression when the third Sp1 site was mutated. Jurkat T cells were cotransfected with linker-scanning LTR-luciferase mutants and vector, constitutively active ROCK (Δ4) or kinase dead ROCK. HIV-1 gene expression was analyzed by luciferase assay at 48 hours post-transfection. The LSM -111/-94 NXS disrupts the 5' proximal NF-κB site, LSM -93/-76 NXS disrupts the 3' proximal NF-κB site and the first 2 bases of the 5' proximal Sp1 site, LSM -75/-58 NXS disrupts the 2 5' proximal Sp1 sites and LSM -57/-40 NXS disrupts the 3' proximal Sp1 site (28). (B) Schematic representing the linker scanning mutation constructs used in this experiment. (C) Schematic depicting the disruption of Nf-κB and Sp1 sites by linker scanning mutants. Blue=NF-κB sites, Red=NFAT site, Green=Sp1 sites and Purple=linker scanning sequence.

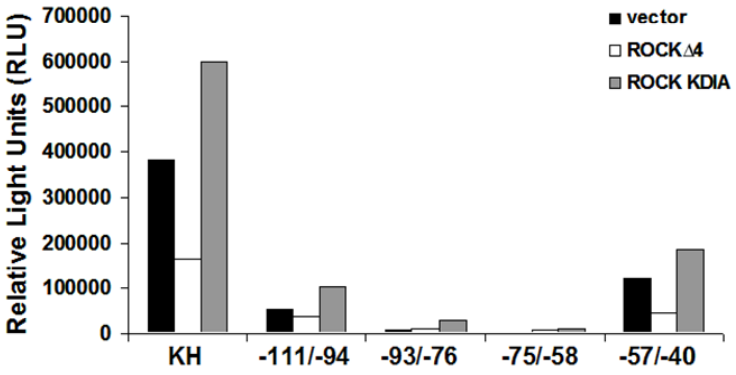

Figure 24: Raw Data Demonstrating that ROCK Inhibits HIV-1 Gene Expression by Affecting Interactions at the NF-κB and the First Two Sp1 Sites. Jurkat T cells were cotransfected with linker-scanning LTR-luciferase mutants and vector, constitutively active ROCK (Δ4) or kinase dead ROCK. HIV-1 gene expression was analyzed by luciferase assay at 48 hours post-transfection. The LSM -111/-94 NXS disrupts the 5' proximal NF-κB site, LSM -93/-76 NXS disrupts the 3' proximal NF-κB site and the first 2 bases of the 5' proximal Sp1 site, LSM -75/-58 NXS disrupts the 2 5' proximal Sp1 sites and LSM -57/-40 NXS disrupts the 3' proximal Sp1 site (28). ROCK still inhibited HIV-1 gene expression when the third Sp1 site was mutated suggesting it was not necessary for ROCK-mediated repression of HIV-1 transcription. The lowest relative light units seen in this experiment were 5000.

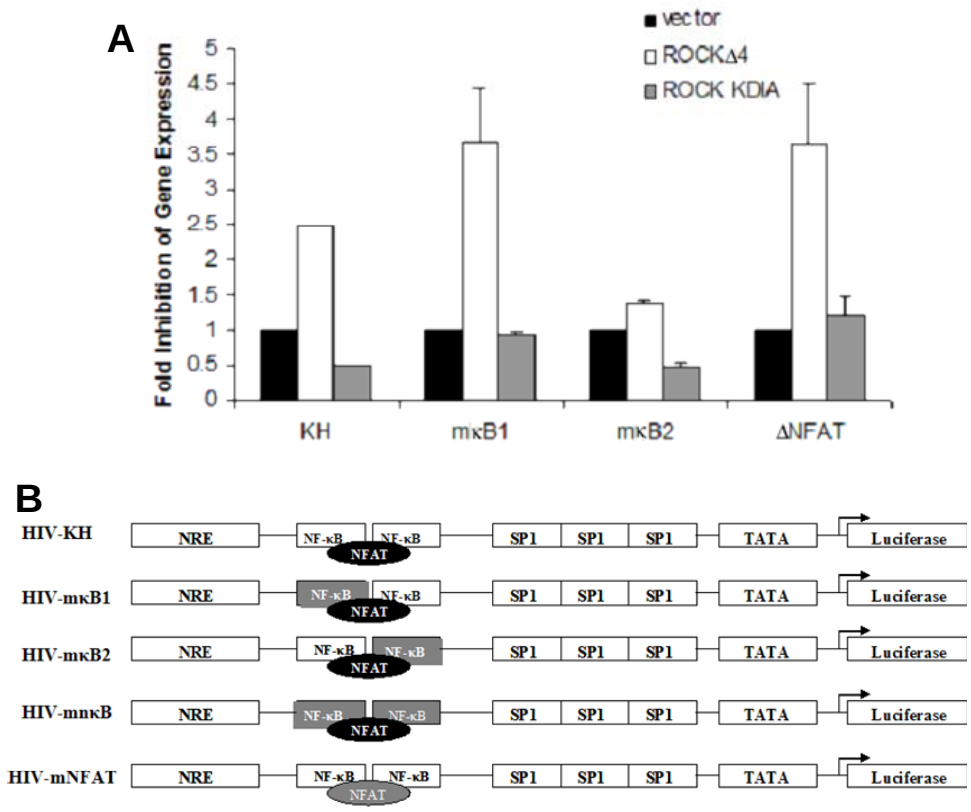

Figure 25: ROCK Inhibits HIV-1 Gene Expression by Affecting Interactions at the Second NF-κB Site. (A) Jurkat T cells were cotransfected with point mutant LTR-luciferase constructs and vector, constitutively active ROCK (Δ4) or kinase dead ROCK. HIV-1 gene expression was analyzed by luciferase assay at 48 hours post-transfection. Deletion of both NF-κB sites resulted in gene expression at background levels (HIV-mnκB). (B) Schematic representing the LTR point mutant constructs used in this experiment. Gray represents the binding site containing a point mutation abrogating binding.

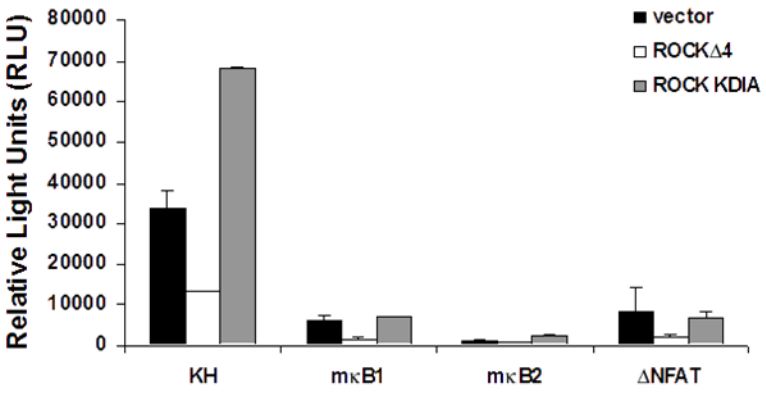

Figure 26: Raw Data Demonstrating that ROCK Inhibits HIV-1 Gene Expression by Affecting Interactions at the Second NF-κB Site. Jurkat T cells were cotransfected with point mutant LTR-luciferase constructs and vector, constitutively active ROCK (Δ4) or kinase dead ROCK. HIV-1 gene expression was analyzed by luciferase assay at 48 hours post-transfection. Deletion of both NF-κB sites resulted in gene expression at background levels (HIV-mnκB). The lowest relative light units seen in this experiment was 800, with the majority of conditions being between 1000-8000.

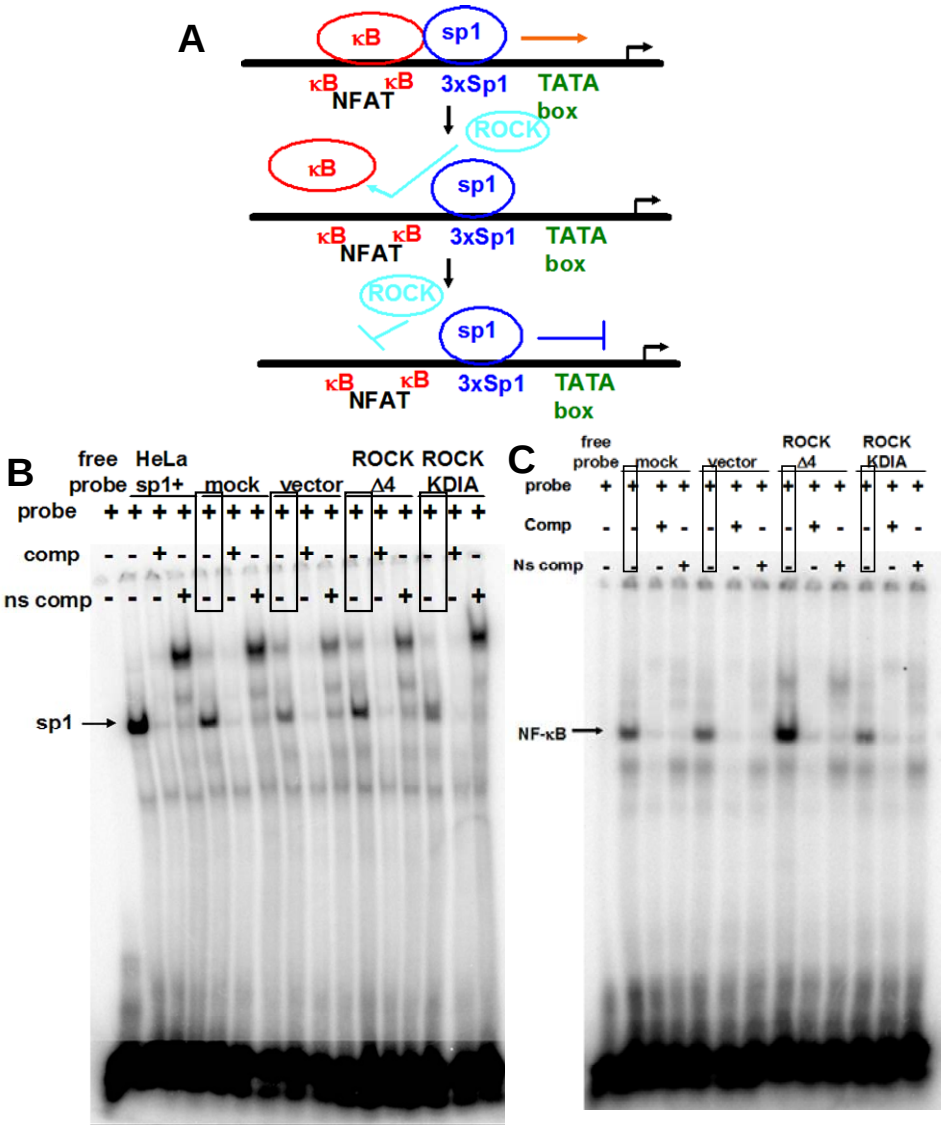

Figure 27: Constitutively Active ROCK Did Not Disrupt Binding at Either the NF-κB or Sp1 Sites. (A) We hypothesized that ROCK may disrupt the cooperative interaction between NF-κB and Sp1 to inhibit HIV-1 gene expression. NF-κB and Sp1 sites are often found adjacently in promoter regions, including those for HIV-1, intracellular adhesion molecule 1, vascular adhesion molecule 1 or granulocyte-macrophage colony-stimulating factor. In HIV-1 LTR, the actions of NF-κB and Sp1 are highly cooperative, involving the effects on DNA binding of both factors to their adjacent binding sites, resulting in increased transcriptional control. (B, C) Electrophoretic mobility shift assays using consensus Sp1 (B) and NF-κB (C) binding site probes, to determine if constitutively active ROCK disrupted/inhibited binding to the Sp1 or NF-κB sites to repress HIV-1 transcription.

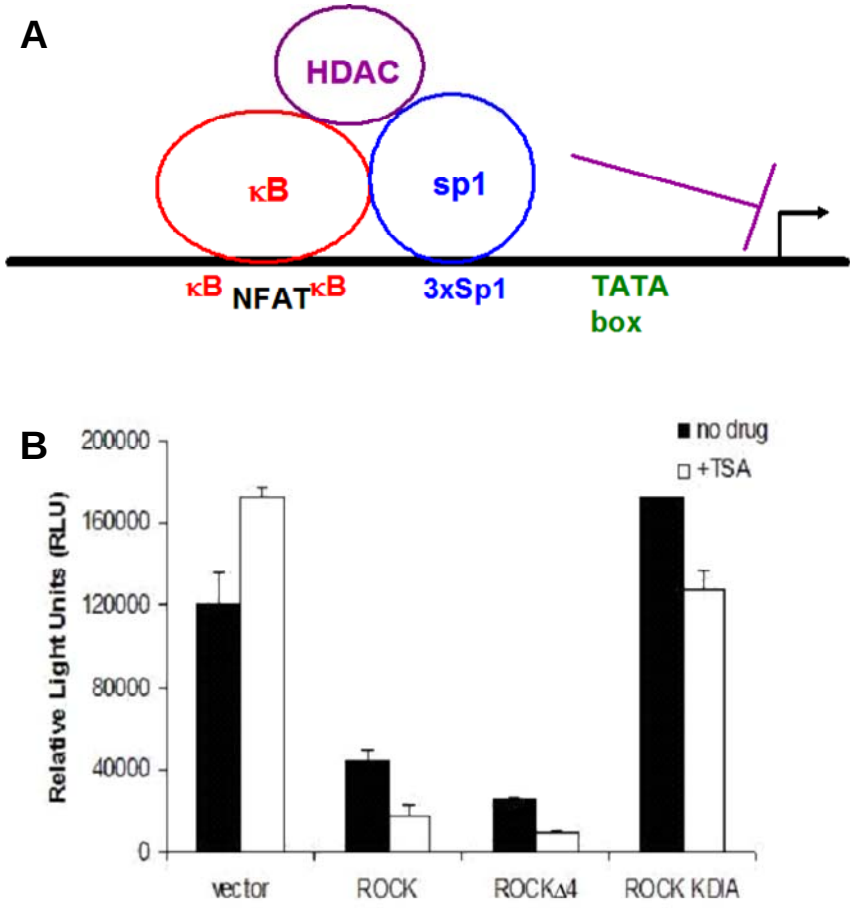

Figure 28: Further Transcriptional Repression was seen with ROCK in the Presence of an HDAC Inhibitor, TSA. (A) We speculated that since ROCK inhibits HIV-1 gene activation, that ROCK enhances recruitment of HDACs to interact with Sp1 and/or NF-κB. Sp1 can be a target for HDAC1-mediated transcriptional repression with the interaction between Sp1 and HDAC1 being direct and requiring the C-terminal domain of Sp1. Deacetylation events are involved in maintaining gene repression. (B) ROCK-mediated HIV-1 gene expression is counteracted by HDAC inhibitor, trichostatin A (TSA). Jurkat T cells were transfected with pNL4.Luc and wild type ROCK, constitutively active ROCK (Δ4) or kinase dead ROCK. TSA was added at 24 hours post-transfection at a concentration of 100 ng/mL. HIV-1 gene expression was analyzed at 48 hours.

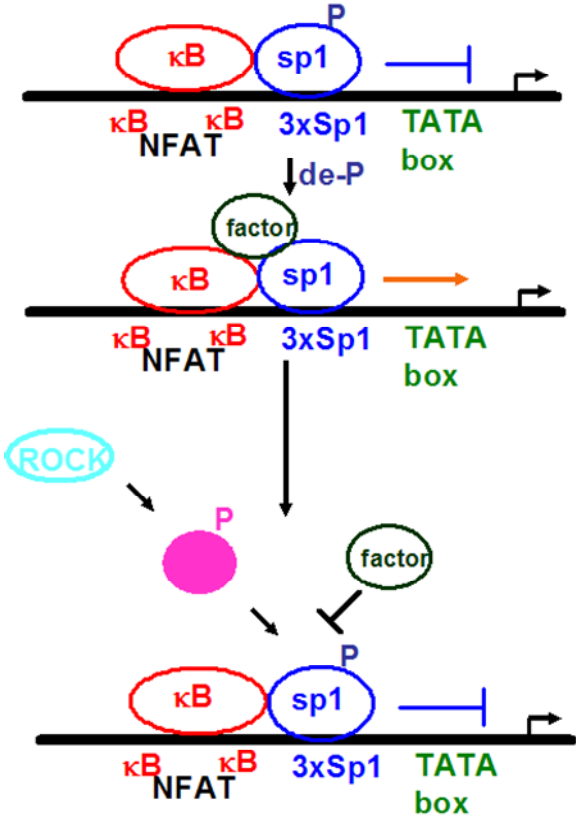

Figure 29: Model for ROCK Mediated Phosphorylation Regulating HIV-1 Repression. The transcriptional activation/repression of the HIV-1 LTR promoter may be regulated through changes in the interaction between Sp1 and other regulatory factors as a result of Sp1 phosphorylation that may be mediated by ROCK**.**

CHAPTER THREE:
PERSPECTIVE ON ROLE OF RHO-ASSOCIATED KINASE ON HIV-1 TRANSCRIPTION

Previously, we reported that the long cytoplasmic tail of the HIV-1 transmembrane envelope glycoprotein, gp41c, interacts directly with the carboxy-terminal regulatory domain of p115RhoGEF, a guanine nucleotide exchange factor and activator of RhoA [195]. Ectopic expression of p115RhoGEF or $G\alpha_{13}$, which activates p115RhoGEF activity, leads to inhibition of HIV-1 replication in a RhoA-dependent mechanism. The RhoA effector activity inhibiting HIV-1 replication is genetically separable from its other known activities [196], suggesting RhoA inhibits HIV-1 regulation via a novel effector activity.

To investigate how RhoA signaling pathways modulate HIV-1 replication, we tested individual RhoA effectors in human T cells. We found that Rho-associated kinase (ROCK), a RhoA effector, to specifically inhibit HIV-1 gene expression in T cells. At very low levels of ROCK overexpression, there was a significant inhibitive effect on HIV-1 virion production (i.e. assembly/release) without a similar effect on HIV-1 gene expression. Ectopic expression of ROCK inhibited viral transcription in a dose-dependent manner with high levels of ROCK efficiently suppressing HIV-1 gene expression. A ROCK specific inhibitor, Y27632, counteracted RhoA inhibition of HIV-1 gene expression. Coexpression of ROCK with constitutively active RhoA further inhibited viral gene expression than when either ROCK or constitutively active RhoA were expressed alone, suggesting RhoA signals through ROCK to inhibit HIV-1 transcription. Mutation or deletion of the second NF-κB

and/or the first two Sp1 sites in the HIV-1 LTR abrogated ROCK-mediated HIV-1 transcriptional repression. Disruption of either NF-κB or Sp1 binding sites was sufficient to abrogate ROCK's effect on HIV-1 gene expression implying that the cooperative interaction required for HIV-1 LTR activation is disrupted in some manner by ROCK. Although we have yet to elucidate the mechanism used by ROCK to inhibit HIV-1 transcription, we have several hypotheses, which will be discussed below.

Rho-associated Kinase (ROCK)

Rho-associated kinase (ROCK) is a 160 kDa protein Ser/Thr kinase with several distinct domains; an N-terminal kinase domain, a 600 amino acid long coiled-coil region in the middle, a pleckstrin homology region and a cysteine-rich zinc-finger like motif in the carboxy-terminus [356]. The kinase domain of ROCK has significant sequence homology to myotonic dystrophy protein kinase [357, 358]. ROCK binds selectively to the GTP-bound form of RhoA and is modestly activated by this binding [358]. ROCK is highly expressed in the heart, lung, skeletal muscle, kidney and pancreas and to a lesser extent in the placenta and liver, with only trace amounts found in the brain [358]. ROCK regulates cytoskeletal organization including stress fiber and focal adhesion formation [329, 356, 359], enhances both myosin light chain (MLC) phosphorylation [360] and c-*fos* SRE gene expression downstream of Rho [361] and is involved in cytokinesis [360, 362].

Phosphorylation of myosin binding subunit (MBS) and MLC by ROCK [121, 360, 363] leads to an increase in MLC phosphorylation, myosin filament assembly and F-actin bundling, thereby enhancing stress fibers. ROCK promotes formation of stress fibers and focal adhesion complexes, which are dependent on both the kinase domain and kinase

activity [359]. ROCK also phosphorylates LIM kinase and adducin. LIM kinase phosphorylates the actin depolymerizing protein, cofilin, inhibiting its function [364]. The inhibition of cofilin stabilizes actin filament arrays such as stress fibers and cell cortex and phosphorylation of adducin by ROCK increases its binding to F-actin [365]. The ROCK specific inhibitor, Y-27632, did not prevent the activation of SRF, transcription of c-*fos* or cell cycle re-entry following serum stimulation, although repeated treatment with Y-27632, substantially disrupted the actin fiber network but did not affect the growth rate [366].

Both RhoA and ROCK are localized to cleavage furrows, participating in cytokinesis. ROCK can directly phosphorylate MLC *in vitro* [360] and *in vivo* [362]. At the same time, ROCK inhibits myosin phosphatase by phosphorylating the MBS of myosin phosphatase, blocking the turnover of MLC phosphorylation. Both activities lead to an increase in MLC phosphorylation. ROCK can take the place of MLC kinase (MLCK) directly phosphorylating the MLC and enhancing myosin activation [360]. ROCK specific inhibitor, Y-27632, does not effectively block cytokinesis suggesting there is another RhoA effector molecule that is a Rho target in cytokinesis.

Latency and Transcriptional Silencing

In infected individuals, viral production is a dynamic process involving continuous rounds of infection of $CD4^+$ T lymphocytes with rapid turnover of both free virus and virus-producing cells that have a half-life of 1–2 days [10, 12]. HIV-1 latency is a natural consequence of the virus replicating in activated $CD4^+$ T cells, cells which are capable of undergoing reversible changes in activation state [8, 342]. After responding to an antigenic challenge, some activated $CD4^+$ T cells return to a resting state and persist as memory cells.

In this state, the cells are not permissive for viral replication, however, integration of the viral genome into a host-cell chromosome guarantees viral persistence for the lifespan of the memory T cell [343]. Upon mitogen stimulation, infectious virus can be recovered from these cells. The lifespan of these cells is long because they provide the cellular basis for immunologic memory.

A major obstacle to HIV-1 eradication is the establishment of a transcriptionally silent viral infection in infected resting memory $CD4^+$ T cells [344, 345]. The extremely long half-life of these cells, combined with the tight control of HIV-1 expression, make this reservoir ideal to maintain hidden copies of the virus. The exact mechanism by which HIV-1 establishes latency is not completely understood, but several models exist; (1) transcriptional repression of the proviral DNA by binding of specific inhibitory DNA binding proteins, (2) modification of the chromatin conformation or (3) methylation of the DNA at the enhancer/modulatory region of the HIV-1 promoter [46, 75, 346-350].

Two key host transcription factors are sequestered in the cytoplasm of resting T cells, NF-κB and NFAT. Both NF-κB and NFAT are only recruited to the nucleus following cellular activation by TCR engagement or cytokine signaling, thereby linking HIV-1 gene expression to the activation state of the host cell. Although nuclear forms of NF-κB and NFAT are clearly involved in upregulation of HIV-1 gene expression following T cell activation, their role in a resting cell when they are located in the cytoplasm, is still unclear. The differential localization of NF-κB and NFAT in resting and activated $CD4^+$ T cells may be important in the maintenance of latent HIV-1 infection. ROCK may facilitate in maintaining a state of latency by preventing activation of transcription from the HIV-1 LTR by acting at the NF-κB and/or Sp1 sites.

Mechanisms by which ROCK May Mediate HIV-1 Repression

First, ROCK could disrupt the cooperative interaction between NF-κB and Sp1 to inhibit gene activation (Figure 27A). In the HIV-1 LTR, the actions of NF-κB and Sp1 are highly cooperative, involving the effects on DNA binding of both factors to their adjacent binding sites, resulting in increased transcriptional control [81]. If this cooperative interaction is disrupted, most likely indirectly, by ROCK then HIV-1 transcription would be inhibited. Our data revealed that there was no change or perhaps an increase in binding to the NF-κB and Sp1 sites, individually, in the presence of constitutively active ROCK (Figure 27B, C). Additional binding studies need to be completed to look at the cooperative binding of the NF-κB and Sp1 sites by using a longer primer to encompass both sites. The LTR deletion/mutation analysis suggests that disruption of either the NF-κB or Sp1 sites abrogated ROCK-mediated inhibition of HIV-1 gene expression, which may ultimately be due to a disruption of the cooperative interaction between the NF-κB and Sp1 sites. ROCK may still inhibit activation of the promoter without affecting binding to either site or the slight increase in binding that we saw in our EMSAs could be due to recruitment of a transcriptional repressor.

Second, ROCK may inhibit HIV-1 gene activation by enhancing HDAC1 (histone deacetylase 1) recruitment for interaction with Sp1 and/or NF-κB (Figure 28A). Sp1 has been shown to directly interact, through its zinc-finger domain, with HDAC1 [340]. In this case, the resulting transcriptional repression did not involve the deacetylase activity of HDAC1 but was accomplished by direct protein-protein interactions between Sp1 and HDAC1, which interferes with GC-box Sp1 binding. Surprisingly, the addition of an HDAC1 inhibitor further repressed HIV-1 gene expression (Figure 28B). One possible

explanation for this unexpected result is that an unidentified repressor involved in controlling HIV-1 transcription through interaction with Sp1 and/or NF-κB is activated by acetylation and its activation promotes transcriptional repression – although that is probably unlikely since acetylation typically enhances transcription. Identification of protein complexes or factors associated with the NF-κB and/or Sp1 binding sites should be completed.

Third, ROCK could affect the state of histone or DNA methylation to prevent NF-κB and/or Sp1 from promoting transcription. DNA methylation is a component of a multilevel control mechanism through which the expression of eukaryotic genes can be regulated. Typically, there is an inverse relationship between gene methylation and gene expression. DNA methylation in the regulatory regions of genes plays a role in silencing of genes either by directly inhibiting interaction of transcriptional factors with their regulatory sequences or by attracting methylated DNA-binding proteins, which then recruit HDACs and histone methyltransferases, resulting in inactive chromatin structure [367]. DNA methylation is catalyzed by DNA methyltransferases (DNMTs) which transfer the methyl moiety from the methyl donor S-adenosylmethionine to the fifth position on the cytosine ring [368]. DNMT1 (DNA methyltransferase I) catalyzes the post-replication methylation of DNA and is responsible for maintaining the DNA methylation pattern during embryonic development and cell division [369, 370].

DNA methylation is known to suppress Sp1 responsive genes by attracting methylated DNA-binding proteins to suppress their activation [371-373]. DNMT1 interacts with HDAC1 [374] and HDAC2 [375] as well as histone methyltransferases [376] suggesting that DNMT1 silences gene expression by recruiting chromatin-modifying enzymes. Knockdown of DNMT1, in lung carcinoma and 293T cells, activated Sp1 response elements

without an increase in either Sp1 or Sp3 protein levels or occupancy of the Sp1 elements within these proteins suggesting that DNMT1 is acting either directly or indirectly on proteins interacting with Sp1 or Sp3 and modulating their trans-activation activity [367]. The regulation of Sp1 responsive elements by DNMT1 is separate from the known epigenetic mechanisms of DNA methylation, histone acetylation or histone methylation because DNMT1 can regulate Sp1 responsiveness in the absence of DNA methylation and acetylation is unaffected by DNMT1 knockdown [367].

Analysis of the EMSAs previously completed demonstrated that constitutively active ROCK minimally enhanced binding at the NF-κB or Sp1 binding sites, implying that the effect on HIV-1 LTR transcription by ROCK may be by an indirect mechanism. ROCK may recruit a DNA methyltransferase to prevent activation of the HIV-1 LTR by Sp1 or in some other way, modulates methylation of the chromatin to prevent transcription. To determine if methylation is important for ROCK to maintain HIV-1 transcriptional repression, we could add a methylation inhibitor, 5-azacytidine, to our transfected cells. 5-azacytidine inhibits the catalytic activity of DNMT1 and would determine how the DNA methylation-dependent roles of DNMT1 contribute to ROCK-mediated HIV-1 repression. RNAi knockdown of DNMT1 [377] would allow us to address the possible DNA methylation-independent regulatory functions of DNMT1 in ROCK-mediated inhibition of HIV-1 gene expression. In both cases, the effect on HIV-1 gene expression, in the context of wild-type and constitutively active ROCK overexpression, could be evaluated.

The last and perhaps, most likely, mechanism for ROCK to mediate HIV-1 gene expression is through phosphorylation. ROCK is a Ser/Thr kinase and Sp1 has a Ser/Thr region with several sites known to be phosphorylated with effects on promoter activity. The

first protein kinase to be identified to phosphorylate Sp1 was DNA-dependent protein kinase (DNA-PK) [378]. Sp1 phosphorylation is predominantly seen in the N-terminal 610 amino acids of Sp1 and is found primarily on Ser residues, with only about 5% of the phosphorylation occurring on Thr residues [378]. In Jurkat and HeLa cells, expression of viral tat was linked to increased Sp1 phosphorylation at Ser^{131} [351]. While phosphorylation of Ser^{59} increased DNA binding implying that the C-terminal region is not the only area in Sp1 responsible for DNA binding and that changes in the very N-terminal region could affect DNA binding either directly or indirectly [340, 352, 353]. Phosphorylation of Thr^{579} (in the second zinc-finger in the DNA binding domain) is essential for CK-II mediated inhibition of binding to a consensus Sp1 binding site [354].

Sp1 phosphorylation in its N-terminus (Ser^{131} and Ser^{59}) [340, 351-353] generally augments transcription whereas phosphorylation of Sp1 at its C-terminus (i.e. Thr^{579}) correlates with Sp1 inactivation [354, 355]. Furthermore, it is possible that transcriptional activation/repression is regulated through changes in the interaction between Sp1 and other regulatory factors as a result of Sp1 phosphorylation (Figure 29). In support of this model, the kinase activity of ROCK was necessary for HIV-1 mediated repression, suggesting that the ability of ROCK to phosphorylate a currently unidentified target plays an important role in HIV-1 gene repression. Additionally, preliminary EMSAs revealed little change in DNA binding to either the Sp1 or NF-κB sites in the HIV-1 LTR promoter. It is necessary to identify possible phosphorylation targets modified, either directly or indirectly by ROCK, in the context of HIV-1 infection, in order to have a more complete understanding of how ROCK inhibits HIV-1 gene expression.

Understanding the role of ROCK in modulating HIV-1 repression, will hopefully provide, not only an increased understanding of how host proteins interact with HIV-1 to mediate replication, but also new host targets for the development of anti-HIV-1 therapeutics.

CHAPTER FOUR:
CITRON KINASE, A RHOA EFFECTOR, ENHANCES HIV-1 VIRION PRODUCTION BY PROMOTING EXOCYTOSIS

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Rebecca J. Loomis performed all experiments.
Derek A. Holmes made the citron N, citron NΔ1, citron NΔPH and citron NΔZnF constructs.
Andrew Elms made the MLV retrovirus in the presence and absence of citron K.
Patricia A. Solski and Channing J. Der provided the citron K and citronΔ1 constructs.

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ABSTRACT:

RhoGTPases play important roles in the regulation of protein transport and membrane recycling. Little is known, however, about how RhoGTPases affect HIV-1 virion production, which is dependent on the endosomal sorting pathway. We report that ectopic expression of citron kinase, a RhoA effector, preferentially enhances HIV-1 virion production. Depletion of endogenous citron kinase inhibits HIV-1 viral production. Citron N, which lacks the kinase domain, also enhances HIV-1 virion production. Additionally, the leucine-zipper, Rho-binding and zinc-finger domains are necessary for the enhancement activity. Citron kinase also enhances murine leukemia viral production and the HIV-1 late domain is not required for the citron kinase-mediated enhancement activity. Ectopic expression of citron kinase leads to the formation of cytoplasmic structures containing citron kinase and HIV-1 Gag proteins. Citron kinase and HIV-1 cooperatively enhance late endosome and lysosome compartments. Finally, citron kinase enhances secretion of exosomes or microvesicles that copurify with HIV-1 virions. We conclude that citron kinase enhances HIV-1 viral production by stimulating exocytic secretion. Collectively, our findings suggest a new mechanism of HIV-1 virion release involving citron kinase-mediated exocytosis and may have important implications for developing novel HIV-1 therapeutics.

INTRODUCTION:

HIV-1 replication is modulated by a number of cellular signaling pathways regulated by both host and viral factors [379]. HIV-1 assembly and release occur in a series of essential steps mediated by the viral Gag precursor protein, $Pr55^{Gag}$ [20, 47]. HIV-1 Gag is organized into four distinct regions: matrix; capsid; nucleocapsid and late domain. The late (L) domain catalyzes the pinching off and detachment of virus particles from the cell surface and each other [47]. The L domain is a highly conserved Pro-rich motif found in enveloped viruses. There are three classes of tetrapeptide motifs; PT/SAP [61, 62], PPXY [217-219] and YPXL [220]. In the case of HIV-1, the L domain is encoded by a small peptide motif, Pro-Thr/Ser-Ala-Pro (PTAP) in the C-terminal, p6 domain of $Pr55^{Gag}$. The L domains of retroviruses, despite differences in amino acid sequence and location within their respective viral structural proteins, are functionally interchangeable, suggesting commonality of function, perhaps as docking sites for host proteins [222, 234, 243]. Mutations in the PTAP motif of p6 or deletion of the p6 domain produced a striking defect in the production of virus particles where viral particles remain tethered to each other and the plasma membrane [61, 62] thereby identifying the p6 domain as playing a critical role in HIV-1 budding.

Viruses like HIV-1 do not encode their own machinery for viral budding and therefore must usurp existing cellular pathways to facilitate viral release. The PTAP motif of HIV-1 Gag^{p6} was found to bind to the ubiquitin enzyme 2 variant (UEV) domain of Tsg101 [204, 240, 248, 261]. Tsg101 is a component of the endosomal sorting complex required for transport (ESCRT-I), a 350 kDa cellular complex essential in the vacuolar protein sorting (VPS) pathway. Point mutations in HIV-1 PTAP motif block virus release at late stages [61, 62] and disrupt binding to Tsg101 [240]. Small inhibitory RNA-mediated Tsg101 depletion

in T cells potently blocks HIV-1 release resulting in the virus forming stalks of tethered virus at the plasma membrane [240]. Additionally, overexpression of the dominant-negative form of VPS4, an ESCRT recycling factor, inhibits particle release of HIV-1 and other enveloped viruses, such as MLV [240]. Additionally, a second region of HIV-1 Gag^{p6} has been defined to contribute to viral release and interacts with AIP1, a host protein [246, 247]. AIP1 interacts with Tsg101 and CHMP proteins of ESCRT-III complex, coupling HIV-1 Gag^{p6} to the early and late-acting endosomal sorting complexes and binds directly to HIV-1 Gag^{p6} LRSL [246, 247]. Taken together, these data suggest that the VPS machinery and perhaps other host factors are involved in the facilitating the budding of retroviruses.

The endosomal sorting pathway controls a variety of cellular processes and plays a role in the sorting of ubiquitinated cargo proteins into the lumen of the multivesicular bodies (MVB) [54, 268]. Ubiquitinated proteins are recognized on the limiting endosomal membrane and sorted resulting in either MVB fusion with the lysosome to degrade contents or release of material into the extracellular environment via exosomal vesicles [269-271]. ESCRT-I, composed of Tsg101, Vps28 and Vps37, recognize the ubiquitinated protein cargo and recruit two more class E protein cargos (ESCRT-II/III) that participate in protein sorting and vesicle formation [272, 273, 327]. HIV-1 may bind Tsg101 and AIP1 to gain access to downstream machinery involved in catalyzing MVB vesicle budding, a mechanism topologically similar to viral budding from the plasma membrane.

RhoGTPases play a pivotal role in the dynamic regulation of actin cytoskeleton [122-124] and through this, control cell morphology [127, 128], motility, adhesion and activation of transcription factors such as NF-κB [126] and serum response factor [125]. Although RhoGTPases have been implicated in various steps of T cell activation [182, 191, 193], little

is known about how RhoGTPases affect HIV-1 replication. We have previously shown that the cytoplasmic tail of the transmembrane glycoprotein, gp41c, interacts directly with the carboxy-terminus of p115RhoGEF [195], a guanine nucleotide exchange factor and activator of RhoA. Activation of p115RhoGEF leads to inhibition of HIV-1 replication in a RhoA-dependent manner [196]. The RhoA effectors involved in modulating HIV-1 replication are not defined.

To investigate how RhoA signaling pathways modulate HIV-1 replication, we tested several RhoA effectors in 293T and human T cells. We determined that citron kinase, a Ser/Thr kinase, enhances both HIV-1 replication and the exocytosis pathway. Ectopic expression of citron kinase preferentially enhanced HIV-1 virion production. Knock-down of citron kinase by shRNA reduced HIV-1 viral production. Citron kinase enhanced murine leukemia viral production, as well as viral production with a GagΔp6 construct suggesting that citron kinase mediates viral production independently of the viral late domain. Citron kinase induced intracellular compartments and colocalized with Gag in these compartments. Citron kinase and HIV-1 cooperatively enhanced late endosome and lysosome compartments. Furthermore, citron kinase enhanced secretion of exosomes and microvesicles that copurified with HIV-1 virions.

Materials and Methods:

Reagents, plasmids and cell lines

The pNL4-3 plasmid encodes the entire HIV-1 genome DNA in pUC18 [327]. The pNL4.Luc.R-E- plasmid was obtained from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program [328]. The pNL4GFP plasmid encodes Gag, Pol, tat and rev and was kindly provided by Dr. Dan Littman, NYU, NY, NY. pCAG (vector), pCAG/citron kinase, pCAG/citronΔ1 plasmids were previously reported [380]. All citron truncation mutants were generated by PCR of the citron N template with an N-terminal Myc-tag added and inserted into a retroviral vector, pHSCG [331]. The control (pSUPER.Retro.Puro, OligoEngine, Seattle, WA) and citron short-hairpin constructs (cit A, cit B and cit C) were a kind gift from Dr. Alan Hall, University College London, London, UK. Gag and GagΔp6 constructs were a kind gift from Dr. Paul Bieniasz, Aaron Diamond AIDS Research Center, NY, NY [381] and GagGFP was a kind gift from Dr. Marilyn Resh, Sloan-Kettering Institute, NY, NY.

293T, HeLa and HeLa-MAGI cells (NIH AIDS Research and Reference Reagent Program; [331]) were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin and L-glutamine. Jurkat T cells were maintained in RPMI 1640 supplemented with 10% FBS, penicillin/streptomycin and L-glutamine. NIH-3T3 cells were maintained in DMEM supplemented with 10% FBS, penicillin/streptomycin and L-glutamine.

HIV-1 Production and Replication in Transfected Human Cells

Transient production of HIV-1 was performed by transfecting NL4-3 (0.1 μg) with pCAG, pCAG/citron kinase, pCAG/citronΔ1 or citron truncation plasmids (0.2 μg) in 293T cells in 48-well plates at $2.5x10^4$ cells/well using Effectene (Qiagen, Santa Clarita, CA) or NL4-3 (0.3 μg) with pCAG, pCAG/citron kinase, pCAG/citronΔ1 or citron truncation plasmids (0.6 μg) in Jurkat T cells in 48-well plates at $4x10^5$ cells/well using GenePorter (Gene Therapy Systems, San Diego, CA). At 48 hours post-transfection, HIV-1 virions in cell supernatant were measured by p24 or RT assays [332] and infectious units were determined by titering the supernatant on HeLa-CD4-LTR-lacZ cells (MAGI) as described previously [331].

To analyze HIV-1 gene expression in transfected cells, pNL4.Luc (0.1 μg) was cotransfected into $2.5x10^4$ 293T cells/well together with pCAG, pCAG/citron kinase, pCAG/citronΔ1 or citron truncation plasmids (0.2 μg) or pNL4.Luc (0.3 μg) was cotransfected into $4x10^5$ Jurkat T cells/well with pCAG, pCAG/citron kinase, pCAG/citronΔ1 or citron truncation plasmids (0.6 μg). Cell extracts were analyzed at 48 hours after transfection for luciferase activity (AutoLumat LB953, EG&G berthold; FluoStar Optima, BMG LabTech). In some experiments, we used pGag, pGagΔp6 or pNL4GFP, in place of pNL4-3 or pNL4.Luc.

Western Blotting

Transfected 293T or Jurkat T cells were lysed and resolved (50-100 μg of total protein) on a 10% SDS-PAGE. Gels were transferred to PVDF membranes (Amersham Biosciences) and blocked with 5% nonfat milk. The membrane was probed with mouse α-

p24 (NIH AIDS Research and Reference Reagent Program), mouse α-Myc (AbCam, Cambridge, MA), rabbit α-citron (a kind gift from Dr. Lola Reid, UNC-CH, as described in [382], mouse α-actin (Sigma-Aldrich), mouse α-tubulin, mouse α-hsc70 (BD Biosciences), mouse α-LAMP1 (Santa Cruz) or mouse α-CD82 (BD Biosciences) specific antibodies and visualized using an ECL Kit (Amersham Biosciences).

shRNA Knockdown of Citron Kinase

The citron kinase short-hairpin RNA (shRNA) and control constructs are in the pSUPER.Retro.Puro vector from OligoEngine. The citron kinase shRNA constructs target different regions of citron kinase. Cit A RNA targets the C-terminus of citron kinase beginning at base pair 6085, Cit B and Cit C RNA target the N-terminus of citron kinase beginning at base pair 359 and 244 respectively (Figure 31A). For experiments in 293T cells, 293T cells were plated in a 24-well plate at $5x10^4$ cells/well and transfected with 0.4 μg control or citron targeted shRNA constructs using Effectene. Twenty-four hours later, another transfection was performed with 0.1 μg pNL4GFP and 0.2 μg control or citron targeted shRNA constructs. Three days after the second transfection, supernatants and cells were harvested for further analysis. For experiments in Jurkat T cells, 0.3 μg of pNL4GFP and 0.6 μg of control or citron targeted shRNA constructs were transfected via GenePorter into $4x10^5$ cells/well. Supernatants and cells were harvested at 48 hours post-transfection for further analysis.

MLV Production and Replication in Transfected Human Cells

293T cells were plated in a 6-well plate at $4x10^5$ cells/well and transfected with 2 μg HSCG, 1.5 μg VSV-G-env, 1.5 μg MLV Gag-Pol and 2 μg vector or citron kinase DNA using the calcium phosphate transfection method as described previously [383, 384]. Retrovirus was harvested 48 hours after transfection. Virus was titered by seeding NIH-3T3 cells at $5x10^4$ cells/well in a 24-well plate, adding serial dilution of virus and counting GFP-positive cells 48 hours after infection on the Guava EasyCyte (Guava Technologies).

Confocal Microscopy

293T cells were plated on coverslips at $1x10^5$ cells/well in a 24-well plate and transfected as described previously (Effectene, Qiagen). For the GagGFP confocal experiments, we used 0.2 μg of GagGFP, 0.02 μg vector, 0.4 μg vector or citron kinase and for the samples with wild-type Gag – 0.2 μg of GagGFP, 0.02 μg of Gag and 0.4 μg vector or citron kinase were used in the transfections. At 48 hours post-transfection, cells were fixed with 4% paraformaldehyde for 15 minutes at 4°C, washed once with cold 1xPBS, permeabilized with ice cold methanol for 30 seconds, washed once with cold 1xPBS, washed three times with Quench Buffer (1% milk, 150 mM NaOAc pH7, 1xPBS), washed three times with wash buffer (1% milk, 1xPBS), incubated with primary antibody for 1 hour at room temperature, washed three times with wash buffer, incubated with secondary antibody for 1 hour at room temperature, washed five times with wash buffer and mounted on slide with PVD. The primary antibody used was chicken α-Myc (Molecular Probes) at 1:1000 dilution. The secondary antibody used was α-chicken-Cy5 (Molecular Probes) at 1:200 dilution.

For LysoTracker experiment, cells were incubated with 60 μM LysoTracker (Molecular Probes) for 90 minutes then fixed with 4% paraformaldehyde 48 hours post-transfection. Images were collected using Olympus confocal microscope and software.

FACS

Each sample (~$4x10^5$ cells) that had been incubated with 60 μM LysoTracker (Molecular Probes) for 90 minutes were washed in PBS with 2% FBS and stained with 7AAD (1 μL/mL) for 15 minutes on ice, then rinsed and resuspended in PBS with 2% FBS. Cells were analyzed using FACScan (Becton Dickinson).

Purification of Exosomal/Microvesicles/HIV-1 Virions

Transfections were performed as described previously (Effectene, Qiagen), except in 100 mm dishes using $1x10^6$ cells/plate of 293T cells or $5x10^5$ cells/plate of HeLa cells. The FBS added to the DMEM media used to culture the cells was depleted of exosomes or microvesicles by spinning at 100,000xg overnight at 4°C. Ten mL of supernatant were collected 48 hours post-transfection. The exosome purification protocol is similar to those described previously [385, 386]. At the time of collection, supernatants were spun at 3000 rpm for 10 minutes to remove cells and large cell debris. Supernatants were then added to Beckman Ultra Clear Centrifuges (11x89 mm) with 2 mL 25% sucrose. Tubes were spun in the Beckman L7 Ultracentrifuge at 100,000xg for 2 hours at 4°C. Supernatant was discarded and exosomal pellet was resuspended in 1% Triton X-100 and loaded onto a 10% SDS-PAGE gel.

RESULTS:

Citron Kinase Preferentially Enhances HIV-1 Viral Replication

To investigate how RhoA signaling pathways modulate viral replication, we tested various RhoA effectors for their effect on HIV-1 viral replication in 293T and human T cells. Ectopic expression of citron kinase (citron-K), a RhoA effector involved in cytokinesis [387] and membrane vesicle transport [388-390], was shown to preferentially enhance HIV-1 viral production without significantly affecting HIV-1 gene expression in 293T (Figure 30A-D), Jurkat T (Figure 30E-G) and HeLa cells (data not shown). Both viral infectious virions (Figure 30A, E) and total virions (virion-associated RT activity or p24, Figure 30B and data not shown) demonstrated a similar enhancement (7-to 15-fold) while expression of HIV-1 reporter gene (Figure 30C, F) and cell-associated viral proteins (Figure 30D, G) showed minimal enhancement (<2.5-fold in 293T cells and no change in Jurkat T cells). The C-terminal truncation mutant of citron kinase, citronΔ1, did not enhance HIV-1 production. We conclude that citron kinase preferentially enhances HIV-1 virion production with minimal effect on HIV-1 gene expression and that activity depends on the C-terminal domains of citron kinase.

To evaluate a role for endogenous citron kinase in HIV-1 virion production, we depleted endogenous citron kinase from 293T and Jurkat T cells using shRNA constructs that targeted various regions of citron kinase (Figure 31A). 293T cells were transfected twice, first with control or citron targeted shRNA constructs only and 24 hours later cells were cotransfected with citron targeted shRNA constructs and pNL4GFP. Supernatant and cells were harvested 72 hours after the second transfection and analyzed. We saw efficient knockdown of citron kinase protein (65-90%, Figure 31B) and a 75-85% reduction in viral

production by all three constructs as determined by p24 ELISA (Figure 31C). HIV-1 gene expression, as measured by cell-associated Gag was not affected by depletion of citron kinase (Figure 31B). Jurkat T cells were also cotransfected with pNL4GFP and the citron-specific shRNA or control constructs. Supernatants and cells were collected 48 hours post-transfection. Virion production was inhibited by 60% in citron kinase depleted cells compared with the control (Figure 31D). The inhibition of viral particle release correlated with reduction in expression of endogenous citron kinase (Figure 31E). Therefore, citron kinase is required for efficient HIV-1 virion production.

Leucine-Zipper, RBD and Zinc-finger but Not Kinase, PH, SH3 or PDZ, Domains of Citron Kinase are Necessary for Enhancing HIV-1 Virion Production

Deletion mutants were generated to map the domain(s) of citron kinase involved in the enhancement of HIV-1 viral production. Citron N, a naturally occurring splice variant of citron kinase lacking the N-terminal kinase domain, showed similar enhancement activity as citron kinase, indicating that the kinase domain was dispensable (Figure 32A). Next, we made a series of C-terminal truncations in the citron N backbone. The PH, SH3 and PDZ domains were not required for the activity. The zinc finger motif of citron was found to be necessary for enhancement of viral production because deletion of the zinc finger domain from citron N (CNΔZnF) abolished its ability to enhance virion production (Figure 32A). The zinc-finger domain alone was unable to enhance virion production (Figure 32A). Further deletion of the N-terminal α-helix demonstrated that a portion of the domain with two copies of the leucine zipper motif and the Rho-binding domain, in addition to the zinc-finger domain were sufficient for citron kinase-mediated viral enhancement (Figure 32A). A

schematic of our findings on the effect of citron mutants on virion production is shown (Figure 32B). A western blot with anti-Myc antibody showed that all citron truncation mutant proteins were efficiently expressed to similar levels as citron kinase or citron N (Figure 33A, B).

Citron Kinase Enhances MLV Release and Enhancement of HIV-1 VLP Production Is Independent of the Late Domain

To determine if citron kinase enhancement of virion production is HIV-1 specific, we evaluated whether citron kinase was able to enhance murine leukemia virus (MLV) production. Citron kinase enhanced MLV virion production to comparable levels as HIV-1 (Figure 34A) suggesting that citron kinase has a general enhancement activity on retroviral production.

The late domain is an important component for retroviral release [61, 62]. It has been shown that HIV-1 viral release is dependent on the unique HIV-1 late domain to interact with the endosomal sorting protein TSG101 and subsequently the endosomal sorting pathway. Release of MLV, which contains a different late domain tetrapeptide sequence (PPPY) than HIV-1 (PTAP), is unaffected by depletion of Tsg101 [240]. To determine the role of HIV-1 late domain in citron kinase-mediated virion production, we transfected 293T cells with Gag or GagΔp6 constructs [381]. The GagΔp6 construct lacks the entire late domain, including the PTAP tetrapeptide sequence. GagΔp6 significantly inhibited virion production compared to full-length Gag (Figure 34B). Citron kinase enhanced VLP production to the same degree with or without the late domain present (5-7 fold, Figure 34B, C). Our results demonstrate that citron kinase enhances HIV-1 virion production independent of the viral late domain.

Citron Kinase Induces the Formation of Intracellular Compartments and Colocalizes with HIV-1 Gag

To define a possible mechanism by which citron kinase enhances HIV-1 viral production, we evaluated by confocal microscopy the subcellular localization of citron kinase and HIV-1 Gag in 293T cells. Citron kinase localized to dense protein aggregates that accumulated in the cytoplasm (data not shown, previously described in [391]). When GagGFP was expressed alone or GagGFP/Gag were coexpressed in 293T cells (Figure 35A, C), Gag exhibited cytoplasmic and plasma membrane localization, the primary site for HIV-1 release. However, when GagGFP or GagGFP/Gag were coexpressed with citron kinase, Gag localized to the same dense protein aggregates that expressed citron kinase (Figure 35B, D). Thus citron kinase relocalized Gag to these citron kinase-induced intracellular structures that could be mediating enhancement of viral production by exploiting an unused or little used intracellular pathway.

Citron Kinase Enhances HIV-1 Viral Production by Promoting Exocytosis

Next, we determined the effect of citron kinase on the late endosomal and lysosomal compartments during HIV-1 replication since all retroviruses are dependent on the late endosomal sorting pathway for productive release. Cells were cotransfected with citron kinase and/or pNL4-3 and labeled with LysoTracker, a dye that marks compartments of low internal pH (i.e. late endosomes and lysosomes). Citron kinase or HIV-1 alone showed no significant effect on these compartments (Figure 36A-C). Citron kinase and HIV-1, however, cooperatively enhanced the late endosomal and lysosomal compartments (Figure 36D). The results from confocal microscopy were supported by FACS analysis of the

LysoTracker signal (Figure 36E). These findings suggest that citron kinase interacts with HIV-1 to enhance activity of the late endosomal sorting pathway to promote HIV-1 virion production.

The endosomal sorting pathway is crucial for a number of key biological processes. Once a protein enters the endosomal sorting pathway, there are three outcomes: (1) internalized receptor/cargo can be recycled to the cell surface; (2) the MVB fuses with the lysosome to degrade its contents or (3) the MVB can be directed to the plasma membrane where its contents are released via an exocytic pathway. We, therefore, hypothesized that citron kinase modulates HIV-1 virion production and the MVB sorting pathway by promoting late endosome trafficking to secretion by the exocytic pathway. To address this, we purified exosomes secreted from cells transfected with HIV-1 proviral DNA in the presence and absence of citron kinase. Our results indicate that citron kinase, alone and with HIV-1, enhanced the secretion of microvesicles with exosomal markers (i.e. hsc70, CD82 and LAMP-1), as well as HIV-1 virions, in HeLa (Figure 37A) and 293T cells (Figure 37C). There was no enhancement of intracellular exosomal markers in the presence of citron kinase and/or HIV-1 (Figure 37B, D). Interestingly, citron kinase and HIV-1 seemed to cooperatively enhance secretion of microvesicles or exosomes by evaluating levels of hsc70, LAMP-1 and CD82. We conclude that citron kinase enhances HIV-1 virion production by enhancing the release of exosome/microvesicle-like virions via the exocytic pathway.

DISCUSSION:

We investigated the role of the RhoA effector, citron kinase, in modulating HIV-1 replication. Ectopic expression of citron kinase and the kinase-deficient splice variant citron N preferentially enhanced HIV-1 virion production. Knock-down of endogenous citron kinase by shRNA inhibited HIV-1 virion production, demonstrating a relevant and important role for endogenous citron kinase in viral budding. Citron kinase also enhanced MLV production and the HIV-1 late domain was not required for the enhancement. Citron kinase forms cytoplasmic structures [391] and when both HIV-1 Gag and citron kinase are co-expressed, Gag is colocalized to these citron kinase-induced cytoplasmic structures. Further analysis of the endosomal sorting pathway indicated that citron kinase, together with HIV-1, enhanced the size and intensity of late endosomes and lysosomes. We demonstrated that production of exosomes and HIV-1 virions were both enhanced by citron kinase. These results suggest citron kinase is involved in the exocytic pathway. HIV-1 interacts functionally with citron kinase and this pathway to cooperatively enhance HIV-1 virion production.

There are two endogenous forms of citron, the kinase form (citron-K) and the nonkinase form (citron N). Citron kinase, a 240 kDa protein, is ubiquitously expressed in most cell-types with cell-cycle dependent cellular localization [380]. RhoA colocalizes with citron kinase in the cell cortex during ana- and telophase and both are enriched in the cleavage furrow and midbody during telophase [387]. The Rho-binding domain of citron kinase is essential for localization to the cleavage furrow and midbody. Citron kinase modulates the contractile motion required for separation of the two daughter cells during cytokinesis. Full-length citron kinase and the kinase domain alone phosphorylated regulatory

light chain (MLC) of myosin II at both Ser^{19} and Thr^{18} during cytokinesis [392]. It is feasible that citron kinase enhances virion budding from host cells in a similar process as cytokinesis. However, our observation that the kinase domain is not required to enhance HIV-1 virion production argues against this mechanism.

The nonkinase form, citron N, is a splice variant of citron kinase and a brain-specific Rho-binding protein [393]. There is a molecular association between citron N and the post-synaptic scaffold protein, PSD-95/SAP-90, a member of the membrane-associated guanylate kinase protein family (MAGUK) [394, 395]. This interaction involves signaling pathways essential for neural plasticity and postsynaptic signal transduction events [394, 395]. Actin polymerization and cytoskeleton are regulated by RhoGTPases and are essential for the organization and dynamics of membrane organelles such as endosomes and the Golgi complex [388-390]. Citron N is enriched and associated with the Golgi apparatus of hippocampal neurons in culture [396]. Suppression of citron N or expression of a mutant lacking Rho-binding activity leads to dispersion of the Golgi apparatus [396] suggesting that citron N functions as a scaffolding molecule on Golgi membranes, organizing Rho-mediated actin polymerization locally by assembling the actin polymerizing complexes together (ROCK II and profilin-IIa) [396]. Therefore, citron N may play a role in organization of the endosomal sorting pathway and regulation of the actin cytoskeleton to facilitate exosome and virion release. We analyzed monomeric and polymerizing actin in cells transfected with citron kinase and/or HIV-1, neither monomeric nor polymerizing actin appear to be altered by citron kinase and/or HIV-1 (Figure 38).

We determined that the region of citron kinase necessary for enhancement of virion production contained the leucine-zipper, the Rho-binding domain and the zinc-finger

domains. It has been speculated that the leucine-zipper domain may be a scaffold for multimeric structures functioning through conformational changes and interactions with additional partners [393]. The zinc-finger (C_6H_2) in citron kinase is believed to bind lipid second messengers or to recruit additional proteins [393]. Taken together, citron kinase may enhance virion production by binding or recruiting other cellular (perhaps endosomal sorting proteins) or viral components to facilitate viral release.

RhoGTPases play a central role in the dynamic regulation of the actin cytoskeleton and through this, control cell morphology, motility and adhesion [120, 288]. RhoGTPases act locally to control individual trafficking events, but also act globally to control the spatial organization of membrane traffic, in response to cues from the extracellular environment. In addition to interacting with the endocytic pathway to modulate clathrin-independent internalization from the cell surface [294, 295]. RhoGTPases are involved in mediating trafficking/sorting decisions at a number of distinct endocytic subcompartments. For example, RhoD is localized to both the plasma membrane and early endocytic vesicles, resulting in activation of an isoform of Diaphanous and tyrosine kinase c-Src [297]. RhoB, which is highly homologous to RhoA, is localized to the plasma membrane and the bounding membrane of MVBs [300, 302] and is activated by internalized epidermal growth factor receptors as they enter the late endosomal compartment [303]. These findings suggest that RhoGTPases interface the actin cytoskeleton with endocytic trafficking events.

Recent evidence has shown that Tsg101, a component of ESCRT-1, binds specifically to the late domain of HIV-1 Gag to promote viral release [204, 240, 261]. Small inhibitory RNA-mediated depletion of Tsg101 potently blocked HIV-1 release but not MLV release [240]. We showed that citron kinase enhanced virion production of MLV. Citron kinase

also enhanced VLP production of HIV-1 GagΔp6, which lacks the late domain. Therefore, this citron kinase activity probably affects a step of HIV-1 viral release that is distinct from the Tsg101-dependent step. Dominant-negative mutants of Vps4 inhibited viral release of both HIV-1 and MLV indicating the essential role of the late endosomal pathway in mediating retroviral release [240]. Coexpression of dominant-negative Vps4 mutants completely blocked HIV-1 virion production in the absence or presence of citron kinase (Figure 39). Therefore, citron kinase enhanced virion production that is still dependent on the late endosomal sorting pathway.

HIV-1 can bud from both the plasma membrane and internal endosomal membranes [197, 240, 247, 280, 381, 386, 397, 398]. Citron kinase may either recruit late endosomal factors to the plasma membrane or promote utilization of the endosomal pathway for viral production. Cells transfected with both citron kinase and HIV-1 lead to larger LysoTracker compartments with increased intensity of signal compared to cells transfected with HIV-1 or citron kinase alone. Based on the localization of citron kinase and colocalization of Gag, it is likely that citron kinase promotes the late steps of the endosomal sorting pathway for viral production.

HIV-1 virions share similar characteristics with exocytosed microvesicles or exosomes [386, 399]. They are similar in size and contain exosome-specific markers, as well as common cellular membrane proteins. Although exosome production occurs more prominently in macrophages, B cells and dendritic cells, recent evidence indicates that the exosomal pathway operates in most blood cell types, including T cells [283]. Our data support a model where citron kinase is involved in modulating the exosomal secretion

pathway and HIV-1 interacts with citron kinase, directly or indirectly, to further enhance exosome and HIV-1 virion production.

Remarkably, citron kinase -/- mice are viable, although they grow at slower rates, are severely ataxic and die before adulthood as a consequence of fatal seizures [400]. Since knock-down of endogenous citron kinase expression or activity in T cells inhibited viral production, the interactions between citron kinase and HIV-1 may provide new host targets (citron kinase and the exosomal pathway) for the development of anti-HIV-1 therapeutics. In addition to affecting HIV-1 virion production, the altered release of exosomes may also have significant immunological consequences as exosomes have been shown to function as APCs to interact with T cells [283, 385, 386, 399].

ADDITIONAL FINDINGS OF INTEREST:

In addition to the findings discussed above, we performed studies in primary T cells. We looked at citron kinase levels in unstimulated and stimulated primary blood mononuclear cells (PBMCs) to elucidate the role of endogenous citron kinase in HIV-1 replication. Unstimulated PBMCs were transfected by Amaxa with pNL4GFP or GFP control vector. PBMCs were stimulated with PHA at 24 hours post-transfection and treated with IL-2 at 48 hours post-transfection. Supernatants and cells were harvested at day five. First, activation of PBMCs induced citron kinase expression. Second, in activated PBMCs, citron kinase expression was further induced by HIV-1 (Figure 40). We have seen this induction or enhancement of citron kinase expression with HIV-1 infection in several cell types besides primary cells including 293T, HeLa and Jurkat T cells (data not shown).

Although many proteins are only expressed when cells are activated, the induction of citron kinase, due to its increased expression in proliferating cells and role in enhancing exocytosis, even in the absence of HIV-1 infection, may be a means for the virus to usurp a necessary cellular component to facilitate its replicative capacity. The further induction of citron kinase with HIV-1 infection could be the result of the virus stabilizing its expression (i.e. preventing degradation), perhaps this is accomplished by citron kinase recruitment to a complex (i.e. with Tsg101 and other endosomal sorting complexes) where it cannot be readily dissociated and turned over. These findings may have important implications for our understanding of HIV-1 infection and in designing HIV-1 therapeutics.

To further address the relevance of citron kinase in HIV-1 infection, it would be of interest to look at whether citron kinase expression is upregulated in HIV-1 positive patients. For this experiment, there would have to be a blood sample from either before infection or

before seroconversion. In a longitudinal study, we would have the opportunity to evaluate an individual through the various phases of HIV-1 infection, perhaps citron kinase expression correlates with phases of higher viral loads.

Acknowledgments:

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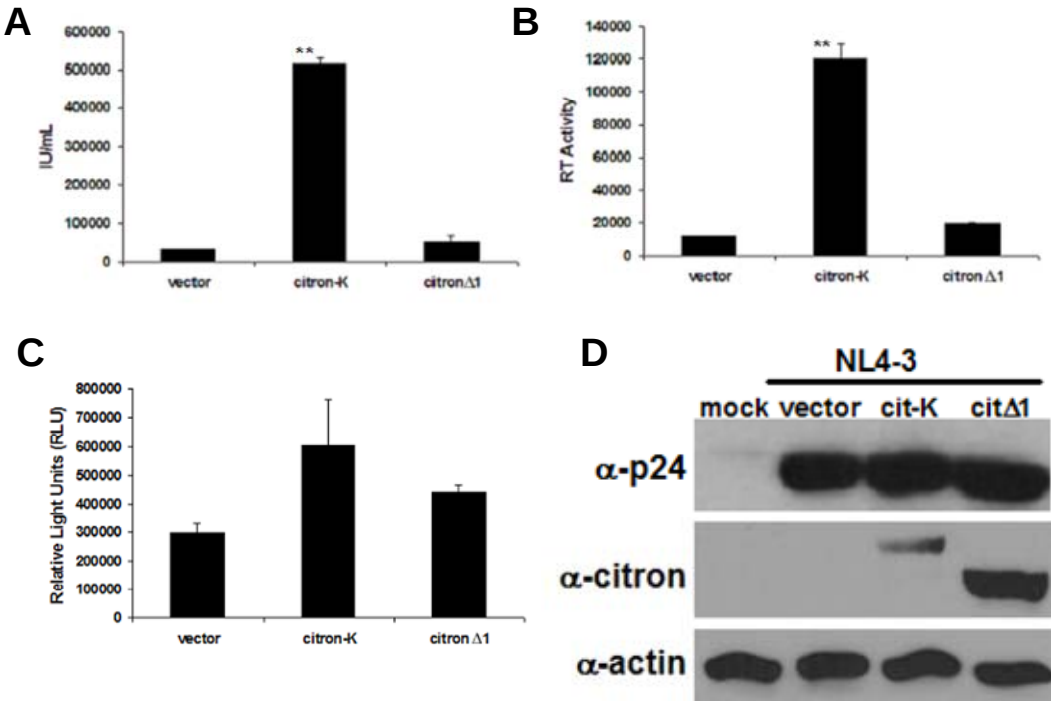

Figure 30: Citron Kinase Preferentially Enhances HIV-1 Virion Production. (A, B) 293T cells were cotransfected with proviral DNA NL4-3 and vector, citron kinase or citronΔ1. Supernatant was harvested at 48 hours post-transfection to determine infectious units/mL (A) or virion-associated RT activity (B). (C, D) 293T cells were cotransfected with pNL4.Luc and vector, citron kinase or citronΔ1. Cells were harvested at 48 hours post-transfection to determine luciferase activity (C). (D) Cell-associated viral Gag proteins, Myc-tagged citron kinase and actin in 293T cells were analyzed using α-p24, α-Myc and α-actin antibodies, respectively. Error bars are standard deviation of duplicate samples and at least 3 independent experiments are performed. **=p value<0.005.

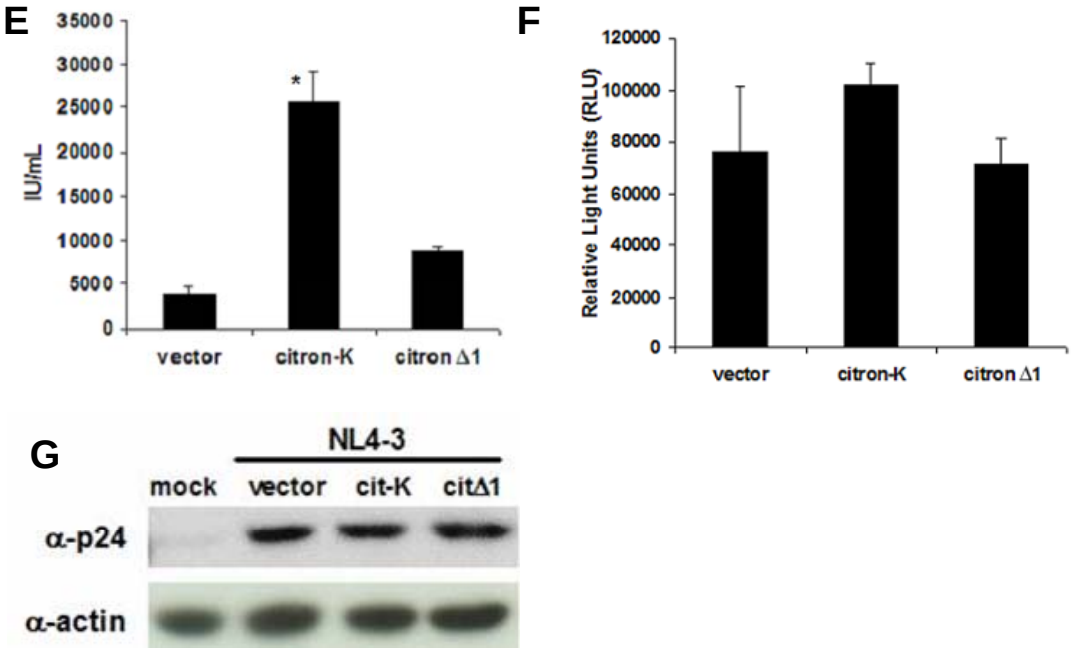

Figure 30: Citron Kinase Preferentially Enhances HIV-1 Virion Production. (E, F) Jurkat T cells were cotransfected with pNL4-3 (E) or pNL4.Luc (F) and vector, citron kinase or citronΔ1. Supernatants and cells were harvested at 48 hours post-transfection to determine infectious units/mL (E) or luciferase activity (F). (G) Cell-associated viral Gag proteins (p55) and actin in Jurkat T cells were analyzed using α-p24 and α-actin antibodies. Error bars are standard deviation of duplicate samples and at least 3 independent experiments are performed. *=p value<0.05.

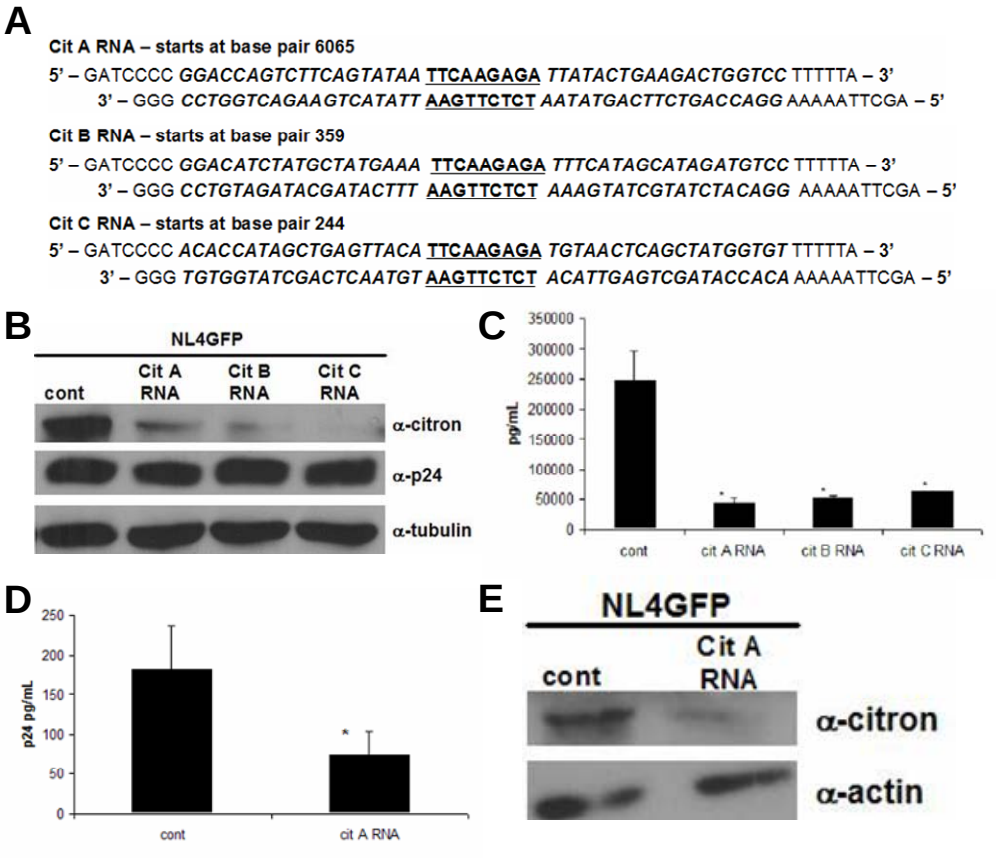

Figure 31: Depletion of Endogenous Citron Kinase Inhibits HIV-1 Virion Production. (A) All citron kinase shRNA constructs were made in the pSUPER.Retro.Puro vector. The start site base pairs are from the beginning of the coding sequence. Italics = target sequence, Underline = hairpin. (B, C) Knock-down of endogenous citron kinase using citron shRNA constructs in 293T cells inhibited HIV-1 viral production. 293T cells were transfected with citron shRNA or control plasmid constructs, 24 hours later, cells were cotransfected with pNL4GFP and citron shRNA or control plasmid constructs. Supernatant and cells were collected 48 hours after the second transfection and analyzed for cell-associated gene expression of citron kinase, p24 and actin (B) and relative HIV-1 virion production in the supernatant (p24 pg/mL) (C). (D, E) Knock-down of endogenous citron kinase in T cells inhibited HIV-1 viral production. Jurkat T cells were transfected with pNL4GFP and citron shRNA or control plasmid constructs. Supernatant and cells were collected at 48 hours post-transfection. The effect of citron kinase knock-down on HIV-1 viral production in the supernatant was measured by p24 ELISA (D). *=p value<0.05. Cells were lysed and analyze with α-citron and α-actin antibodies (E).

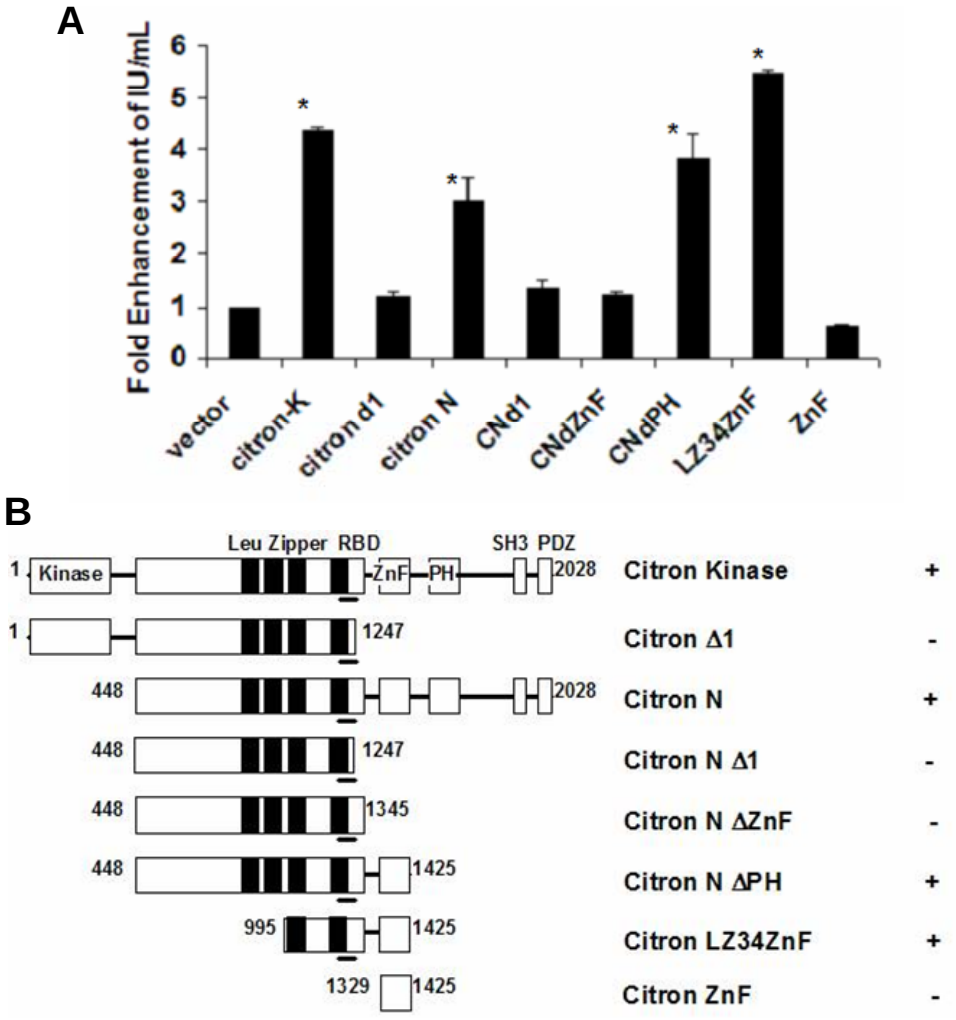

Figure 32: Leucine Zipper, Rho-binding Domain and Zinc-finger Motifs, but Not the Kinase Domain, were Necessary for Citron Kinase to Enhance HIV-1 Viral Release. (A) 293T cells were cotransfected with pNL4-3 and citron truncation constructs. Supernatants and cells were collected at 48 hours for infectious unit assays. Graph depicts fold enhancement of infectious units by various citron truncations compared to vector control. *=p value<0.05. (B) Schematic diagram of the citron kinase constructs and of results shown in (A). + = enhancement of viral release.

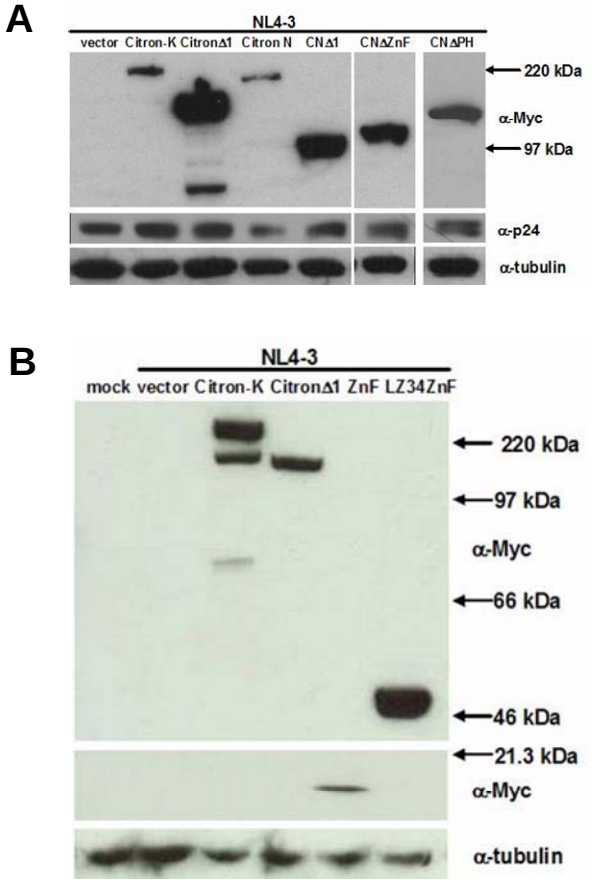

Figure 33: Protein Expression of Citron Kinase Mutants. (A, B) Expression of citron constructs was verified by analyzing cell-associated proteins with α-Myc, α-p24 and α-actin antibodies. All citron constructs are Myc-tagged on the N-terminus.

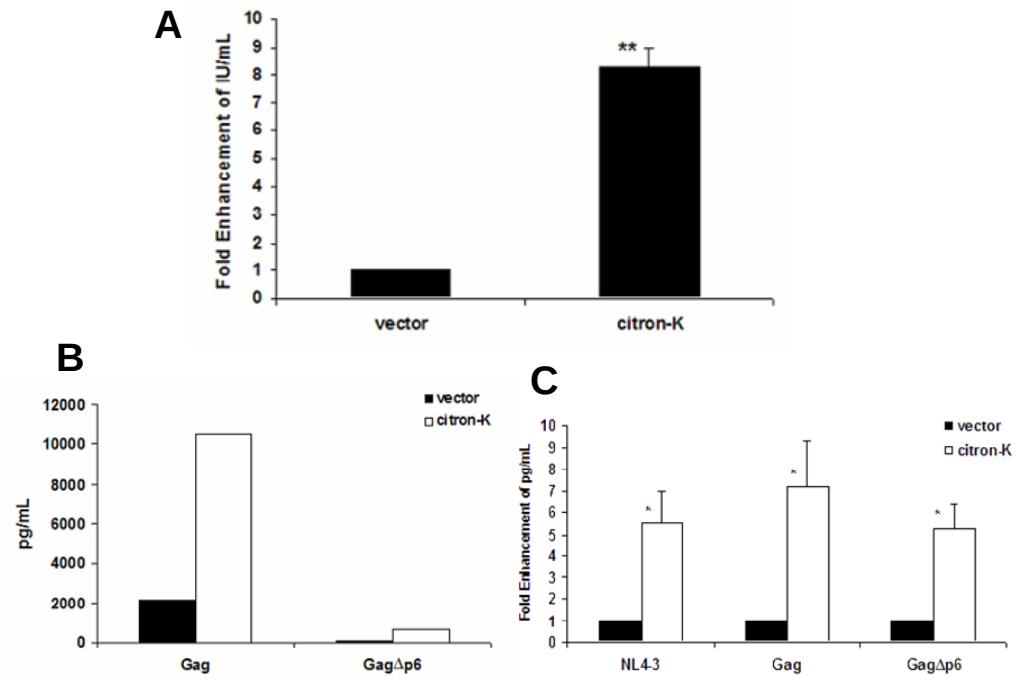

Figure 34: Citron Kinase also Enhanced Release of MLV and HIV-1 GagΔp6 (A) MLV virus-like particle production was similarly enhanced by citron kinase. 293T cells were cotransfected with MLV Gag-Pol, VSV-G-env and HSCG (an MLV-based retroviral vector expressing GFP) with citron kinase or control vector. Supernatant was collected at 48 hours post-transfection and titered for GFP expression on 3T3 cells to determine infectious units/mL. (B, C) Citron kinase enhancement of virion production is late domain independent. 293T cells were cotransfected with Gag or GagΔp6 and vector or citron kinase. Supernatant and cells were collected 48 hours post-transfection for analysis. (B) Citron kinase enhances virion production of both HIV-1 Gag and GagΔp6. Virion production of GagΔp6 alone is significantly reduced compared to Gag but is enhanced by citron kinase. (C) Citron kinase similarly enhances virion production of HIV-1 Gag, GagΔp6 and NL4-3. Shown is relative enhancement of virion production of Gag, GagΔp6 and NL4-3 from two independent experiments. **=p value<0.005, *=p value<0.05.

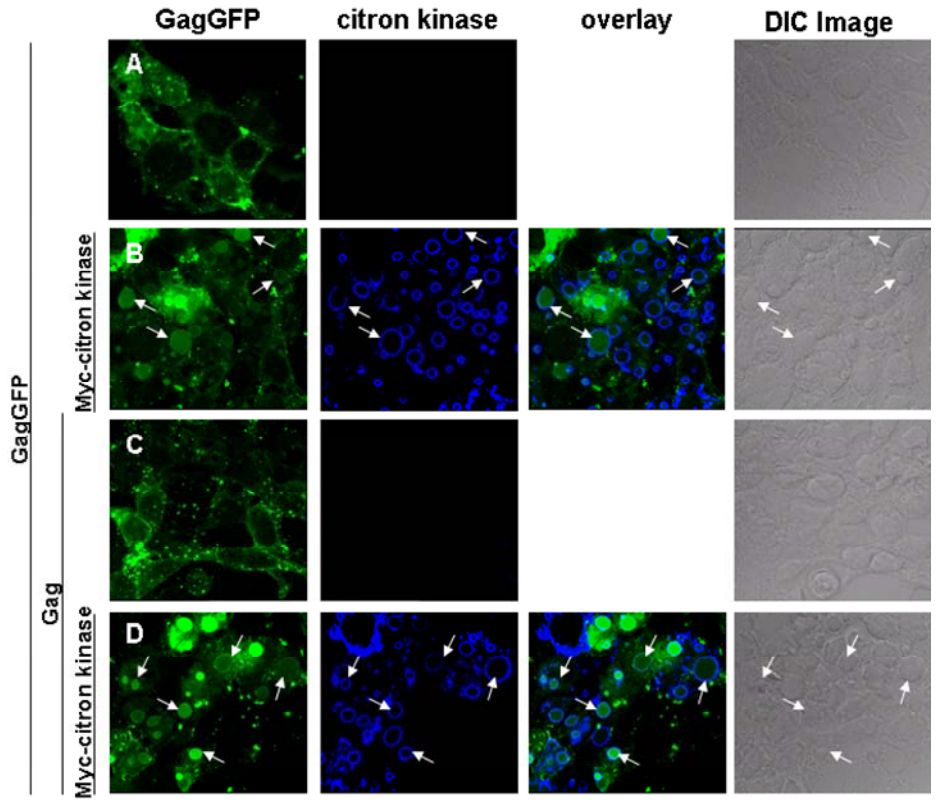

Figure 35: Citron Kinase and Gag Colocalize to Citron Kinase Induced Cytoplasmic Compartments. (A, C) 293T cells were transfected with GagGFP or GagGFP/Gag in a 10:1 ratio and protein localization was determined by confocal microscopy. GagGFP localizes primarily to the plasma membrane in the absence of exogenous citron kinase. (B, D) 293T cells were cotransfected with GagGFP or GagGFP/Gag combination and citron kinase. The localization of GagGFP is altered in the presence of citron kinase. Instead of localizing to the plasma membrane, GagGFP localized intracellularly with citron kinase into "citron induced cytoplasmic compartments". Bar represents 17.5 μm.

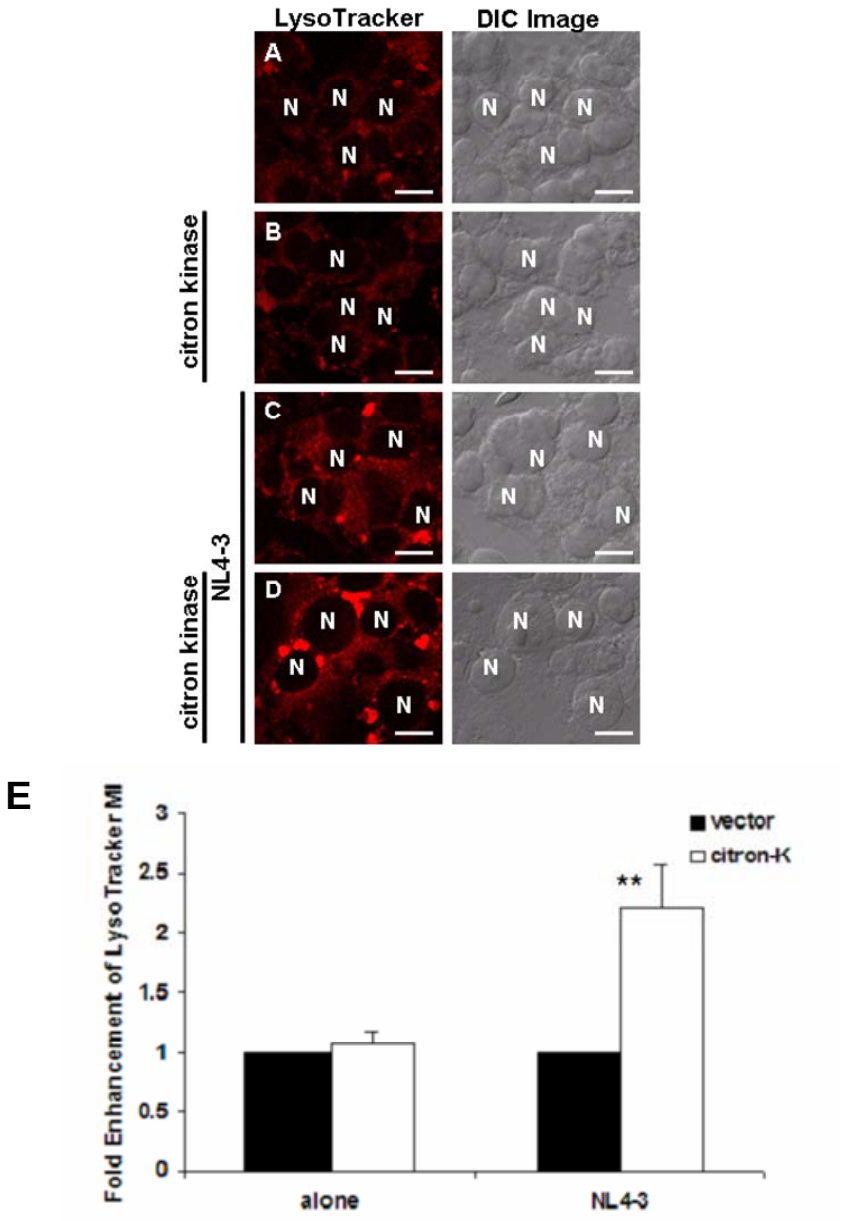

Figure 36: Citron Kinase and HIV-1 Cooperatively Enhance Late Endosomal and Lysosomal Compartments. 293T cells were transfected with pNL4-3 and vector or citron kinase. Cells were incubated with 60 μM LysoTracker for 90 minutes before fixation at ~48 hours post-transfection. (A-D) Images of LysoTracker signals were collected via confocal microscopy. N= nucleus. Bar represents 7 μm. (E) Relative LysoTracker signals of 293T cells were measured by FACS analysis (average of four independent experiments). **=p value<0.005.

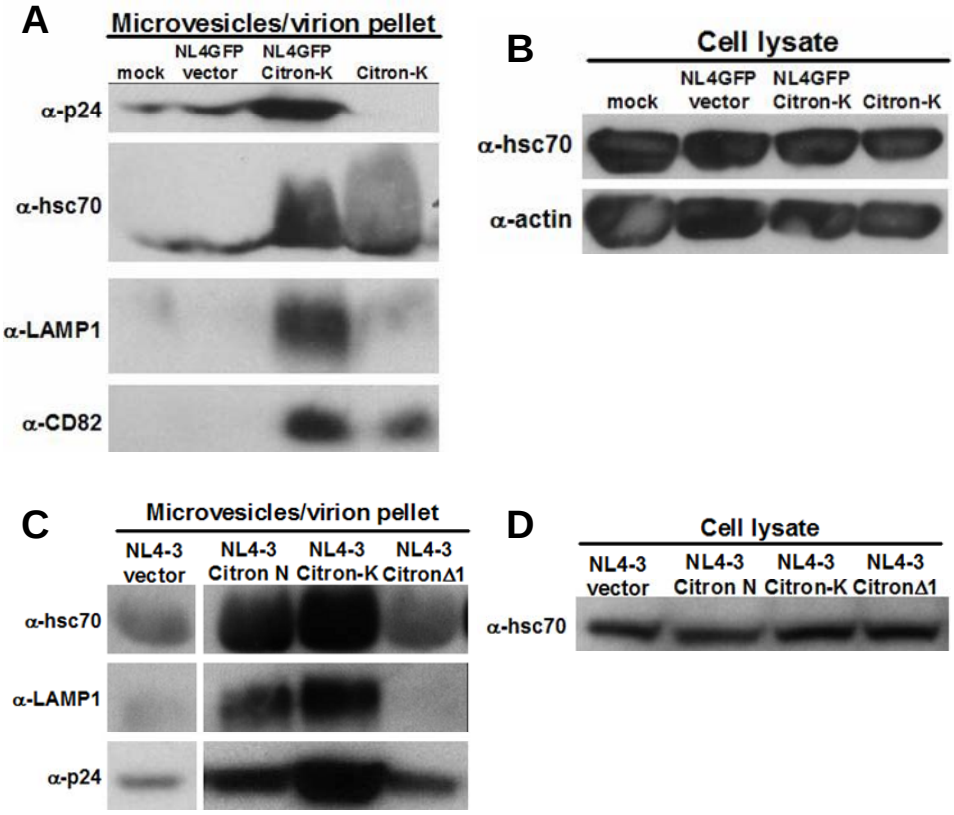

Figure 37: Citron Kinase Enhances the Exocytic Pathway. (A, B) HeLa cells or (C, D) 293T cells were cotransfected with pNL4-3 and vector or citron kinase. Supernatant and cells were collected 48 hours post-transfection for analysis. The microvesicles and virion pellets in the supernatant were purified and analyzed for late endosomal/exosomal markers and HIV-1 Gag p24 (A, C). Exosomal markers analyzed include hsc70, LAMP-1 and CD82. Cell-associated proteins were analyzed for late endosomal/exosomal markers (B, D).

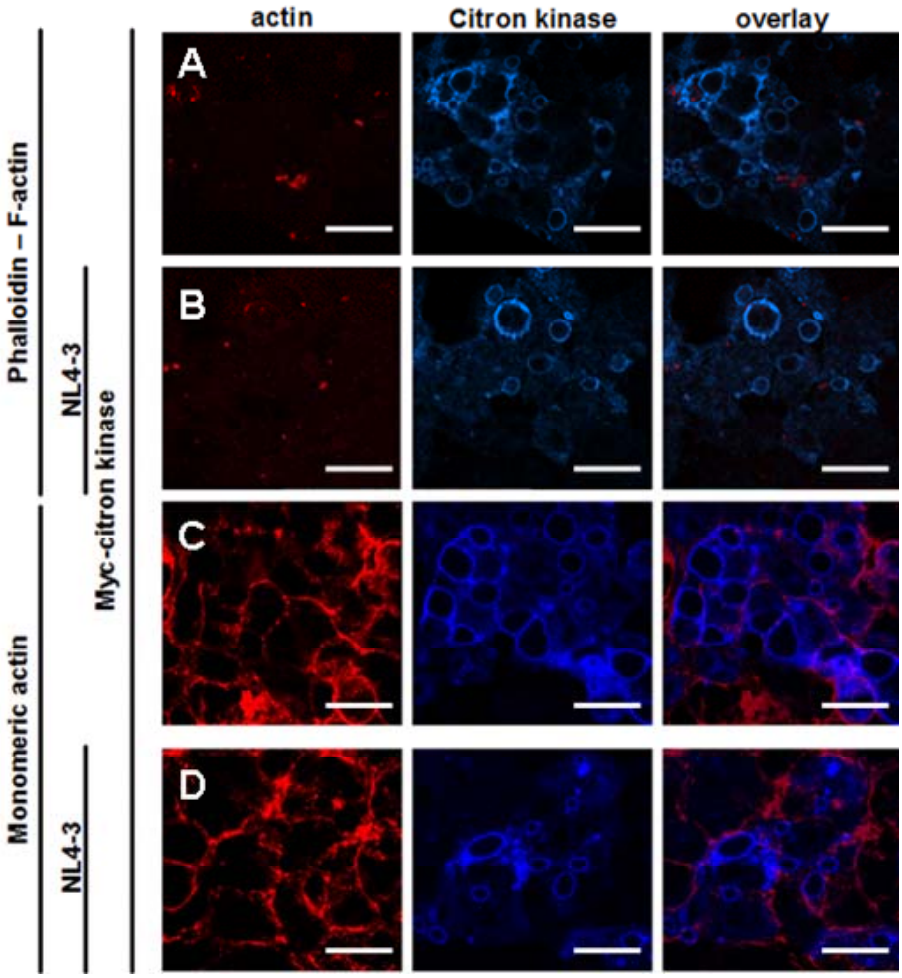

Figure 38: Citron Kinase and/or HIV-1 Does Not Alter Monomeric or Polymerizing Actin. 293T cells were transfected with proviral DNA, pNL4-3 and vector or citron kinase. Cells were fixed, permeabilized and stained with (A, B) phalloidin to analyze polymerizing actin or (C, D) mouse α-actin to analyze monomeric actin. Bar represents 17.5 μm.

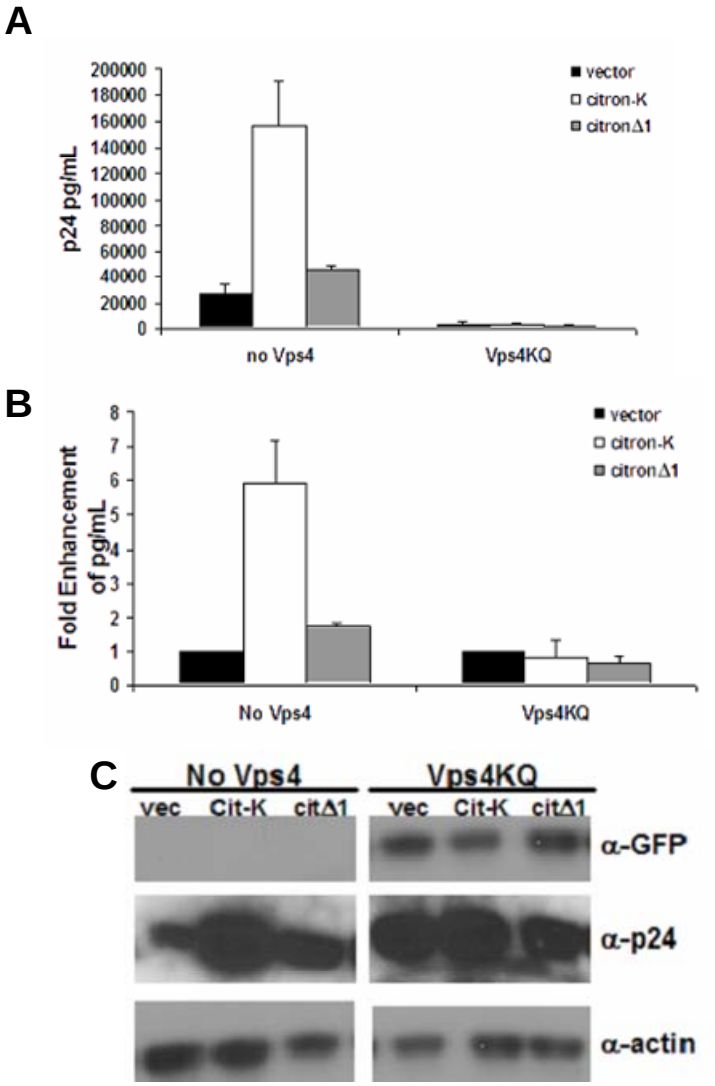

Figure 39: Citron Kinase Requires an Intact Late Endosomal Sorting Pathway to Enhance HIV-1 Virion Production. (A, B, C) 293T cells were cotransfected with pNL4-3 and vector or citron kinase and/or Vps4EQ. Supernatant and cells were collected 48 hours post-transfection for analysis. (A, B) Supernatants were analyzed for viral production by p24 ELISA. Raw data is shown in (A) and fold enhancement is shown in (B). (C) Cell-associated proteins were analyzed for Vps4 expression (mouse α-GFP) and intracellular p24 expression.

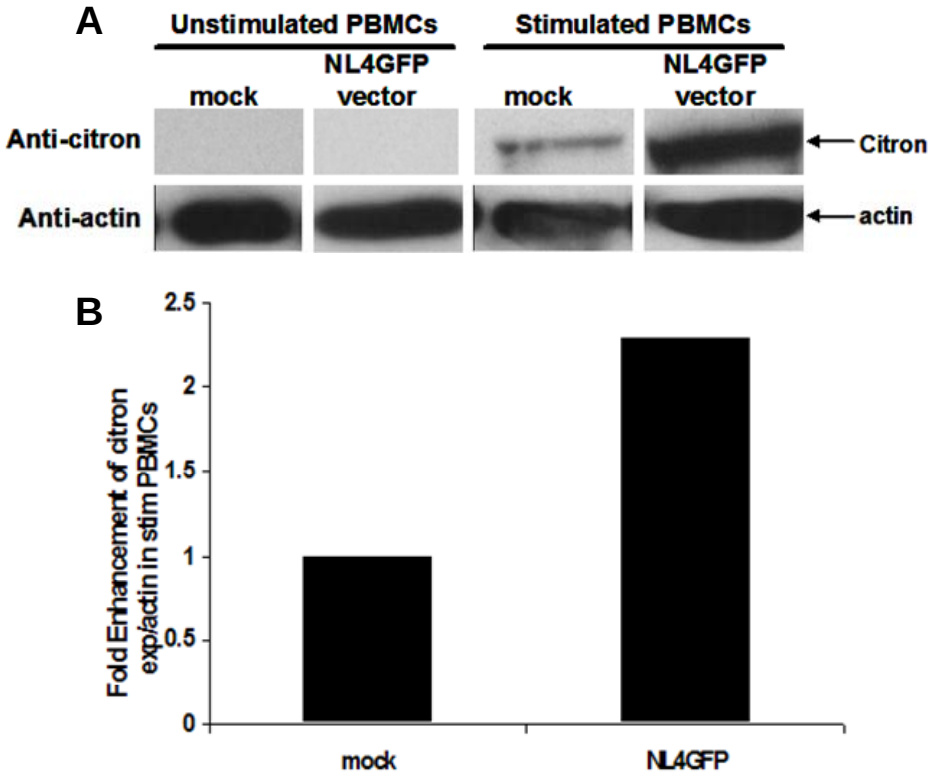

Figure 40: Endogenous Citron Kinase Expression is Induced by T Cell Activation and Further Induced by HIV-1 Infection. (A, B) Primary blood mononuclear cells were Amaxa treated with NL4GFP, stimulated with PHA 24 hrs post-transfection, IL-2 was added 48 hrs post-transfection and cells were collected at day 5. Cells were lysed and analyzed for citron kinase expression (A). (B) Fold enhancement of citron kinase expression compared to actin in stimulated PBMCs.

CHAPTER FIVE:
CHARACTERIZATION OF THE CITRON KINASE-TSG101 INTERACTION AND ITS EFFECT ON VIRAL RELEASE

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Rebecca J. Loomis performed all experiments.

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ABSTRACT:

RhoGTPases play crucial roles in modulating the actin cytoskeleton, controlling cell processes and activating transcription factors. Previously, we showed that the cytoplasmic tail of the HIV-1 envelope glycoprotein, gp41c, interacted directly with the carboxy-terminus of p115RhoGEF, an activator of RhoA, to inhibit HIV-1 replication via a RhoA-dependent mechanism. Recently, we identified citron kinase, a RhoA effector, to preferentially enhance HIV-1 replication by promoting exocytosis. This enhancement was HIV-1 late domain independent and required an intact late endosomal sorting pathway. In this study, we wanted to identify possible component(s) of the endosomal sorting pathway that may facilitate citron kinase's role in mediating HIV-1 virion production.

We identified a functional interaction between citron kinase and Tsg101, a component of the ESCRT-I complex, that inhibited HIV-1 virion production. The kinase activity of citron kinase was necessary and sufficient to mediate the functional interaction between citron kinase and Tsg101. Disruption of the PTAP-binding region of Tsg101 (Tsg101 TYN$^-$) prevented citron kinase and Tsg101 from functionally interacting. Immunoprecipitation studies indicated that when citron kinase and Tsg101 functionally interact, the Tsg101-Gag^{p6} interaction was undisrupted suggesting a requirement for the Tsg101-Gag^{p6} interaction.

Taking these findings and those previously described (Chapter 4) suggest a disparate role for citron kinase; one involving its protein-protein interaction domains to enhance HIV-1 production by promoting exocytosis and the other, involving the kinase activity of citron kinase which functionally interacts with Tsg101 to inhibit HIV-1 virion production.

INTRODUCTION:

RhoGTPases play pivotal roles in the dynamic regulation of actin cytoskeleton [122-124] and through this, control cell morphology [127, 128] and activation of transcription factors such as serum response factor [125] and NF-κB [126]. Although RhoGTPases have been implicated in various steps of T cell activation [182, 191, 193], little is known about how RhoGTPases affect HIV-1 replication. We have previously shown that the cytoplasmic tail of the HIV-1 envelope glycoprotein, gp41c, interacts directly with the carboxy-terminus of p115RhoGEF [195], a guanine nucleotide exchange factor and activator of RhoA. Activation of p115RhoGEF leads to inhibition of HIV-1 replication in a RhoA-dependent manner [195]. The RhoA effector activity inhibiting HIV-1 replication is genetically separable from its other known activities, suggesting RhoA inhibits HIV-1 replication by a novel effector activity [196]. We found citron kinase, a RhoA effector, to preferentially enhance HIV-1 replication by promoting exocytosis (manuscript under review, Chapter 4).

There are two endogenous forms of citron that result from differential splicing [391, 393]; the kinase form (citron-K) and the nonkinase form (citron N). Citron kinase is a 240 kDa Ser/Thr kinase resembling the ROCK family of proteins in its overall domain structure [401]. Citron kinase is ubiquitously expressed in most tissues and cell-types with a cell-cycle dependent localization and plays a key role in cytokinesis [380, 387]. The nonkinase form, citron N, is a splice variant of citron kinase and a brain-specific Rho-binding protein [393]. It has been suggested that citron N functions as a scaffolding molecule on Golgi membranes, organizing Rho-mediated actin polymerization locally by assembling the actin polymerizing complexes together (ROCK II and profilin-IIa) [396]. Actin polymerization and cytoskeletal

rearrangements are regulated by RhoGTPases and are essential for the organization and dynamics of membrane organelles such as endosomes and the Golgi complex [388-390].

HIV-1 replication is modulated by a number of cellular signaling pathways regulated by both host and viral factors [379]. HIV-1 assembly and release occur in a series of essential steps mediated by the viral Gag precursor protein, $Pr55^{Gag}$ [20, 47]. The late (L) domain catalyzes the pinching off and detachment of virus particles from the cell surface and each other [47]. In the case of HIV-1, the L domain is encoded by a small peptide motif, Pro-Thr-Ala-Pro (PTAP) in the C-terminal, p6 domain of $Pr55^{Gag}$. The L domains of retroviruses, despite differences in amino acid sequence and location within their respective viral structural proteins, are functionally interchangeable, suggesting a commonality of function, perhaps as docking sites for host proteins [222, 234, 243].

Viruses like HIV-1 do not encode their own machinery for viral budding and therefore must usurp existing cellular pathways to facilitate viral release. The PTAP motif of HIV-1 Gag^{p6} was found to bind to the ubiquitin enzyme 2 variant (UEV) domain of Tsg101 [204, 240, 248, 261]. Tsg101, a component of the endosomal sorting complex required for transport (ESCRT-I), is a 350 kDa cellular complex essential in the vacuolar protein sorting (VPS) pathway. Point mutations in HIV-1 PTAP motif block virus release at late stages [61, 62] and disrupt binding to Tsg101 [240]. Small inhibitory RNA-mediated Tsg101 depletion in T cells potently blocks HIV-1 release resulting in the virus forming stalks of tethered virus at the plasma membrane [240]. Additionally, overexpression of the dominant-negative form of VPS4, an ESCRT recycling factor, inhibits particle release of HIV-1 and other enveloped viruses, such as MLV [240] indicating the importance of a functional endosomal sorting pathway in mediating HIV-1 viral release.

The endosomal sorting pathway controls a variety of cellular processes and functions in the sorting of ubiquitinated cargo proteins into the lumen of multivesicular bodies (MVB) [54, 268]. Ubiquitinated proteins are recognized on the limiting endosomal membrane and sorted resulting in either MVB fusion with the lysosome to degrade contents or release of material into the extracellular environment via exosomal vesicles [269-271]. ESCRT-I, composed of Tsg101, Vps28 and Vps37, recognizes the ubiquitinated protein cargo and recruits two more class E protein cargos (ESCRT-II/III) that participate in protein sorting and vesicle formation [272, 273, 327]. HIV-1 may bind Tsg101 and other host proteins to gain access to the downstream machinery involved in catalyzing MVB vesicle budding, a mechanism topologically similar to viral budding from the plasma membrane.

Previously, we found citron kinase to preferentially enhance virion production of both HIV-1 and MLV, in a late domain independent manner. The kinase domain of citron kinase was not necessary for this enhancement activity. Citron kinase enhanced secretion of exosomes and microvesicles that copurified with HIV-1 virions. However, citron kinase was unable to enhance HIV-1 virion production when a dominant-negative Vps4 mutant was expressed indicating that a functional late endosomal sorting pathway was required for citron kinase to enhance HIV-1 virion production.

To more clearly understand the role of citron kinase in modulating HIV-1 virion production, we wanted to identify possible component(s) of the endosomal sorting pathway that may interact with citron kinase to help facilitate its role in enhancing HIV-1 virion production. In this study, we identified a functional interaction between citron kinase and Tsg101. The kinase activity of citron kinase was necessary and sufficient to mediate the functional interaction with Tsg101. Disruption of the PTAP-binding region of Tsg101

(Tsg101 TYN$^-$) abrogated the functional interaction between citron kinase and Tsg101. Immunoprecipitation studies indicated that when citron kinase and Tsg101 functionally interact, the Tsg101-Gag^{p6} interaction was undisrupted whereas under conditions where citron kinase enhanced HIV-1 virion production, the Tsg101-Gag^{p6} interaction was reduced. This data, in conjunction with the inability of the Tsg101 TYN$^-$ mutant to maintain a functional interaction with citron kinase, suggests that the Tsg101-Gag^{p6} interaction is required for the functional interaction between citron kinase and Tsg101. Our findings demonstrate that (1) the kinase activity of citron kinase is necessary and sufficient to mediate the functional interaction with Tsg101 and (2) the functional interaction between citron kinase and Tsg101 requires the PTAP-binding region of Tsg101 to remain intact.

Materials and Methods:

Reagents, plasmids and cell lines

The pNL4-3 plasmid encodes the entire HIV-1 genome DNA in pUC18 [327]. The pNL4.Luc.R-E- plasmid was obtained from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program [328]. pCAG (vector), pCAG/citron kinase, pCAG/citronΔ1, CRIK SK, CRIK SKKD and CRIK KD plasmids were previously reported [380, 391]. Citron N was generated by PCR with an N-terminal Myc-tag added and inserted into a retroviral vector, pHSCG [331]. Tsg101-FLAG was a kind gift from Dr. Wes Sundquist (University of Utah, Salt Lake City, Utah). TsgF and TsgF TYN$^-$ constructs were a kind gift from Dr. Paul Bieniasz (Aaron Diamond AIDS Research Center, NY, NY).

293T and HeLa-MAGI cells (NIH AIDS Research and Reference Reagent Program; [331]) were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin and L-glutamine.

HIV-1 Production and Replication in Transfected Human Cells

Transient production of HIV-1 was performed by transfecting NL4-3 (0.1 μg) with vector, citron-K or other citron mutant (0.2 μg) and Tsg101-FLAG, TsgF, TsgF TYN$^-$ or vector (0.05 μg) in 293T cells in 48-well plates at $2.5x10^4$ cells/well using Effectene transfection reagent (Qiagen, Santa Clarita, CA). At 48 hours post-transfection, HIV-1 virions in cell supernatant were measured by infectious units, determined by titering the supernatant on HeLa-CD4-LTR-lacZ cells (MAGI) as described previously [331].

For the Tsg101-FLAG titration experiment, 293T cells were cotransfected with NL4-3 (0.1 μg) and increasing amounts of Tsg101-FLAG (0.01 μg-0.2 μg), with vector being

added to keep the total DNA transfected in each case at 0.3 μg in 48-well plates at $2.5x10^4$ cells/well using Effectene transfection reagent (Qiagen, Santa Clarita, CA). At 48 hours post-transfection, HIV-1 virions in cell supernatant were measured by infectious units as described previously [331].

For the citron kinase titration experiment, 293T cells were cotransfected with NL4-3 (0.05 μg) and increasing amounts of citron-K (0.01 μg-0.5 μg), with vector being added to keep the total DNA transfected in each case at 0.6 μg in 24-well plates at $5x10^4$ cells/well using Effectene transfection reagents (Qiagen, Santa Clarita, CA). At 48 hours post-transfection, HIV-1 virions in cell supernatant were measured by p24 ELISA assay.

Western Blotting

Transfected 293T were lysed and resolved (50-100 μg of total protein) on a 10% SDS-PAGE. Gels were transferred to PVDF membranes (Amersham Biosciences) and blocked with 5% nonfat milk. The membrane was probed with mouse α-p24 (NIH AIDS Research and Reference Reagent Program), mouse α-Myc (AbCam, Cambridge, MA), mouse α-actin (Sigma-Aldrich), rabbit α-citron [382] specific antibodies and visualized using an ECL Kit (Amersham Biosciences).

Immunoprecipitations

Transfections were performed as described previously (Effectene, Qiagen), except in 100 mm dishes using $1x10^6$ cells/plate of 293T cells and cells were transfected with 1 μg pNL4-3, 2 μg vector or citron-K and 0.5 μg TsgFLAG or vector. At 48 hours post-transfection, supernatants and cells were collected for analysis. Cells were lysed in a buffer

containing 0.1% Triton X-100, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 50 mM NaF, 1mM Na_3VO_4) with protease inhibitors (0.1 mM each PMSF, aprotinin, leupeptin and pepstatin) at 4^{o}C for 1 hour, followed by 10 minutes centrifugation in a microcentrifuge (10,000xg) at 4^{o}C. Lysates were quantitated using BCA Protein Assay Reagent Kit (Pierce, Rockford, IL). One mg protein/sample was pre-cleared using 15 μL CL-4B beads, rotated at 4^{o}C for 30 minutes and microcentrifuged at 3000xg for 3 minutes. Supernatant was removed to new tube where 3 μg mouse α-Tsg101 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was added to each sample and incubated overnight at 4^{o}C with rocking. Proteins were incubated using pre-washed protein A beads (15 μL/tube, Pierce, Rockford, IL), rotated at 4^{o}C for 1 hour, washed 5 times with lysis buffer. Bound proteins were eluted by boiling in sodium dodecyl sulfate (SDS) sample buffer. Both whole cell lysates (50 μg) and the Tsg101-bound fraction were analyzed by Western blotting (described above) with immunoblot detection with rabbit α-p24 (NIH AIDS Research and Reference Reagent Program) or rabbit α-citron [382] antibodies.

RESULTS:

Citron Kinase and Tsg101 Functionally Interact

Previously, we found that ectopic expression of citron kinase preferentially enhanced HIV-1 virion production and although, this activity required an intact endosomal sorting pathway, it was late domain independent. To further evaluate the role of citron kinase and its effect on virion production, we wanted to identify component(s) of the endosomal sorting pathway that may facilitate citron kinase in mediating HIV-1 virion production. We transfected Tsg101 and citron kinase, individually and in combination, in 293T cells. Tsg101 overexpression exhibited a dose-dependent response on HIV-1 virion replication; at low levels it enhanced virion production (2-fold) but at higher levels, Tsg101 inhibited virion production (Figure 41A) which is in agreement with previous findings [251]. The stability and expression of intracellular Gag was unaffected by Tsg101 overexpression, even at high levels (Figure 41B) indicating that Tsg101 affected a step of the viral life cycle downstream of HIV-1 gene expression. Overexpression of citron kinase enhanced HIV-1 virion production in a dose-dependent manner (Figure 41C) however, at higher levels was unable to enhance virion production. Intracellular Gag levels were unaffected by citron kinase overexpression (Figure 41D). Although ectopic expression of either citron kinase or Tag101 enhanced HIV-1 virion production, coexpression of citron kinase and Tsg101 inhibited HIV-1 replication (Figure 41E) with intracellular Gag levels remaining unaltered (Figure 41F). These findings indicate that citron kinase and Tsg101 functionally interaction.

Kinase Activity of Citron Is Necessary and Sufficient for Functional Interaction with Tsg101

Truncation/deletion mutants were used to evaluate the domain(s) of citron kinase important for the functional interaction with Tsg101. Citron N, a truncation lacking the N-terminal Ser/Thr kinase domain and a naturally occurring splice variant of citron kinase, showed no functional interaction with Tsg101 (Figure 42A). CitronΔ1, a C-terminally truncated citron kinase mutant, demonstrated functional interaction with Tsg101 (Figure 42A) suggesting an important role for the kinase domain of citron in the functional interaction of citron kinase with Tsg101.

To further characterize the role of citron's kinase domain in the interaction with Tsg101, we used a kinase-dead citron construct (citron KD), the kinase domain alone (citron SK) and a kinase-dead kinase domain construct (citron SKKD) (Figure 42B). The kinase domain alone was capable of functional interaction with Tsg101, however, the kinase-dead kinase domain demonstrated no functional interaction with Tsg101 (Figure 42A) indicating that the activity of the kinase domain is necessary for the functional interaction with Tsg101. The kinase-dead mutant in the context of wild-type citron kinase yielded a similar loss of functional interaction with Tsg101, as the kinase-dead kinase domain, further supporting the finding that the activity of the kinase domain is necessary and sufficient to mediate the functional interaction of citron kinase and Tsg101.

Functional Interaction of Tsg101 with Citron Kinase is Disrupted with Mutation of Tsg101's PTAP-binding Region

After mapping the region of citron kinase necessary for the functional interaction with Tsg101, we wanted to determine the domain(s) of Tsg101 involved. Mutation of the PTAP-binding region of Tsg101 (Tsg101 TYN$^-$) leads to the disruption of not only the Tsg101-Gag^{p6} interaction but also interaction of Tsg101 with AIP1, another ESCRT protein and prevents homodimerization of Tsg101. Disruption of the PTAP-binding region inhibited the ability of Tsg101 to functionally interact with citron kinase (Figure 43A). Citron kinase, alone, was able to enhance HIV-1 virion production (Figure 43A) and this enhancement was not inhibited by exogenous Tsg101 TYN$^-$ mutant. Previously, we found that citron kinase enhanced HIV-1 virion production independently of the HIV-1 viral late domain (manuscript under review, Chapter 4). These findings suggest that binding of proteins to the PTAP-binding region of Tsg101 is inhibitory to the ability of citron kinase to enhance HIV-1 virion production and may be due to the disruption of the Tsg101-Gag^{p6} interaction. Additionally, citron mutants such as citronΔ1, which do not enhance HIV-1 viral release in the absence of exogenous Tsg101 but do functionally interact with Tsg101, did not demonstrate any functional interaction with the Tsg101 TYN$^-$ mutant suggesting that citron kinase can enhance HIV-1 virion production independently of the Tsg101-Gag^{p6} interaction. However, the functional interaction of citron kinase with Tsg101 is dependent on the Tsg101-Gag^{p6} interaction because if that interaction is disrupted with the Tsg101 TYN$^-$ mutant, there is no functional interaction seen between citron kinase and Tsg101.

Our results demonstrate that the functional interaction between Tsg101 and citron kinase is dependent on the integrity of the PTAP-binding region of Tsg101. To investigate

whether citron kinase affects the Tsg101-Gag^{p6} interaction, we immunoprecipitated Tsg101 from cell lysates containing HIV-1, citron-K and exogenous Tsg101 with mouse α-Tsg101 antibody (Santa Cruz) comparing the amount of Gag associated with Tsg101 under each condition. Under conditions where citron kinase enhanced HIV-1 virion production (no exogenous Tsg101), citron kinase reduced the interaction of Tsg101 with HIV-1 Gag (Figure 43C) indicating citron kinase either inhibits the Tsg101-Gag^{p6} interaction or promotes the dissociation of Tsg101 and HIV-1 Gag to complete viral release. When both Tsg101 and citron kinase were overexpressed and HIV-1 virion production was inhibited, there was no change in the Tsg101-Gag^{p6} interaction (Figure 43C), suggesting that Tsg101-Gag^{p6} interaction is necessary for the functional interaction of citron kinase and Tsg101. Additional immunoprecipitation studies need to be completed looking at the effect of a Tgs101 mutant defective in Gag^{p6} binding (Tsg101 TYN^{-}) with various citron kinase constructs including the kinase domain alone (citron SK) and the kinase-dead citron kinase (citron KD) on the Tsg101-Gag^{p6} interaction.

We further mapped the domain(s) of Tsg101 that were important for the functional interaction with citron kinase. The N-terminal region of Tsg101 is involved in mediating interactions with other proteins such as HIV-1 Gag^{p6} and AIP1 and in homodimerization [204, 240, 246-248, 402] whereas the C-terminal region of Tsg101 mediates interactions with other components of the ESCRT-I complex, most notably Vps28 [381]. The results of further domain mapping of Tsg101 were inconclusive (Figure 44), probably due to the multiple roles of the each region of Tsg101.

Collectively, our results suggest that the ability of citron kinase to enhance HIV-1 virion production is a late domain independent function whereas the functional interaction of

Tsg101 and citron kinase which inhibits HIV-1 virion production is a late domain dependent function.

DISCUSSION:

Citron kinase was previously found to preferentially enhance HIV-1 virion production by promoting exocytosis. Despite this enhancement by citron kinase being HIV-1 late domain independent, it required an intact late endosomal sorting pathway. In this report, we wanted to identify possible component(s) of the endosomal sorting pathway that may facilitate citron kinase in mediating HIV-1 virion production. We identified a functional interaction between citron kinase and Tsg101 that required the kinase activity of citron kinase and the PTAP-binding region of Tsg101 suggesting a possible HIV-1 Gag late domain dependent function.

Rho family proteins have been implicated in cell shape regulation, cell adhesion, cell division and for effects on actin structures, most notably, stress fibers [122-128]. These proteins are regulated by nucleotide binding, with the GDP-bound form being inactive and the GTP-bound form being active. When Rho family proteins are activated, they are able to interact with downstream effector proteins, such as citron kinase.

There are two endogenous forms of citron resulting from differential splicing [391, 393]; the kinase form (citron-K) and the nonkinase form (citron N). Citron kinase and citron N interact with GTP-bound forms of Rho and Rac1, but not Cdc42 [393]. Citron kinase is a 240 kDa Ser/Thr kinase resembling the ROCK family of proteins in its overall domain structure. The kinase domain of citron has ~50% sequence identity to the sequences of ROCK, myotonic dystrophy protein kinase and the Cdc42 effector known as MRCK or GEK [401, 403]. Citron kinase is ubiquitously expressed in most tissues and cell-types with cell-cycle dependent subcellular localization [380, 387]. The Rho-binding domain of citron kinase is essential for localization to the cleavage furrow and midbody [380] where citron

kinase modulates the contractile motion required for the separation of the two daughter cells during cytokinesis [380, 387]. Kinase active mutants of citron caused abnormal contractions during cytokinesis but a kinase-dead citron mutant abrogated the abnormal contractile movements observed with a C-terminal citron truncation mutant. These findings suggest that the kinase domain of citron plays an important role in mediating cytokinesis [380].

Full-length citron kinase and the kinase domain alone phosphorylated regulatory light chain (MLC) of myosin II at both Ser^{19} and Thr^{18} during cytokinesis [392]. MLC is currently the only known phosphorylation target for citron kinase. Unlike ROCK, which also phosphorylates MLC, citron kinase does not phosphorylate the myosin binding subunit of myosin phosphatase and therefore, does not prevent turnover of MLC phosphorylation [392]. Expression of the kinase domain of citron increased MLC di-phosphorylation and this di-phosphorylation restored stress fiber assembly even when ROCK was inhibited with a specific inhibitor. Both mono- and di-phosphorylated MLC were found in cleavage furrows, but di-phosphorylated MLC had a more constrained localization than did mono-phosphorylated MLC [392].

The functional interaction between citron kinase and Tsg101 was dependent on the kinase activity of citron kinase, because when it was deleted (citron N) or a point mutation was made to abrogate kinase activity (citron KD) in citron kinase, no functional interaction was observed. The kinase domain alone maintained a functional interaction with Tsg101 while the kinase-dead kinase domain mutant lost its ability to functionally interact with Tsg101, suggesting that the activity of the kinase domain is necessary and sufficient for mediating the functional interaction with Tsg101. It is unlikely that Tsg101 is a direct phosphorylation target for citron kinase since there is no evidence that Tsg101 or its yeast

homologue, Vps23, are phosphorylated. Citron kinase may phosphorylate another protein that is in the endosomal sorting pathway.

In T cells and monocyte-derived myeloid cells, inhibition of myosin light chain kinase (MLCK), through the use of wortmannin, an inhibitor of MLCK leading to inhibition of MLC phosphorylation, suppressed HIV-1 release [315]. Wortmannin did not disrupt the transport of viral components to the plasma membrane, but rather inhibited budding only, since even at high levels of wortmannin, viral components were seen with myosin at sites of viral budding. These findings suggest that after localization to sites of viral budding, the subsequent actin-myosin interactions participate in the release of viral particles from host cells [315]. MLC is the only identified phosphorylation target for citron kinase. It is unlikely that phosphorylation of MLC by citron kinase plays a role in the functional interaction of citron kinase with Tsg101 and subsequent inhibition of HIV-1 viral production. The aforementioned study found that inhibition of MLC phosphorylation suppressed HIV-1 release [315], whereas we found citron kinase phosphorylation to be necessary for inhibition, which may correlate with an increase in MLC phosphorylation.

Tyrosine phosphorylation is the most prevalent phosphorylation event in regulating various aspects of endosomal trafficking. Coordinated phosphorylation and dephosphorylation events are often necessary to mediate association of proteins and to facilitate vesicle transport [404]. Protein kinase activity is important in endocytosis. When protein kinase C βII (PKCβII) was mutated to be deficient in phosphorylation, it specifically blocked EGF receptor trafficking and degradation without affecting transferrin receptor recycling [405], perhaps by forming a nonfunctional late endosome. Also, Hrs, a hepatocyte growth factor-regulated tyrosine kinase substrate and interacting protein of Tsg101, is

phosphorylated by binding of a variety of growth factors and cytokines to their receptors, as a means of coordinating endosomal receptor sorting and signaling. Hrs phosphorylation was prevented when endocytosis was inhibited [406]. Collectively, these findings indicate the importance of phosphorylation events in regulation of the endosomal sorting pathway.

Additionally, a second region of HIV-1 Gag^{p6} has been defined to contribute to viral release and interacts with AIP1, a host protein [246, 247]. AIP1/Alix is an adaptor protein that interacts with Tsg101 and CHMP proteins of ESCRT-III complex, coupling HIV-1 Gag^{p6} to the early and late-acting endosomal sorting complexes and binds directly to HIV-1 Gag^{p6} LRSL [246, 247]. The Tyr^{319} of Alix binds to Src's SH_2 domain leading to AIP1/Alix phosphorylation at the C-terminal tyrosine rich region by Src [407]. Phosphorylation of AIP1/Alix causes translocation from the membrane and cytoskeleton to the cytoplasm and a reduction in its interaction with binding partners [408-410]. Src antagonizes the effects of Alix phosphorylation of its C-terminus, leading to disruption of interaction with target proteins, negatively regulating its biological function [407].

Even though the majority of these known interactions linking phosphorylation with trafficking events involve tyrosine phosphorylation, it does not preclude a role for a Ser/Thr kinase in regulation. It is possible that citron kinase phosphorylates AIP1/Alix, a known component in linking early and late endosomal sorting complexes to HIV-1, thereby inhibiting its function in binding to Tsg101, Gag or CHMP proteins and ability to act as a scaffold protein, leading to inhibition of HIV-1 virion production. Alternatively, citron kinase may phosphorylate an intermediary protein which then phosphorylates AIP1/Alix or there may be an unidentified phosphorylation target for citron kinase. We have yet to identify a potential downstream phosphorylation target for citron kinase. Elucidation of a

phosphorylation target would shed light on how citron kinase and Tsg101 interact to mediate HIV-1 virion production.

This paper suggests a disparate role for citron kinase in regulating HIV-1 virion replication (Figure 45). Previously, we identified citron kinase to preferentially enhance HIV-1 virion production by promoting exocytic secretion. The enhancement of HIV-1 virion production by citron kinase required the leucine zipper, the Rho-binding and zinc-finger domains but not the kinase domain and was late domain independent. This activity of citron kinase is most likely due to it acting as a scaffolding molecule, linking host proteins to the endosomal sorting pathway. In this paper, we found that the kinase activity of citron was necessary and sufficient to mediate the functional interaction with Tsg101 and required Tsg101's PTAP-binding region to be intact, suggesting a potential HIV-1 Gag late domain dependent function. This activity is significantly different from the role citron kinase plays in enhancing exocytic release and may be involved in mediating interaction with additional host factors through regulation of phosphorylation or by activating an already associated factor. Studies on how these two distinct functions of citron kinase are regulated during HIV-1 infection are underway.

These findings imply an increasing complexity in the interactions of HIV-1 viral proteins with host proteins and host proteins with each other to modulate viral release. The interactions between citron kinase and the endosomal sorting pathway in regulation of HIV-1 viral release may provide new host targets for the development of anti-HIV-1 therapeutics.

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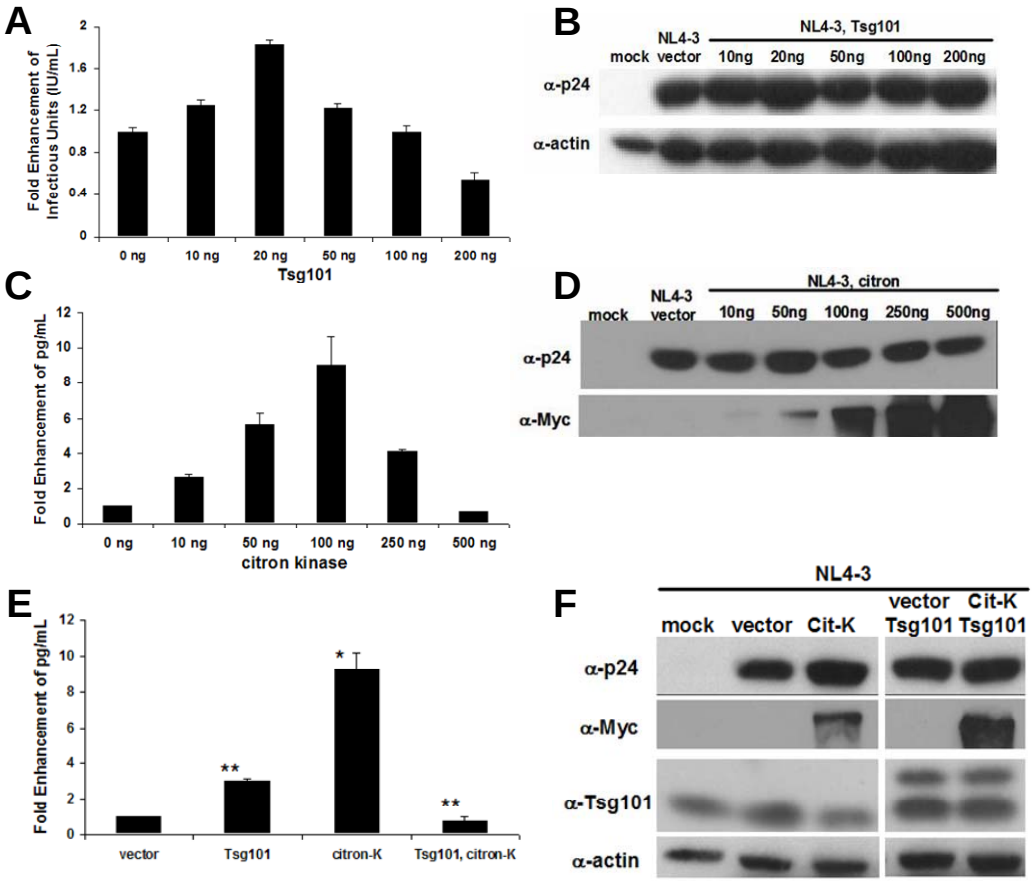

Figure 41: Citron Kinase and ESCRT-I Component, Tsg101, Functionally Interact. (A, B) Dose dependent response of Tsg101 expression on HIV-1 virion production. 293T cells were cotransfected with increasing amounts of Tsg101-FLAG and constant amount of NL4-3. Supernatants and cells were collected 48 hours post-transfection for analysis by measuring infectious virion production (A) and cell-associated protein expression (B). (C, D) Dose dependent response of citron kinase expression on HIV-1 virionl production. 293T cells were cotransfected with increasing amounts of citron kinase and constant amount of NL4-3. Supernatants and cells were collected 48 hours post-transfection for analysis by measuring infectious virion production (C) and cell-associated protein expression (D). (E, F) Under conditions where expression of Tsg101 and citron kinase individually enhanced HIV-1 virion production, overexpression, in combination, negatively affected HIV-1 virion production. 293T cells were cotransfected with Tsg101-FLAG, citron kinase and/or NL4-3. Supernatants and cells were collected 48 hours post-transfection for analysis by measuring infectious virion production (E) and cell-associated protein expression (F). **=p value<0.005 and *=p value<0.05.

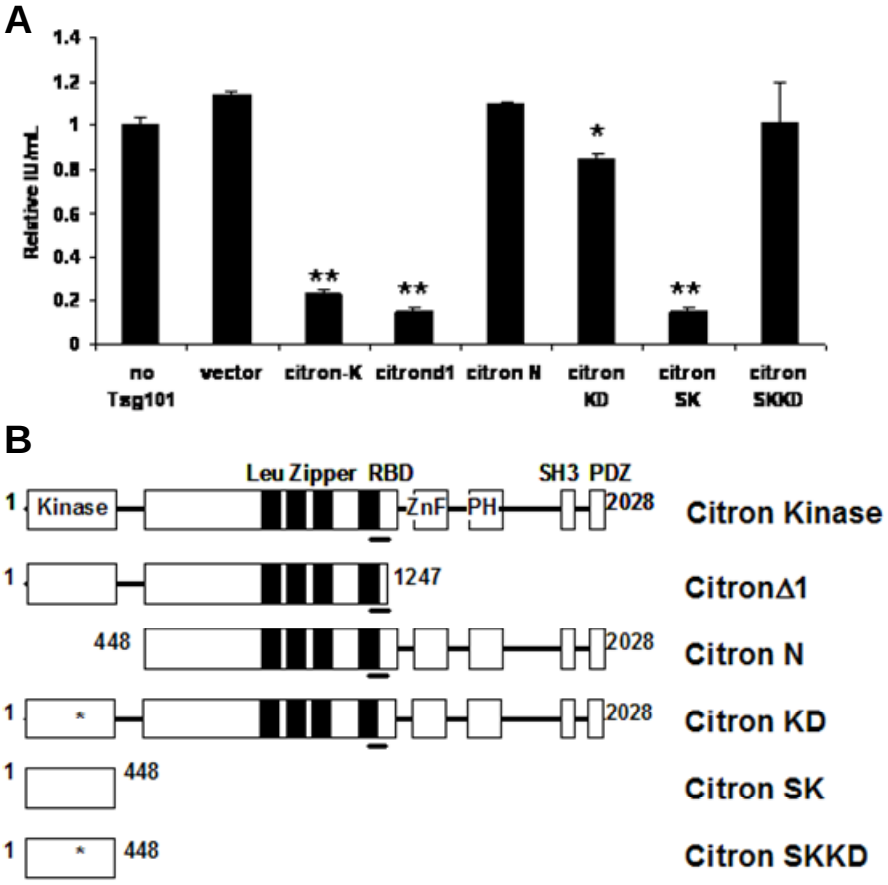

Figure 42: The Kinase Activity of Citron is Necessary and Sufficient for the Functional Interaction with Tsg101. (A) 293T cells were cotransfected with NL4-3, Tsg101-FLAG and vector, citron kinase or various citron truncation constructs. Supernatants were collected at 48 hours post-transfection and infectious virion production measured. (B) Schematic of citron kinase constructs used in this experiment. **=p value<0.005 and *=p value<0.05.

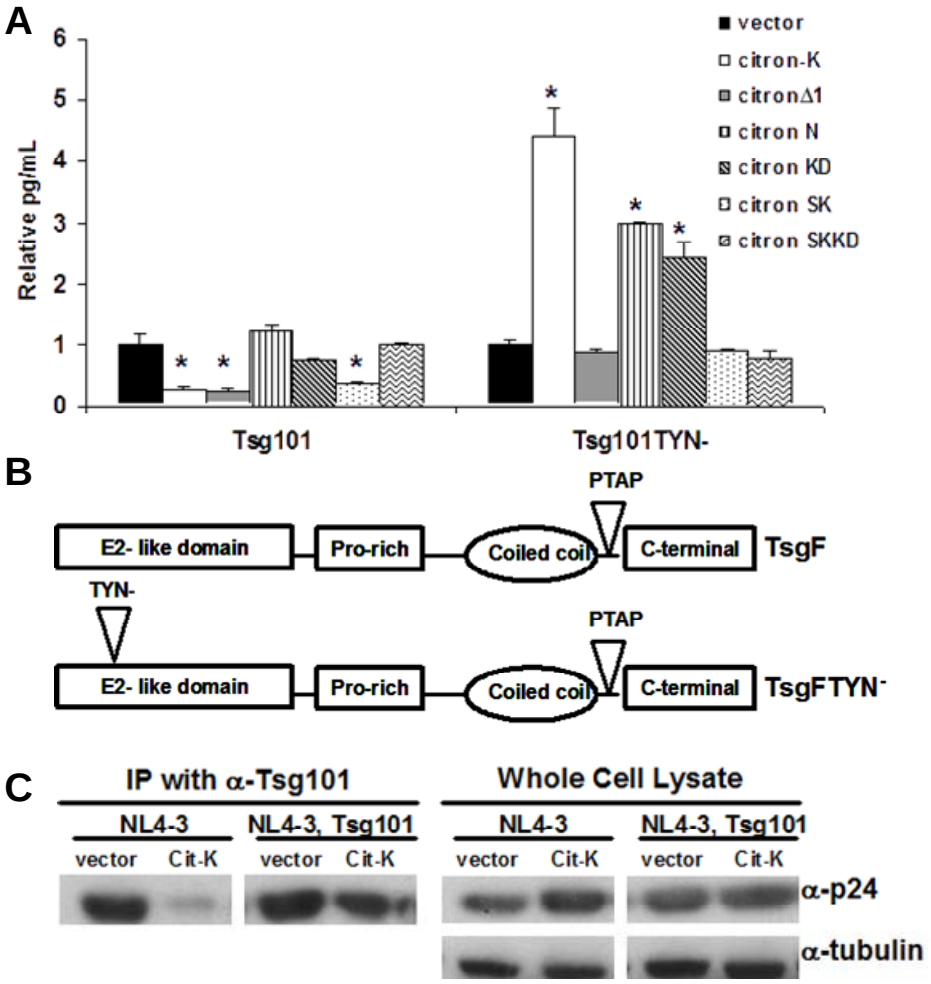

Figure 43: The Functional Interaction of Tsg101 and Citron Kinase is Disrupted with Mutation of the PTAP-binding Region. (A) 293T cells were cotransfected with vector, citron kinase or various citron truncation mutants, NL4-3 and either wild-type Tsg101 or Tsg101 with a mutated Gag^{p6} binding site, Tsg101 TYN^-. Supernatants were collected at 48 hours post-transfection and analyzed. (B) Schematic of Tsg101 constructs used in this experiment. **=p value<0.005, *=p value<0.05. (C) 293T cells were cotransfected with pNL4-3 and vector or citron kinase and/or Tsg101. Supernatant and cells were collected 48 hours post-transfection for analysis. Cells were lysed and immunoprecipitated with mouse α-Tsg101. Immunoprecipitated complexes (left panel, 1 mg protein immunoprecipitated) and whole cell lysate (right panel, 50 μg whole cell lysate) were analyzed by western blot with mouse α-p24 and mouse α-tubulin antibodies.

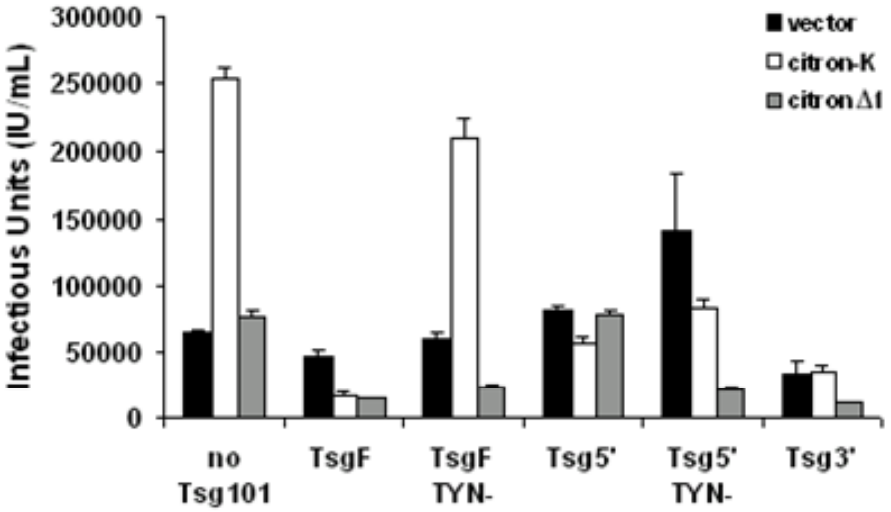

Figure 44: The PTAP-binding Region of Tsg101 is Important for Its Functional Interaction with Citron Kinase. 293T cells were cotransfected with vector, citron kinase or citron∆1, NL4-3 and either wild-type Tsg101 or Tsg101deletion mutants (described in Goila-Gaur *et al.* 2003). Supernatants were collected at 48 hours post-transfection and analyzed by infectious unit assay.

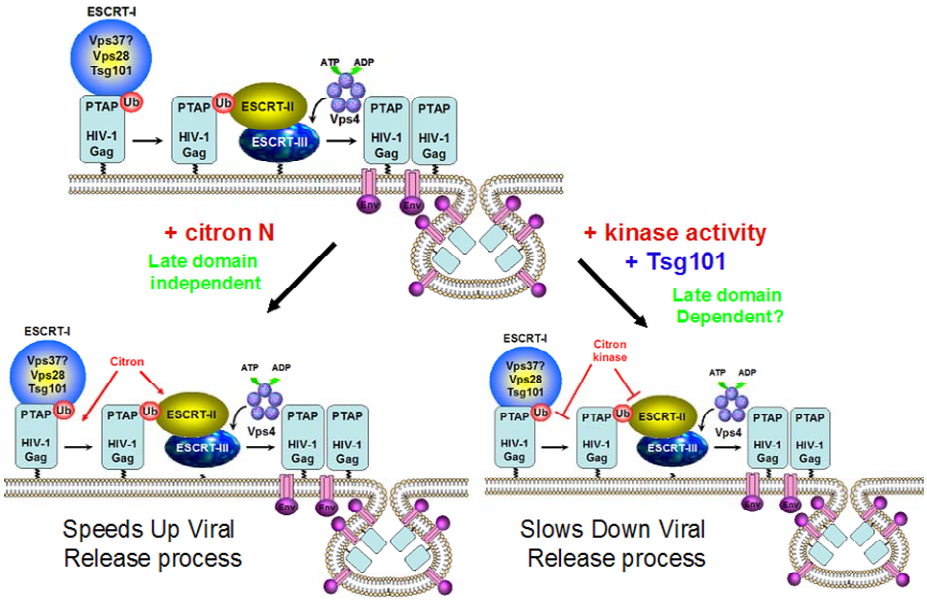

Figure 45: Model for the Disparate Role of Citron Kinase in Regulating HIV-1 Virion Production. HIV-1 replication is dynamically regulated by citron kinase. On the left side, citron N enhances HIV-1 viral replication, is late domain independent and requires the leucine zipper, Rho-binding and zinc-finger domains for its enhancement activity. On the right, the citron kinase domain inhibits HIV-1 viral replication by functionally interacting with Tsg101 which requires the kinase activity of citron and is late domain dependent.

CHAPTER SIX:
PERSPECTIVE ON THE ROLE OF CITRON KINASE IN HIV-1 REPLICATION

Previously, we reported that the long cytoplasmic tail of the HIV-1 transmembrane envelope glycoprotein, gp41c, interacts directly with the carboxy-terminal regulatory domain of p115RhoGEF, a guanine nucleotide exchange factor and activator of RhoA [195]. Ectopic expression of p115RhoGEF or $G\alpha_{13}$, which activates p115RhoGEF activity, leads to inhibition of HIV-1 replication in a RhoA-dependent mechanism. The RhoA effector activity inhibiting HIV-1 replication is genetically separable from its activities in activation of SRF and actin stress fiber formation [196], suggesting RhoA inhibits HIV-1 regulation via a novel effector activity. To investigate how RhoA signaling pathways modulate HIV-1 replication, we tested individual RhoA effectors in 293T and human T cells.

We demonstrated that ectopic expression of citron kinase and citron N preferentially enhanced HIV-1 virion release without significantly affecting HIV-1 gene expression in 293T and Jurkat T cells. Depletion of endogenous citron kinase inhibits HIV-1 virion production. Additionally, the leucine-zipper, Rho-binding and zinc-finger domains, but not the kinase domain, were necessary for the enhancement activity. Citron kinase also enhances MLV virion production and the enhancement activity is late domain independent. Ectopic expression of citron kinase leads to the formation of cytoplasmic structures containing citron kinase and HIV-1 Gag proteins. Citron kinase and HIV-1 cooperatively enhance late endosome and lysosome compartments. Finally, citron kinase enhances secretion of

exosomes or microvesicles that copurify with HIV-1 virions. We conclude that citron kinase enhances HIV-1 virion production by stimulating exocytosis. Collectively, our findings suggest a novel mechanism of HIV-1 virion release that is late domain independent and involves citron kinase-mediated exocytosis.

Citron kinase mediated enhancement of virion production is dependent on an intact late endosomal sorting pathway therefore we wanted to identify possible endosomal sorting pathway components that may facilitate citron kinase in enhancing HIV-1 virion production. Although ectopic expression of either citron kinase or Tsg101 enhanced HIV-1 virion production, coexpression of citron kinase and Tsg101 inhibited HIV-1 replication. The kinase activity of citron kinase was necessary and sufficient for the functional interaction. We have yet to identify a potential phosphorylation target for citron kinase. Disruption of the PTAP-binding region of Tsg101 (Tsg101 TYN$^-$) prevented citron kinase and Tsg101 from functionally interacting suggesting a potential role for the Tsg101-Gag^{p6} interaction in mediating the functional interaction with citron kinase. Immunoprecipitation studies indicated that when citron kinase and Tsg101 functionally interact, the Tsg101-Gag^{p6} interaction was undisrupted. This data, in conjunction with the inability of the Tsg101 TYN$^-$ mutant to maintain a functional interaction with citron kinase, suggests that the Tsg101-Gag^{p6} interaction is required for the functional interaction between citron kinase and Tsg101.

Citron Kinase

There are two endogenous forms of citron; the kinase form (citron-K) [380] and the nonkinase form (citron N) [393]. Citron kinase is a 240 kDa Ser/Thr kinase resembling the ROCK family of proteins in its overall domain structure [356, 358, 411]. The kinase domain

of citron has ~50% sequence identity to the sequences of ROCK and myotonic dystrophy protein kinase and the Cdc42 effector known as MRCK or GEK [401, 403]. Citron kinase and citron N interact with GTP-bound forms of Rho and Rac1, but not Cdc42 [393]. Activity of citron kinase is stimulated by activated RhoA and has been implicated in the control of cytokinesis downstream of Rho [380, 387] and in the di-phosphorylation of myosin light chain (MLC) [392]. Citron kinase is ubiquitously expressed in most tissues and cell-types with cell-cycle dependent subcellular localization [380, 387].

RhoA colocalizes with citron kinase in the cell cortex during ana- and telophase and both are enriched in the cleavage furrow and midbody during telophase [387]. The Rho-binding domain of citron kinase is essential for localization to the cleavage furrow and midbody [380] where citron kinase modulates the contractile motion required for the separation of the two daughter cells during cytokinesis [380, 387]. Kinase active mutants of citron caused abnormal contractions during cytokinesis but a kinase-dead citron mutant abrogated the abnormal contractile movements observed with a C-terminal citron truncation mutant. These findings suggest that the kinase domain of citron plays an important role in mediating cytokinesis, specifically contractile movements [380].

Full-length citron kinase and the kinase domain alone phosphorylated the regulatory light chain (MLC) of myosin II at both Ser^{19} and Thr^{18} during cytokinesis [392]. MLC is currently the only known phosphorylation target for citron kinase. Unlike ROCK, which also phosphorylates MLC, citron kinase does not phosphorylate the myosin binding subunit of myosin phosphatase and therefore, permits turnover of MLC phosphorylation [392]. Expression of the kinase domain increased MLC di-phosphorylation which was able to restore stress fiber assembly even when ROCK was inhibited with a specific inhibitor.

Although both mono- and di-phosphorylated MLC were found in cleavage furrows, di-phosphorylated MLC showed more constrained localization than did mono-phosphorylated MLC [392].

The nonkinase form, citron N, is a splice variant of citron kinase and a brain-specific Rho-binding protein [393]. There is a molecular association between citron N and the post-synaptic scaffold protein, PSD-95/SAP-90, a member of the membrane-associated guanylate kinase protein family (MAGUK) [394, 395]. This interaction involves signaling pathways essential for neural plasticity and postsynaptic signal transduction events [394, 395]. Actin polymerization and cytoskeleton are regulated by RhoGTPases and are essential for the organization and dynamics of membrane organelles such as endosomes and the Golgi complex [388-390]. Citron N is enriched and associated with the Golgi apparatus of hippocampal neurons in culture [396]. Suppression of citron N or expression of a mutant lacking Rho-binding activity leads to dispersion of the Golgi apparatus [396] suggesting that citron N functions as a scaffolding molecule on Golgi membranes, organizing Rho-mediated actin polymerization locally by assembling the actin polymerizing complexes together (ROCK II and profilin-IIa) [396].

Possibilities for Localization to the Endosomal Pathway

In our studies, we found that (1) citron kinase relocalized HIV-1 Gag from the plasma membrane into cytoplasmic structures, where citron kinase also localized (Figure 36) and (2) citron kinase stimulated the exocytic pathway (Figure 38), consequently enhancing HIV-1 virion production. This is of interest because in other cell types such as macrophages, HIV-1 buds almost exclusively into MVBs and is released into the extracellular environment when

the MVB fuses with the plasma membrane [280, 386, 399]; although this mechanism of viral release is rarely seen in cell types such as T cells. Citron kinase may divert HIV-1 from release at the plasma membrane to release by the exocytic pathway, perhaps because it is a more efficient viral release process or promote exploitation of an unused or little used pathway in most cell types as a means to enhance virion production. The subcellular localization of citron kinase and HIV-1 Gag implies that viral release occurs predominantly into intracellular compartments, most likely of endosomal origin, instead of at the plasma membrane. In T cells, HIV-1 buds primarily from the plasma membrane, although its endosomal sorting pathway (exocytic pathway) is operational, suggesting that some factor, whether viral or host, prefers the plasma membrane as the site of budding. Neither is mutually exclusive and citron kinase-mediated enhancement is probably the result of both.

One unanswered question is what causes citron kinase to localize to compartments of the endosomal sorting pathway/intracellular origin? In an initial paper describing citron kinase, the authors demonstrated that the Rho-binding region of citron kinase was necessary for localization to the cleavage furrow and midbody [380]. Deletion of the Rho-binding domain led to an increasingly cytosolic pattern of expression. One possibility is that citron kinase is activated by RhoB.

Higher vertebrates have 3 RhoGTPases, RhoA, RhoB and RhoC which share 85% amino acid sequence identity with most of the sequence divergence being at the C-terminus. The C-terminus of Rho family GTPases is essential for correct localization of the proteins. RhoA plays a key role in the regulation of actinomyosin contractility and demonstrates predominantly membrane localization. RhoB which is localized primarily on endosomes has been shown to regulate cytokine trafficking and cell survival while RhoC is more important

in cell locomotion. In addition, these three Rho isoforms have very different expression profiles [412], although they are expressed in most tissues, expression levels varied significantly depending on tissue type.

RhoA, RhoB and RhoC are post-translationally modified by prenylation of a conserved C-terminal cysteine followed by methylation and proteolytic removal of the last three amino acids [413]. Prenylation of Rho proteins appears to be important for their stability, as inhibitors of enzymes that synthesize prenyl groups induce a decrease in RhoA or RhoB protein levels and their function [414]. The length of the prenyl group differs between Rho proteins; RhoB can be prenylated either with a 15-carbon farnesyl or a 20-carbon geranylgeranyl group, whereas RhoA and RhoC are only geranylgeranylated. This difference is reflected in their localization because when RhoB is only geranylgeranylated, it shows a predominant membrane localization [415].

There is a small degree of sequence divergence in and around the Switch I region (the region necessary for effector interaction) between RhoA, RhoB and RhoC, suggesting there could be differences in their affinities for regulators or effector proteins. Protein interaction studies suggest that ROCK and citron kinase have a higher affinity for RhoC compared to either RhoA or RhoB, however the VFSKD sequence in Switch I, essential for binding PRKs, ROCK and citron kinase [416, 417], is identical in RhoA, RhoB and RhoC with PRK, ROCK and citron kinase binding to all three isoforms of Rho.

RhoGTPases are critical regulators of actin dynamics and have been involved in the control of endocytosis. RhoB has been localized to the cytoplasmic face of endosomal membranes [300, 302] suggesting a role in endosomal trafficking regulation. Once activated by PRK1, RhoB delays the transport of internalized EGF-receptor to lysosomes [301] and

promotes, with Dia1, the polymerization of an actin coat around endosomes and their association to subcortical actin cables effectively inhibits further endosomal transport [305]. Overexpression of wild-type RhoB had no effect on endosome transport, suggesting that endogenous RhoB transiently inhibits endocytosis under the regulation of the GTPase cycle. Dia1 and PRK1 may cooperate in regulating endosomal trafficking downstream of RhoB because a dominant-negative Dia1 reversed the inhibition of EGFR degradation caused by activated RhoB [305].

Citron kinase may be activated by RhoB, instead of RhoA, causing it to localize to the endosomal sorting pathway and once localized there, may interact with other components/complexes of the endosomal sorting pathway to promote exocytosis. RhoB seems to only transiently inhibit endocytosis trafficking; *rhoB* transcripts have a half-life of about 30 minutes, significantly shorter than either *rhoA* or *rhoC* transcripts [418] therefore it is plausible that citron kinase transiently interacts with RhoB localize to the late endosome and to come into close proximity with additional binding partners. To determine if citron kinase is activated by RhoB to promote exocytosis and HIV-1 virion production, we can perform a series of transfections with citron kinase, constitutively active RhoA and dominant-negative RhoB or citron kinase, constitutively active RhoB and dominant-negative RhoA. Analysis of HIV-1 virion production under each condition and immunofluorescence to determine localization of citron kinase under each condition may shed light on what induces citron kinase to localize to the endosomal sorting pathway.

Citron Kinase May Act as a Scaffolding Molecule

We identified a portion of the leucine zipper, the Rho-binding and zinc-finger domains to be necessary for citron kinase to enhance HIV-1 virion production (Figure 32). Citron kinase promoted secretion of microvesicles and virions from the exocytic pathway (Figure 37). The domains of citron kinase necessary of enhancement are often associated with protein-protein interactions and the endosomal/exocytic pathway involves coordinated interactions with a number of complexes to complete its sorting processes, suggesting that citron kinase is a scaffolding molecule.

Citron kinase may act as a scaffold for a multimeric structure functioning through conformational changes induced by Rho binding and interactions with additional partners via the leucine zipper and zinc-finger domains. The presence of several leucine zippers is consistent with such a model and coiled-coil regions usually serve as dimerization domains, suggesting that native citron is a dimer [393]. Rho binding may partially open this structure, causing a major conformational change in citron dimer thereby affecting the properties of the scaffold, as is established for myosin [419]. Additionally, the zinc-finger domain which consists of six cysteines and two histidines, is not a classical zinc-finger and is thought to bind to lipid second messengers [393, 420].

An attractive model for the enhancement of HIV-1 virion production by citron kinase is that citron kinase acts as a scaffold, linking components of the endosomal sorting pathway together. It is unlikely that citron kinase binds directly to HIV-1 Gag since citron kinase can, in the absence of a viral infection, promote secretion of microvesicles and exosomes. It is much more likely that citron kinase binds to cellular factors, recruiting them to the site(s) of viral release. Depletion of endogenous citron kinase inhibits virion production implying that

citron kinase is necessary for a productive viral infection (Figure 31). Additionally, in primary T cells, there was an induction of endogenous citron kinase expression with HIV-1 infection (Figure 40).

Tsg101 interacts with a number of proteins via its UEV domain by binding the PTAP sequences of Hrs, Tsg101 itself, AIP1 and HIV-1 Gag^{p6} [204, 240, 248, 261, 421-424]. Tsg101 is also thought to multimerize thereby allowing it to interact with several different proteins via the same UEV region [247]. AIP1 was identified to act as a scaffolding molecule linking complexes that act early (Tsg101/ESCRT-I) and late (CHMP/ESCRT-III) to the endosomal sorting pathway [247]. Additionally, it was found that mutations disrupting the Tsg101-Gag^{p6} interaction inhibited HIV-1 release to a greater extent than mutations disrupting the AIP1-Gag^{p6} interaction [247]. One of the primary purposes of both Tsg101 and AIP1 in interacting with HIV-1 Gag^{p6} is to act as scaffolds to recruit additional components and complexes to facilitate in viral egress.

It is commonly believed that the Tsg101-Gag^{p6} interaction is required for efficient viral release and that disruption of this interaction, either by depletion of Tsg101 or mutations removing the HIV-1 late domain, inhibits viral release. We found citron kinase to enhance HIV-1 virion production even when the PTAP-binding region of Tsg101 is disrupted – a novel and unprecedented finding (Figure 35). Citron kinase enhanced virion production of not only MLV, which uses the PY- late domain and is Tsg101 independent, but also GagΔp6, a Gag mutant which completely lacks the viral late domain. Collectively, these findings indicate that citron kinase's ability to enhance HIV-1 virion production is independent of the Tsg101-Gag^{p6} interaction, although it does not rule out the necessity of Tsg101 to recruit other components of the ESCRT complexes.

Citron kinase enhances virion production in a manner capable of circumventing the Tsg101-Gag^{p6} interaction, suggesting that citron kinase acts a scaffold to recruit the necessary factors for viral release. If citron kinase is acting as a scaffolding molecule, it is necessary to identify possible proteins that it interacts with directly. It has already been found, via yeast two-hybrid, that citron kinase binds to Tsg101 (W. Sundquist, personal communication), making citron kinase likely to interact with other components of the endosomal sorting pathway.

Phosphorylation Target(s) for Citron Kinase

We identified a functional interaction between citron kinase and Tsg101 which was dependent on (1) the kinase activity of citron and (2) the PTAP-binding region of Tsg101. We have not identified a potential phosphorylation target for citron kinase. Currently, the only known phosphorylation target for citron kinase is MLC. MLC can also be phosphorylated by myosin light chain kinase (MLCK) and ROCK. It was found that in T cells and monocyte-derived myeloid cells, inhibition of MLCK by wortmannin, an inhibitor of MLCK which inhibits MLC phosphorylation, suppressed HIV-1 release [315]. Even at high levels of wortmannin, viral components localized with myosin at sites of viral budding implying that wortmannin did not disrupt transport of viral proteins to the plasma membrane but rather inhibited budding. These findings suggest that after localization to the plasma membrane, release of viral particles is dependent on the subsequent actinomyosin interactions at the cell surface [315]. It is unlikely that phosphorylation of MLC by citron kinase plays a role in the functional interaction of citron kinase and Tsg101 or the subsequent inhibition of HIV-1 virion production. The previous study found that inhibition of MLC

phosphorylation suppressed HIV-1 release [315], whereas we found citron kinase phosphorylation to be necessary for inhibition, which may correlate with an increase in MLC phosphorylation.

Tyrosine phosphorylation is the most prevalent phosphorylation event in regulating various aspects of endosomal trafficking. Two cellular events normally follow binding of a growth factor to its cell surface receptor; the receptor transmits an intracellular signal, typically initiating a phosphorylation cascade and it becomes internalized by endocytosis. Following endocytosis, the receptor–ligand complex is delivered to an early endosome, from which it may be recycled back to the plasma membrane or transported further along the endocytic pathway to lysosomes for degradation [425]. Coordinated phosphorylation and dephosphorylation events are often necessary to mediate association of proteins and to facilitate vesicle transport [404].

Protein kinase activity plays an important role in endocytosis. Among such kinases, the protein kinase C (PKC) superfamily is responsible for diverse regulatory roles in many cellular processes [426, 427]. PKC inhibition has been shown to affect endocytosis [428] with normal trafficking of EGF requiring PKCβII activity. When protein kinase C βII (PKCβII) was mutated to a phosphorylation deficient form, it specifically blocked EGF receptor trafficking and degradation without affecting transferrin receptor recycling [405]. PKCβII may be a specific regulator of late endosome function with defective PKCβII phosphorylation leading to a nonfunctional late endosome.

Also, Hrs, a hepatocyte growth factor-regulated tyrosine kinase substrate and interacting protein of Tsg101, is phosphorylated by binding of a variety of growth factors and cytokines to their receptors [406]. Since Hrs is localized to endosomes and has been

implicated in the regulation of signal transduction as well as membrane trafficking, it is regarded as a potential coordinator of endosomal receptor sorting and signaling. Although the functional consequence of Hrs phosphorylation is not known, it may be of significance that Hrs phosphorylation is prevented when endocytosis is inhibited [429].

Additionally, PACS-1 (phosphofurin acidic cluster sorting protein-1) is a sorting connector that links furin to the AP-1 clathrin adaptor and is required for localization of furin to the TGN [430, 431]. The phosphorylation state of PACS1 acidic cluster regulates the ability of PACS-1 to bind to and sort cargo proteins to the TGN. Disruption of PACS-1 phosphorylation by a Ser^{278} to Ala substitution results in an interfering mutant that inhibits the PACS-1-directed endosome to TGN sorting suggesting a coordination of signaling events to regulate transport within the TGN/endosomal system through the phosphorylation state of both cargo and sorting machinery [432]. Collectively, these findings indicate the importance of phosphorylation events in regulation of the endosomal sorting pathway.

A second region of HIV-1 Gag^{p6} has been defined to contribute to viral release and it interacts with AIP1, a host protein [246, 247]. AIP1/Alix is an adaptor protein involved in regulating the function of receptor and cytoskeleton-associated tyrosine kinases. AIP1 interacts with Tsg101 and CHMP proteins of ESCRT-III complex, coupling HIV-1 Gag^{p6} to the early and late-acting endosomal sorting complexes and binds directly to HIV-1 Gag^{p6} LRSL [246, 247]. The Tyr^{319} of Alix binds to Src's SH_2 domain leading to AIP1/Alix phosphorylation at the C-terminal tyrosine rich region by Src [407]. Phosphorylation of AIP1/Alix causes translocation from the membrane and cytoskeleton to the cytoplasm and a reduction in its interaction with binding partners SETA/CIN85 [408], EGF [409] and Pyk2 [410]. Src antagonizes the effects of Alix phosphorylation of its C-terminus, leading to

disruption of interaction with target proteins, negatively regulating its biological function [407].

Despite the majority of known interactions linking phosphorylation with trafficking events involve tyrosine phosphorylation; it does not preclude a role for a Ser/Thr kinase in regulation. It is unlikely that citron kinase phosphorylates Tsg101 since there is no evidence to indicate that either Tsg101 or its yeast homologue, Vsp23, was phosphorylated. However, the functional and/or physical (W. Sundquist, personal communication) may be necessary to recruit citron kinase to a complex where it phosphorylates another component, such as AIP1/Alix. It is possible that citron kinase phosphorylates AIP1/Alix, a known component in linking early and late endosomal sorting complexes to HIV-1, thereby inhibiting its function in binding to Tsg101, Gag or CHMP proteins and ability to act as a scaffold protein, leading to inhibition of HIV-1 virion production. Alternatively, citron kinase may phosphorylate an intermediary protein which then phosphorylates AIP1/Alix (or another component of the ESCRT network) or there may be an unidentified phosphorylation target for citron kinase.

Elucidation of a phosphorylation target for citron kinase could potentially explain the mechanism of how citron kinase and Tsg101 interact to mediate HIV-1 virion production. These findings imply an increasing complexity in the interactions of HIV-1 viral proteins with host proteins and host proteins with each other to modulate viral release.

The Disparate Roles of Citron Kinase in Mediating HIV-1 Replication

An interesting finding is the disparate role that citron kinase appears to play in regulating HIV-1 replication (Figure 45). Previously, we identified citron kinase to preferentially enhance HIV-1 virion production by promoting exocytosis. The enhancement

activity of citron kinase required a portion of the leucine zipper, the Rho-binding and zinc-finger domains but not the kinase domain. Citron kinase may act as a scaffold, linking host proteins of the endosomal sorting and exocytic pathways. Additionally, the kinase activity of citron, although not necessary for its enhancement activity, was necessary and sufficient to mediate the functional interaction with Tsg101. Additionally, if the PTAP-binding region of Tsg101 was disrupted, citron kinase and Tsg101 no longer functionally interacted to inhibit HIV-1 virion production. Disruption of Tsg101's PTAP-binding region prevents interaction with Gag^{p6}, AIP1, another component of the ESCRT pathway and homodimerization. The functional interaction between citron kinase and Tsg101 may be involved in mediating interactions with additional host factors through phosphorylation regulation or activation of an already associated factor or to promote the Tsg101-Gag^{p6} interaction. The two activities of citron kinase involved in mediating HIV-1 virion production are distinct and independent of one another.

In the context of wild-type citron kinase, both activities are able to function, therefore how are these seemingly opposing functions balanced, not only by the cell but by the virus? One possibility is that these two activities are temporally regulated, with the enhancement activity of citron kinase occurring first. At early time points in HIV-1 infection, citron kinase would be acting as a scaffolding molecule, linking components of the endosomal sorting pathway to facilitate exocytic secretion of microvesicles and HIV-1 viral particles. The peak of citron kinase-mediated enhancement of virion production occurs at about 32-40 hours post-transfection (Figure 46). After that point, citron kinase loses its ability to enhance virion production and at later time points (greater than 48 hours post-transfection), appears to slightly inhibit virion production. This inhibition at later time points may be due to

functional interaction of citron kinase with Tsg101 and a phosphorylation event that facilitates in slowing down viral release, perhaps as a means of keeping virion production under control. Citron kinase may enhance viral secretion so significantly, that the cell becomes overwhelmed and/or overworked. Both the cell and the virus ideally strive to maintain a balance between virion production and health of the cell. If virion production outpaces the ability of the cell to translate a necessary protein, production would be inhibited.

The second possibility involves the protein levels/accumulation of citron kinase. Citron kinase has a very slow turnover rate, as implied from our time course experiment (Figure 46) – we see an increased accumulation of citron kinase protein over time. At late time points (48 hours post-transfection), we saw high levels of citron kinase overexpression and a slight inhibitive effect on virion production (Figure 41C-D, Figure 46). When analyzing the dose-dependent response of citron kinase overexpression on HIV-1 virion production, we found that citron kinase protein levels were increased proportionately to the amount of citron kinase transfected (Figure 41D). At very high level of citron kinase expression, virion production was not enhanced (Figure 41C). These findings suggest that high levels of citron kinase are inhibitory to its enhancement activity. Additionally, when we coexpress Tsg101 and citron kinase, we see an upregulation of citron kinase expression, compared to when an equal amount of citron kinase is transfected in the absence of exogenous Tsg101 (Figure 41F). Again, this suggests that high levels of citron kinase are inhibitory to its enhancement activity. Perhaps citron kinase is upregulated to control virion production or because the phosphorylation activity of citron kinase requires higher protein expression levels.

Regardless, it is possible that citron kinase's two distinct functions are temporally regulated and are ultimately controlled by the amount of citron kinase that has accumulated within the cell. Further analysis will have to be completed to determine if this is, in fact, a correct mechanism.

CHAPTER SEVEN:
PERSPECTIVE ON RHOA ACTIVITIES AND HIV-1 REGULATION

The HIV-1 life cycle involves regulation on many levels by both viral and host factors. The cellular changes essential for a productive HIV-1 viral infection require coordinated regulation of processes such as membrane trafficking, actin cytoskeleton, and activation of transcription. RhoGTPases are critical regulators of actin dynamics and have been linked to control of various endocytic pathways. Additionally, the Rho family of proteins have been implicated in cell shape regulation, cell adhesion, cell division and actin cytoskeleton reorganization, most notably the formation of stress fibers [122-128]. RhoGTPases are known to be involved in a diverse array of cellular processes and are poised to be used by the virus to facilitate its replicative cycle.

We identified two RhoA effectors, ROCK and citron kinase to be involved in regulation of HIV-1 replication. Although we have yet to identify the mechanisms used by these two effectors, we have linked RhoA signaling pathways to regulate more than one step in the HIV-1 life cycle. ROCK specifically inhibited HIV-1 gene expression in T cells. A ROCK specific inhibitor, Y27632, counteracted RhoA inhibition of HIV-1 gene expression suggesting that RhoA signals through ROCK to regulate HIV-1 replication. Citron kinase, however, preferentially enhanced HIV-1 virion production by promoting exocytosis. This activity was found to be HIV-1 late domain independent, but still required an intact late endosomal sorting pathway. Additionally, we found a functional interaction between citron kinase and Tsg101 that inhibited HIV-1 virion production. The kinase activity of citron

kinase was necessary and sufficient for this interaction. Disruption of the PTAP-binding region of Tsg101, using a Tsg101 mutant that is defective in Gag^{p6} binding, interaction with AIP1 and homodimerization, abrogated the functional interaction between citron kinase and Tsg101.

It is interesting that two different RhoA effectors effect HIV-1 replication in two distinct fashions. A primary function of RhoA signaling pathways is to regulate the actin cytoskeleton. ROCK promotes the formation of stress fibers and focal adhesion complexes, which are dependent on both the kinase domain and kinase activity [359]. A kinase-defective mutant was unable to induce focal adhesions and stress fibers [329].

In our study, the kinase-defective ROCK mutant did not affect HIV-1 gene expression (Figure 19A, B), suggesting a role for its kinase activity in mediating HIV-1 replication. One possibility for why ROCK inhibits HIV-1 viral release at very low levels may be due to the disruption of the actin cytoskeleton. The actin cytoskeleton is an important component in the regulation of vesicle transport. In the case of constitutively active ROCK there is an induction of stress fiber formation that results in thicker bundles than when ROCK is not ectopically expressed. These thicker stress fiber bundles could inhibit vesicle or protein transport from the nucleus or other cytoplasmic compartment to the plasma membrane where viral budding is believed to occur in T cells. The addition of a ROCK specific inhibitor, Y27632, abrogated RhoA-mediated inhibition of HIV-1 gene expression through ROCK (Figure 20). The inhibitor, Y27632, has been shown to prevent stress fiber bundling and focal adhesion assembly [329, 359, 366, 433]. The loss of RhoA-mediated inhibition of HIV-1 gene expression when a ROCK specific inhibitor is added,

suggests that the integrity of the actin cytoskeleton is an important component of HIV-1 replication modulation.

Citron N, a naturally occurring splice variant lacking the N-terminal Ser/Thr kinase domain, is enriched and associated with the Golgi apparatus of hippocampal neurons in culture [396]. Suppression of citron N led to the dispersion of the Golgi apparatus [396] suggesting that citron N functions as a scaffolding molecule on Golgi membranes, organizing Rho-mediated actin polymerization locally by assembling the actin polymerizing complexes together (ROCK II and profilin-IIa) [396]. Therefore, citron N may play a role in organization of the endosomal sorting pathway and regulation of the actin cytoskeleton to facilitate exosome and virion release.

Additionally, when we added an actin depolymerizing agent, latrunculin B, in cells overexpressing citron kinase, we were able to prevent the enhancement of virion production by citron kinase (Figure 47). Latrunculin B induces shortened and thicker stress fiber bundles to form that inhibit vesicle transport. We believe that citron kinase enhances HIV-1 virion production by promoting exocytosis therefore it is not surprising that disrupting actin cytoskeleton and inhibition of vesicle transport would prevent citron kinase from enhancing viral production since actin plays a key role in controlling vesicle transport. This finding also suggests that citron kinase uses a novel pathway (because latrunculin B did not disrupt viral release in the absence of citron kinase overexpression) – one that is not used or seldom used in 293T cells, perhaps using components of the endosomal sorting pathway in their native location instead of at the plasma membrane. It is equally possible that the addition of latrunculin B prevents citron kinase from organizing Rho-mediated actin polymerization

locally or inhibits the ability of citron kinase to function as a scaffolding molecule, thereby abrogating the enhancement of HIV-1 production by citron kinase.

Despite two very distinct functions for ROCK and citron kinase in regulating HIV-1 replication, it is likely that these functions are linked through regulation of the actin cytoskeleton by RhoA. Understanding the mechanisms RhoA signaling pathways employ to modulate HIV-1 replication will most likely link to its ability to regulate the actin cytoskeleton and will provide an increasingly clear picture of the complex viral and host protein interactions necessary to have a productive viral infection.

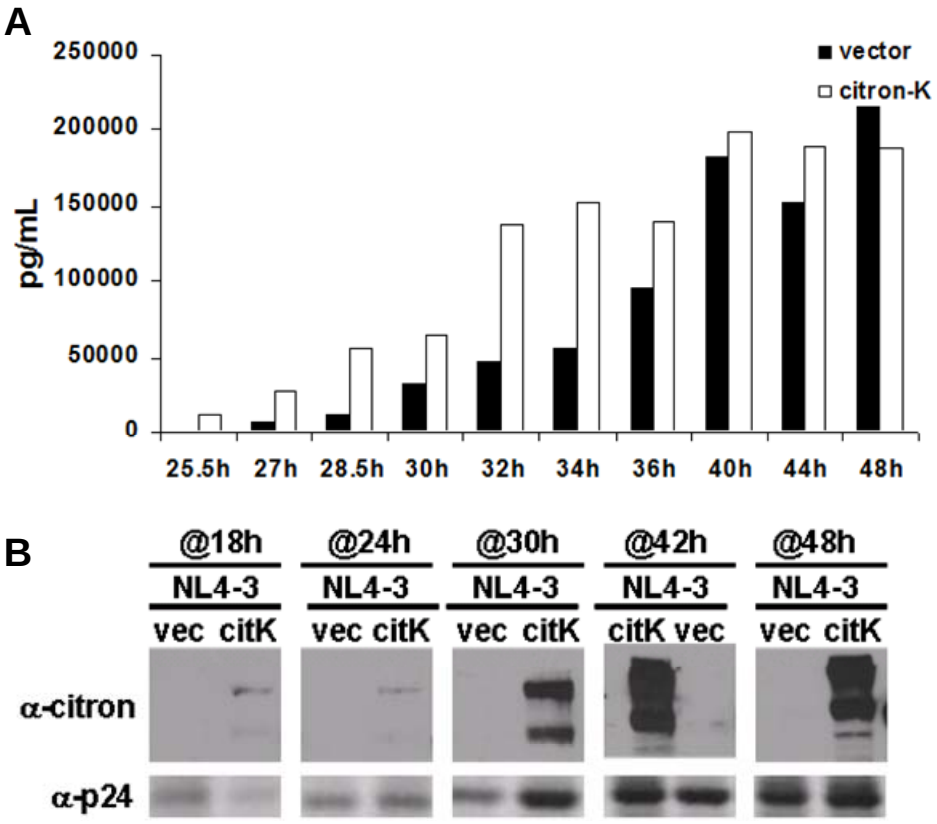

Figure 46: HIV-1 Virion Production Over Time in the Presence of Citron Kinase. (A, B) 293T cells were cotransfected with pNL4-3 (0.1 μg) and vector or citron kinase (0.2 μg) in a 48-well plate at $2.5x10^4$ cells/well. Supernatants and cells were collected at various time points post-transfection to analyze viral production by p24 ELISA (A) and cell-associated protens (B) by α-citron and α-p24 antibodies.

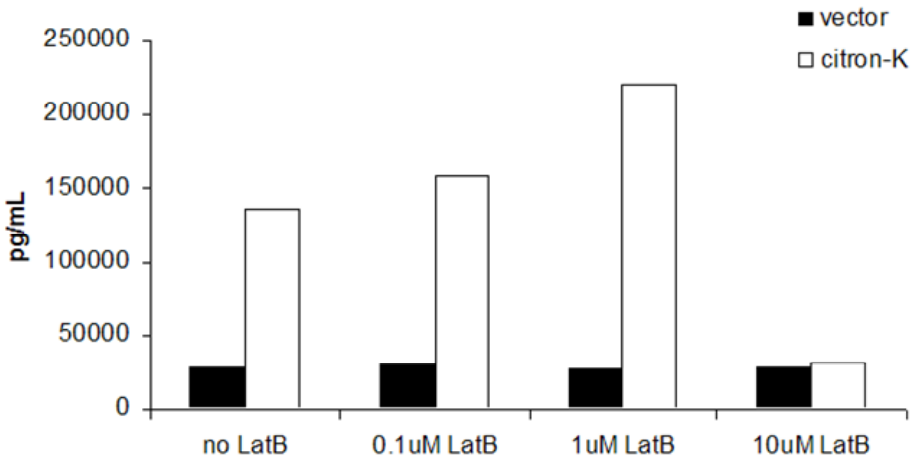

Figure 47: Disruption of the Actin Cytoskeleton with Latrunculin B Inhibitor Prevented Citron Kinase from Enhancing Virion Production. 293T cells were cotransfected with pNL4-3 (0.1 μg) and vector or citron kinase (0.2 μg) in a 48-well plate at $2.5x10^4$ cells/well. Various amounts of latrunculin B, an inhibitor of actin polymerizion and disrupter of microfilament organization, was added at 0μM, 0.1μM, 1μM or 10μM at 36 hours post-transfection. Supernatants were collected at 40 hours post-transfection and analyzed for viral production by p24 ELISA.

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