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Characterization of Binding and Fusion Efficiencies Mediated by the V1-V5 Env Derived from Transmitted and Non-transmitted Viruses Isolated from a Perinatal Transmission Cohort from Zambia

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**CHARACTERIZATION OF BINDING AND FUSION EFFICIENCIES
MEDIATED BY THE V1-V5 REGION OF HIV-1 ENV DERIVED FROM
TRANSMITTED AND NON-TRANSMITTED VIRUSES ISOLATED FROM A
PERINATAL TRANSMISSION COHORT FROM ZAMBIA**

by

Mackenzie Waltke

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University of Nebraska, 2012

Advisor: Charles Wood

Abstract: Human Immunodeficiency Virus Type 1 (HIV-1) is the etiological agent of acquired immunodeficiency syndrome (AIDS), which affects over 34 million people worldwide. In sub-Saharan Africa where access to antiretroviral therapies (ART) is limited, mother-to-child transmission (MTCT) rates remain high and represent a major concern in the global HIV/AIDS epidemic. Little is known about the biological properties of viruses that are transmitted perinatally, including how the biological functions of envelope (Env) influence transmissibility. Previously, transmitted viruses were found to have an advantage in replicative fitness mediated by Env V1-V5. In this study viruses derived from transmitting mother infant pairs (MIPs) were used to determine if the binding and fusion activities are influenced by Env V1-V5 and if any differences correlate to replicative fitness and transmission. Fusion and binding assays were used to measure the biological functions of Env from individual clones of five MIP. **RESULTS:** Neither binding nor fusion is predictive of transmission. However, clonal variation in both functions is observed, demonstrating V1-V5 is capable of influencing fusion and binding. Fusion correlates with infectivity, but does not correlate with binding. The

results of this study have provided new insights to better understand the functional properties of Env and its role in MTCT.

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Chapter 1: Literature Review

Overview of HIV-1 and the Global AIDS epidemic

Human immunodeficiency virus (HIV) is the etiological agent of acquired immunodeficiency syndrome (AIDS). The United Nations Program on AIDS (UNAIDS) estimated that as of 2010 there were 34 million people living with AIDS. Due to successes in increasing access to antiretroviral therapies (ART) worldwide, both the number of new HIV infections and AIDS-related deaths have decreased over the last decade. Another success has been an increase in access to HIV testing and counseling. However, testing in low- and middle-income countries still remains problematic. Women and children remain under-served in terms of testing and access to ART. Women also remain more affected by HIV than men in sub-Saharan Africa. Several other populations, for example men who have sex with men, remain also underserved. Low- and middle-income countries with HIV/AIDS epidemics have made progress in increasing the availability of ART, but ultimately remain insufficient. Moving forward in the effort to combat the global HIV/AIDS epidemic, early detection of infection and early access to ART remained vital [WHO, 2011]. HIV-1 is a lentivirus within the retroviridae family. The HIV-1 genome has three major genes (gag, pol, and env) and a number of accessory genes. HIV-1 infects primarily CD4+ T cells and monocyte/macrophage lineage cells. Viral entry into host cells is initiated by the engagement of the receptor, CD4, and the coreceptor, CCR5 or CXCR4, by the envelope glycoprotein (Env) spike, a heterotrimer of three glycoprotein 120 molecules (gp120) bound to three transmembrane glycoprotein

41s (gp41). Following the binding events a conformational change induces fusion of the viral and cellular membranes that allows the HIV-1 core to be delivered to the cytoplasm. Once in the cytosol, the viral RNA is reverse transcribed into double-stranded DNA. The viral DNA is translocated into the nucleus and integrated into the host cell chromosomal DNA. This integrated DNA drives the transcription of viral RNA which is then transported to the cytoplasm to be translated. Newly synthesized viral proteins and two copies of the full length genomic RNA are assembled into new viral particles. As these particles are released, cleavage of the viral protein into the mature form takes place to generate the mature virions capable of initiating new infection.

Transmission of HIV-1 occurs through sexual and non-sexual routes. Sexual transmission remains the major route of HIV-1 transmission in sub-Saharan Africa and globally [WHO, 2011]. Both heterosexual and homosexual transmission across mucosal epithelial cells is common. Many factors contribute to the risk of sexual transmission including the disease status of the transmitting partner, the susceptibility of the recipient partner, the number exposures, the type of sexual act, and characteristics of the transmitted virus. Viral load of the index partner is a major risk factor in sexual transmission. Viral load in genital secretions is also a determinant during sexual transmission. Often viral levels in both the female genital tract and in semen correlate with plasma viral loads. Acute infection, late-stage disease, and other infections are all factors that can increase HIV-1 concentrations in the blood and are risk factors for sexual transmission. The presence of STIs or other conditions that are capable of disrupting the mucosal layer in the genital tract that leads to an increase in sexual transmission [Galvin and Cohen, 2004].

Mother to child transmission and the HIV/AIDS Epidemic

Another major route of HIV transmission is perinatal transmission. UNAIDS estimated in 2008 that there were over two million children living with HIV. Approximately 90% of these children were infected during pregnancy, birth, or through breastfeeding [Ahmad, 2000; UNAIDS, 2008]. Recent programs to prevent mother to child transmission (MTCT) as well as access to ART have achieved a measure of success at limiting new child infections of HIV and child deaths due to AIDS [WHO, 2011]. However, as of 2010 there were still 390,000 new HIV infections in children under 15, only 35% of pregnant women were tested for HIV, and 48% of HIV positive pregnant women were receiving some form of antiretroviral treatment. A majority of these new pediatric infections occurred in sub-Saharan Africa [WHO, 2011]. In countries with the highest HIV prevalence, it is the underlying cause of one-third of the deaths of children under the age of five [Bennett and Rogers, 1991; WHO, 2011]. In many developing countries and in the absence of ART, perinatal transmission rates remain between 30-45% [Bulterys and Lepage, 1998; Lathey et al, 1999]; this is of particular concern in sub-Saharan Africa where access to ART is still limited and HIV-1 infection is epidemic [Cooper et al, 2002]. MTCT remains a major concern in the global fight against HIV/AIDS.

Routes and risk factors for perinatal transmission

HIV perinatal transmission occurs through three routes; intrauterine, intrapartum, and during breast feeding. However, these routes are hard to distinguish and mechanisms underlying perinatal are not well-understood. It is likely that interplay between host and viral factors will play important roles in perinatal transmission. Maternal parameters that

could influence MTCT include advanced clinical stages of the disease, a low CD4+ lymphocyte count, a high level of circulating HIV-1, and disease progression. Also, maternal immune response to HIV-1 and recent infection have been implicated as risk factors for perinatal transmission [Adjorlolo-Johnson et al, 1994; Newell, 1995; Ryder et al, 1989; Study, 1994]. Studies indicate that an elevated viral load in the mother plays a role in MTCT [Cao et al, 1997; Garcia et al, 1999; Newell and Peckham, 1993]. Acute infection during pregnancy, the presence of sexually transmitted diseases or other chronic infections, and the disruption of placental integrity are also associated with perinatal transmission [Newell, 1993; Report, 1992]. Some studies provide evidence that vitamin A deficiency, malnutrition that lead to immunodeficiency, and disruption of mucosal integrity, are all associated with an increased risk of perinatal transmission of HIV-1 [Semba, 1997].

Intrauterine transmission

It is often difficult to distinguish between the routes of perinatal transmission; while some studies point to specific risk factors associated with each route, these results still remain controversial. A number of studies have shown that HIV is capable of infecting the placenta at all stages of pregnancy. HIV-1 has been isolated from aborted fetuses as early as 8 weeks of gestation, indicating that early in *utero* transmission also takes place [Lewis, 1990]; however, the risk of placental infection appears to be more likely during late pregnancy. Placental disruption, secondary to chorioamnionitis or smoking, has been associated with increased risk of transmission [Newell, 1995]. Generally, diagnosis of in *utero* infection involves HIV-1 that can be detected by virus culture or PCR in peripheral blood within 48 hours of birth [Lewis, 1990]. Intrauterine

transmission could also occur during late pregnancy, and HIV-1 may not be detectable in infant plasma for several days to a week after birth, and therefore a negative PCR test at birth cannot exclude *in utero* transmission [Dunn et al, 2000].

Intrapartum transmission

Many factors important in intrauterine transmission can also increase the risk for intrapartum transmission. They include maternal plasma viral load, lack of antiretroviral use during pregnancy, and ascending infections from the vagina/cervix to fetal membranes and amniotic fluid [Newell, 1995]. HIV has been cultured from cellular and cell-free fractions of vaginal and endocervical secretions. There is some evidence that the excretion of HIV in these secretions is higher in pregnant women [UNAIDS, 2008], thus enhancing the risk of intrapartum transmission. Overall, intrapartum transmission rates range from ~10-20% and transmission during delivery accounts for ~2/3 of infant infections. Obstetrical considerations, including mode of delivery, may alter the duration of ruptured membranes and was shown to have an effect on the risk of HIV transmission [Newell and Peckham, 1993]. Unique risk factors influencing transmission during the intrapartum period include genital tract HIV-1 levels, genital ulcer disease, delivery complications, and breaks in the placental barrier. Another recently reported risk factor during delivery is placental microtransfusions, which were found to be associated with increased intrapartum but not in *in utero* transmission [Kwiek et al, 2006].

Transmission during breastfeeding

Infection via breastfeeding is difficult to determine closely following parturition, because it is hard to distinguish breast milk transmission from late intrauterine or intrapartum infections. It is estimated that as many as one-third to one-half of the infants

infected with HIV-1 were infected via breast milk [UNAIDS, 2008]. In countries where alternatives to breastfeeding are not available, overall transmission risk increases to 30-45% [Rousseau et al, 2004; Thorne and Newell, 2003]. A meta-analysis of nine cohorts of HIV-1 infected breastfeeding mothers reported that infant infections attributable to breastfeeding during the first month following birth was 24~42% [Coutsoudis et al, 2004]. Two of the most important determinants of breast milk transmission risk are duration of breastfeeding and HIV-1 viral levels in breast milk. In general, breast milk HIV-1 RNA concentrations are 2-3 log₁₀ lower than levels in plasma, ranging from undetectable to greater than 10⁵ log₁₀ copies/ml, and are highly correlated with plasma HIV-1 viral load [Rousseau et al, 2004]. Local inflammation resulting from mastitis, breast abscess, or other pathology may increase breast milk virus levels and the risk of transmission [John et al, 2001]. It is still unclear if transmission occurs through HIV infected cells in breast milk or through cell-free HIV in breast milk. Chronic or acute maternal infection does not appear to play a role in the risk of transmitting the virus to offspring via breastfeeding [Newell, 1995].

Host genetic factors

Maternal and infant genetic factors also play a role in MTCT. Human leukocyte antigen (HLA) concordance between the mother and infant increases the risk of transmission [Mackelprang et al, 2008; Polycarpou et al, 2002]. Certain variations in HLA have been shown to be associated with MTCT. Mothers with certain HLA- B (1302, 3501, 3503, 4402, 5001) variants were shown to transmit to their children even with low viral loads. Mothers with HLA-B 4901 and 5301 showed a lower risk of transmission even with high viral loads [Winchester et al, 2004]. Another study found

that mother-infant pairs that were discordant for HLA-G variants 3743 C/T, 634 C/G, and 714insG/G had a decreased risk for transmission [Aikhionbare et al, 2006].

The structure and function of CCR5 in both mothers and infants also influences MTCT. Modifications of CCR5, such as a 32-bp deletion from the coding region ($\Delta 32$) provide protection from infection. Individuals homozygous for $\Delta 32$ have almost complete protection from infection. Some studies support protection from MTCT in heterozygous infants [Kostrikis et al, 1999], while others do not [Misrahi et al, 1998]. The chemokine CCL3 is a natural ligand for CCR5 and acts as a potent agonist against HIV-1. CCL3 produced by both infants and mothers affects transmission. Infants that produce less CCL3 were more susceptible to HIV-1 infection than infants that were exposed to HIV-1 and produced high amounts of CCL3. Similarly, mothers that produced less CCL3 were more likely to transmit [Meddows-Taylor et al, 2006].

Up regulation of CCR5 in the placenta may be related to transmission. Behbahani et. al. [2000] found a four-fold increase in the ratio of CCR5:CXCR4 expression in the placenta from transmitting mothers compared to non-transmitting mothers. Studies carried out with a MTCT cohort from Malawi showed that polymorphisms in the CCR5 gene were related to an increase in CCR5 expression in the placenta; however, they were unable to show that this increase in expression led to an increase in transmission [Joubert et al, 2010]. At low maternal viral loads, SNP in CCR5 (2459G and 2135T) were associated with lower CCR5 expression in the placenta and provided some protection against transmission [Pedersen et al, 2007].

HIV-1 Env synthesis, structure, and function

While host genetic factors may contribute to perinatal transmission, there is also evidence that viral factors may play a key role. Env is important in both infection and transmission and is likely an important viral factor in perinatal transmission. An understanding of the structure, function, and genetic diversity of Env is important in determining its role in MTCT.

The Env glycoproteins are synthesized as a precursor glycoprotein (gp160) on the rough endoplasmic reticulum (RER) [Hunter and Swanstrom, 1990; Freed et al, 1995]. While still on the ER, gp160 is modified by glycosylation with primarily N-linked oligosaccharide side chains. Oligomerization of gp160 into trimers also occurs in the ER and is thought to facilitate trafficking to the Golgi complex. Further modification to the oligosaccharide side chains continues as gp160 traffics through the trans-Golgi network [Leonard 1990].

Env gp160 is proteolytically cleaved by cellular proteases at a highly conserved K/R-X-K/RR motif in the Golgi [Freed et al, 1989]. This cleavage, which yields the surface subunit gp120 and the transmembrane subunit gp41, is essential for viral infectivity. Three molecules of gp120 and three molecules of gp41 form a heterotrimeric spike. Relatively weak noncovalent interactions maintain the association between gp120 and gp41 [Zhu et al, 2003]. The spike proteins are trafficked to the plasma membrane. It is thought that two factors contribute to the low cell surface expression and virion incorporation of the Env proteins. First, Env is rapidly recycled via endocytosis from the PM [Egan et al, 1996; Rowell et al, 1995]. Second, the weak interactions between gp120 and gp41 allow for shedding of gp120. The resultant low expression of the glycoproteins is thought to contribute to immune evasion by HIV-1.

gp120

Based on high genetic diversity, gp120 is organized into five variable regions (V1-V5) separated by five more constant regions (C1-C5) [Starcich et al, 1986; Willey et al, 1986]. Within these regions, the V1/V2 loop has the most diversity in length and glycosylation. The V3 loop as well as C2, C3, and C4 have the least variation in length. It is likely that variation in the loop length, particularly V1/V2, plays a role in immune evasion by masking antibody binding sites [Chohan et al, 2005; Kitrinis et al, 2003; Masciotra et al, 2002; Palmer et al, 1996; Sagar et al, 2006; Shioda et al, 1997]. The major role of gp120 is binding of the virion to the target cell. Both the CD4 and CCR5/CXCR4 binding sites are located within gp120. CD4 binding is determined by the tertiary structure, where discontinuous conserved residues found in C1, C3, and C4 are brought into proximity [Lasky et al, 1987; Kowalski et al, 1987; Olshevsky et al, 1987]. The coreceptor binding site is located in the V3 loop [Cann et al, 1992; Chesebro et al, 1991; Hwang et al, 1991; O'Brien et al, 1990; Shioda et al, 1991]. Specific mutations within V3 facilitate the switch from CCR5 to CXCR4 usage [Fouchier et al, 1992; Pollakis et al, 2004]. V3 also plays a role in membrane fusion [Freed et al, 1991] and contains epitopes for neutralizing antibodies [Goudsmit et al, 1988; Matsushita et al, 1988; Palker et al, 1988; Rusche et al, 1988]. Binding to CD4 produces a large number of conformational changes within the Env that exposes the R5/ X4 binding sites. Thus, the unbound form of Env is able to mask the vulnerable V3 region until coreceptor binding occurs [Chen et al, 2005].

Tertiary structure is also highly influenced by glycosylation and a number of conserved Cysteine residues capable of forming disulfide bonds [Leonard et al, 1990].

The gp120 is primarily modified with N-linked glycans [Allan et al, 1985] with a smaller number of O-linked glycans [Bernstein et al, 1994]. There are approximately 20-35 potential N-glycosylation sites (PNGS) in gp120. Glycosylation plays a role in protein folding, immune evasion, and virion binding [Montefiori et al, 1988; Li et al, 2008].

Proper tertiary structure is also dependent on the formation of disulfide bridges.

Generally, 18 Cys residues form 9 disulfide bridges. The V1 and V2 loops are separated by two disulfide bridges and contained within a larger loop formed by another disulfide bond. The V3 and V4 loops are also formed by disulfide bonds. Disruption of these bonds changes Env structure and may have effects on viral properties [Gao et al, 1996; Jobes et al, 2006].

gp41

As compared to gp120, gp41 is more conserved and arranged into three functional domains: the extracellular domain, the transmembrane domain (TMD), and the C-terminal cytoplasmic tail (CT). The major function of the gp41 subunit is to mediate fusion and the major fusion determinants are contained within the extracellular domain [Bosch et al, 1989; Freed et al, 1990; Freed et al, 1992]. This domain has the N-terminus fusion peptide that is hydrophobic as well as two hydrophobic heptad repeat regions (HR1 and HR2) [Chan et al, 1997; Dubay et al, 1992; Lu et al, 1995; Tan et al, 1997; Weissenham et al, 1997], a polar region, and a membrane-proximal external region (MPER) [Munoz-Barroso et al, 1999; Salzwedel et al, 1999]. Fusion is primarily driven by interactions between the HR regions and exposure of the fusion peptide. Upon binding of CD4 and the coreceptor, a conformational change is induced that exposes the fusion peptide. The exposed peptide is able to penetrate the cellular membrane of target cells,

causing destabilization of the membrane and leading to the formation of the fusion pore [Brasseur et al, 1990]. Next a stable six-helix bundle is formed by the interaction of 3 HR1 motifs and 3 HR2 motifs and brings the cellular and viral membranes together for fusion to occur. To form the six-helix bundle, the HR1 motifs form a core bundle and fold over a hydrophobic groove with the HR2 motifs [Chan et al, 1997; Weissenham et al, 1997]. HR1 and HR2 are both targets for antiretrovirals; peptides-derived from either heptad repeat are able to disrupt the formation of the six-helix bundle and prevent fusion [Furuta et al, 1998; Kilby et al, 1998; Wild et al, 1994].

In addition to the fusion peptide, the MPER is also found within the extracellular domain and is involved in promoting fusion. It has been shown to be necessary for infectivity and fusogenicity, but the mechanism underlying the actions is unclear [Munoz-Barroso et al, 1999; Salzwedel et al, 1999]. It is known that this region is highly conserved and is a target of neutralizing antibodies [Cao et al, 1993; Zwick 2001].

A second domain in gp41, the TMD, anchors Env in the lipid bilayer and consists of approximately 25 highly conserved amino acids. The TMD is necessary for Env function and mutations within this domain have been shown to interrupt fusion [Shang et al, 2008; Shang and Hunter, 2010; Kondo et al, 2010]. The traditional model for gp41 topology suggests that the TMD forms an alpha helix that spans the membrane one time. With this scenario the entire CT would be contained within the virion. However, another model suggests gp41 spans the membrane three times exposing portions of the CT to the extracellular space [Kennedy et al, 1986; Steckbeck et al, 2010]. Current evidence favors the single pass model, but both models may exist [Hollier and Dimmock, 2005].

The third functional domain, the CT has multiple functions. Like other lentiviruses HIV-1, HIV-2, and SIV have a long CT when compared to other retroviruses [Rushlow et al, 1986; Rice et al, 1990]. The length of the CT contributes to its function as it has been shown that truncations in the CT of SIV suppress viral replication in rhesus macaques [Shacklett et al, 2000]. The HIV-1 CT has an impact on several properties of Env. Viral infectivity, Env incorporation into virions, gp120 shedding, Env cell-surface expression, gp120 and gp41 conformation, and fusion are all influenced by the CT. Evidence that the CT plays a role in modulating gp120 conformation is that mutations in the CT affect antibody recognition and neutralization, and truncations in the CT has been associated with CD4 independence [Edwards et al, 2001; Edwards et al, 2002; Kalia et al, 2005].

There is also evidence that potential interactions between gp120 and gp41 affect fusogenicity. Wang et. al. [2011] found that mutations that conferred resistance to a fusion inhibitor targeting HR1 clustered in both gp41 and gp120. Resistance was mapped primarily to the gp41 mutations, but the gp120 mutations were capable of enhancing fusion and this was related to the ability to utilize CD4 and CCR5. Another study found mutations in the gp41 ectodomain that produce an unstable gp120-gp41 complex correlate to a decrease in fusion function [Maerz et al, 2001]. Additionally, viruses that contain adaptive mutations in gp120 that allowed for usage of mutant forms of CCR5 were capable of fusing at a quicker rate due to reduced constraints holding gp41 [Platt et al, 2007]. Given this evidence it is reasonable to suggest that differences in gp120 may play a role in Env-mediated fusion.

Characteristics of HIV Env in perinatal transmission

Several studies have highlighted the importance of Env in MTCT. The ability of variants to use CCR5 may play a role in transmission; newly transmitted viruses from a subtype C mother-infant-pair (MIP) MTCT cohort [Zhang et al, 2002] and a subtype A MIP cohort [Rainwater et al, 2007] were found to use CCR5 exclusively. Even minor variants with CCR5 phenotypes are selected for transmission in HIV-1 infected mothers during perinatal transmission and/or are amplified after transmission [Matala et al, 2001].

Previous studies have demonstrated that, despite a complex viral population in the mother, only viruses of a restricted subset were typically transmitted to the infant [Ahmad et al, 1995; Contag et al, 1997; Lamers et al, 1993; Mulder-Kampinga et al, 1995; Samleerat et al, 2008; Scarlatti et al, 1993a; Scarlatti et al, 1993b; Scarlatti et al, 1993c; Verhofstede et al, 2003; Wolinsky et al, 1992; Zhang et al, 2002]. Controversy in the field of pediatric HIV-1 infection has involved whether selective transmission of a specific, homogeneous maternal viral population to the infant occurs *in utero* or intrapartum; studies have failed to pinpoint a specific timing for transmission [Contag et al, 1997; Mulder-Kampinga et al, 1995]. In some cases the transmitted strain was a minor variant of the maternal viral pool, and was due to selective pressures against V3 (Ahmad et al, 1995; Wolinsky et al, 1992). Despite evidence of restrictive transmission as a characteristic of MTCT, several other groups have also reported a more random pattern of transmission, including of multiple and/or major maternal HIV-1 genotypes from mother to infant [Dickover et al, 2001; Lamers et al, 1994; Pasquier et al, 1998]. Mothers acutely infected with HIV-1 that underwent near simultaneous seroconversion with their infants transmitted a number of closely related viral variants [Hoffmann et al, 2008].

Similarly, mothers with primary infection during pregnancy showed a random pattern of transmission [Ceballos et al, 2008].

One source of selective pressure in perinatal transmission of HIV-1 is maternal antibodies, which could play a role in limiting transmission of neutralization sensitive variants. Studies have shown that nontransmitting mothers had more frequently detectable and/or higher levels of neutralizing antibody responses than transmitting mothers [Bongertz et al, 2002; Guevara et al, 2002; Lathey et al, 1999]. It has also been suggested that the virus variants more resistant to neutralization are preferentially transmitted perinatally [Scarlatti et al, 1993b]. A recent study found that variants from infants were more resistant to neutralization by maternal plasma than the overall maternal virus population [Rainwater et al, 2007; Wu et al, 2006]. Another study found that newly transmitted viruses from a cohort of subtype C MIP were more resistant to neutralization by paired maternal plasma than contemporaneous maternal plasma and more resistant to IgG b12, but were sensitive to pooled plasma [Zhang et al, 2010b]. In contrast, others have shown no notable differences between mother and infant viruses in sensitivity to heterologous plasma or the antibodies 2G12 and b12. A direct association between maternal antibodies against the V3 domain of the Env and a lower rate of transmission of HIV-1 has been suggested by some studies [Y Devash et al, 1990; Wu et al, 2006], but others have not found a correlation [Halsey et al, 1992; Parekh et al, 1991].

The number and location of PNGS may favor certain variants for transmission. Several studies have shown newly transmitted viruses have shorter variable loops and fewer PNGS [Russell et al, 2011; Wu et al, 2006; Zhang et al, 2010b]; however, others do not observe this trend [Kishko et al, 2011; Samleerat et al, 2008; Thenin et al, 2009]. A

study of a cohort in Thailand examined the gp120 sequences from variants of CRF01_AE clade and found that the number of PNGS was not related to transmission. However, PNGS at positions N301 and N384 were conserved among all infant sequences and variable among mother sequences, suggesting a potential role in transmission [Samleerat et al, 2008]. Another study examined the V1-V5 and PNGS at N339 was found at a higher frequency in transmitting mothers than non-transmitting mothers. They also found that PNGS at N295 was in lower frequency in children than mothers [Baan et al, 2011]. The role of PNGS in MTCT is likely subtype specific.

Despite evidence that Env is playing a key role in MTCT, the functional role of Env in MTCT remains controversial. Neither Env processing nor incorporation into virions was found to predict transmission [Zhang et al, 2010b; Thenin et al, 2009]. Viral entry, which is dependent on Env, did not differ between mother and infant variants as measured by the response to entry inhibitors such as soluble CD4, PSC-Rantes, and TAK779 [Rainwater et al, 2007; Thenin et al, 2009]. However, replicative fitness mediated by V1-V5 was shown to be higher in infant viruses from chronically infected MIP [Kong et al, 2008]. The role of the biological functions of Env, such as binding and fusion, during MTCT still remains unclear and provides an avenue for further inquiry.

Objective

Based on the previous data that demonstrated a role for V1-V5 in the higher replication fitness of transmitted viruses [Kong et al, 2008], our overall objective was to determine if this region had an effect on other biological properties of Env including fusion and binding. Our specific aims were:

- 1) To determine if V1-V5 is capable of influencing fusion and if any differences that might exist correlate to replicative fitness and predict transmission. This was carried out by using a fluorescent resonance energy transfer (FRET) based virus-to-cell fusion assay to measure the fusion efficiency in a transmitting MIP cohort from Zambia, and determined if any differences in fusion efficiency correlated to transmission.
- 2) To determine if V1-V5 is capable of influencing CD4 binding and if any differences that might exist correlate to replicative fitness and predict transmission. This was carried out by a virus-to-cell binding assay to measure the binding efficiency of transmitting MIP from Zambia. We also correlated any differences to fusion and transmission.

Our results demonstrated a positive correlation between fusion and infectivity, and there were variations between different clones in binding and fusion efficiencies. This variation suggests that differences in fusion and binding exist in a clone-to-clone manner, and are likely to be influenced by the V1-V5 region of Env. However, there was no correlation between binding and fusion activities of various Env clones and variations in fusion and binding among clones are not predictive of their ability to transmit from mother to infant.

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Chapter 2: Virus to cell fusion of HIV-1 is influenced by envelope V1-V5, but does not predict transmission.

Introduction

Despite recent efforts and successes in reducing the incidence of perinatal transmission of HIV-1, MTCT still remains the dominant source of pediatric HIV-1 infections and presents a serious concern in countries with the highest HIV prevalence. In these countries HIV-1 is the underlying cause of death in one-third of childhood mortalities for children under five years of age [Bennett and Rogers, 1991; UNAIDS, 2008]. In the absence of ART, perinatal transmission rates remain between 30-45% in many developing countries [Bulterys et al, 1998; Lathey et al, 1999]. In sub-Saharan Africa, where HIV-1 infection is epidemic and access to ART is limited, perinatal transmission remains a major pediatric concern [Cooper et al, 2002]. Since prevention of MTCT remains a key goal in the global fight against HIV/AIDS, a better understanding of the possible mechanisms underlying MTCT, including functional properties of transmitted viruses, remains critical.

Many of the studies investigating transmitted viruses from mother-infant-pair (MIP) cohorts are contradictory. In general, restrictive transmission of a small viral subset appears to be a hallmark of MTCT in mothers chronically infected with HIV-1. In some cases where infection in the infant was established by a minor variant of the maternal viral pool; selection for the minor variant is attributable to pressures against V3

[Ahmad et al, 1995; Wolinsky et al, 1992]. Coreceptor usage, a function of V3, may play a role in transmission. In both a subtype C MIP cohort [Zhang et al, 2002] and a subtype A MIP cohort [Rainwater et al, 2007] newly transmitted viruses were found to use CCR5 exclusively. Others have observed selection for minor variants with CCR5 phenotypes in HIV-1 infected mothers during perinatal transmission and/or amplification of CCR5 using variants after transmission [Matala et al, 2001]. This data suggests a role for Env in determining perinatal transmission and that there is functional difference between the Env of transmitted and non-transmitted viruses. Neutralization sensitivity likely plays a role in transmission. Multiple studies have shown a relationship between low levels of neutralizing antibody response in mothers [Bongertz et al, 2002; Guevara et al, 2002; Lathey et al, 1999] and neutralization resistance [Scarlati et al, 1993; Rainwater et al 2007; Wu et al, 2006] and transmission. Some studies have proposed a direct association between maternal antibodies against the V3 domain of the Env and a lower rate of transmission of HIV-1 [Y Devash et al, 1990].

The number and location of potential N-glycosylation sites (PNGS) in Env may also be a target of selection for certain variants during transmission. Newly transmitted viruses with shorter variable loops and fewer PNGS have been observed in many studies [Russell et al, 2011; Wu et al, 2006; Zhang et al, 2010]. PNGS at positions N301, N384 [Samleerat et al, 2008] and N339 [Baan et al, 2011] are all associated with transmitted viruses. There also appears to be selection against PNGS at N295 [Baan et al, 2011].

Sequential evidence suggests Env is playing a key role in MTCT; however, the functional role of Env in MTCT remains unknown. Neither Env processing nor incorporation into virions is predictive of transmission [Zhang et al, 2010; Thenin et al,

2009]. Env mediated-viral entry, measured by the response to entry inhibitors such as soluble CD4, PSC-Rantes, and TAK779, did not differ between transmitted and nontransmitted variants [Rainwater et al, 2007; Thenin et al, 2009]. However, our previous studies observed replicative fitness mediated by V1-V5 to be higher in infant viruses from chronically infected MIP [Kong et al, 2008]. Based on the data available we decided to further examine the functional role of Env during viral entry into target cells as a potential determinant of transmissibility.

Entry of virus into target cells is determined mainly by Env function. Entry has two distinct stages, binding and fusion. While the fusion domain is found within gp41, fusion is subsequent to binding and may be influenced by both the structure and function of gp120. Thus, we hypothesized that gp120 may also be playing a role in influencing fusion, in addition to binding, which could contribute to the fitness advantage found in transmitted viruses.

Materials and Methods

Patient information

The subjects of this study were selected from a Zambian perinatal transmission cohort followed for HIV-1 infection. We chose five MIP (2669, 2617, 1084, 1449, and 2873) for our current study. All the pairs chosen represented chronically infected mothers, mothers infected at delivery likely via sexual transmission, which transmitted to their infants. Venous blood was obtained from the mothers before delivery and HIV-1 serological status was determined by two rapid assays, Capillus (Cambridge

Biotech, Ireland) and Determine (Abbott laboratories), and confirmed by immunofluorescence assay. All mothers were HIV-1 positive at delivery and this was subsequently defined as baseline for mothers.

Children from these MIPs represented both rapid (2669, 2617, 1449, 2873) and slow (1084) progressors as determined by clinical outcome and survival. Rapid progressors died within the first year of life due to HIV-related complications, while slow progressors were followed for more than four years and remained clinically asymptomatic. All children remained anti-retroviral naïve throughout the study. Venous blood was also obtained from the infants within 24 hours of birth and follow-up was maintained at 2, 4, and 6/8 months and at regular intervals through 48 months (for slow progressors). HIV-1 infection in the infants was detected by sequential viral isolation from the infants' peripheral blood mononuclear cells (PBMC), as previously described [Zhang et al, 2005] and by PCR of the HIV-1 provirus *env* gene from genomic DNA. HIV-1 isolation was unsuccessful from all samples and all infants were PCR negative at birth, suggesting either intrapartum or postpartum transmission. HIV-1 *env* sequences were amplified from infant PBMC at different postpartum time points. Due to the limited sample volume, viral isolation was prioritized over PCR when required (e.g., infant 1084, viral isolation was positive by 4 month and the first PCR was performed 6 month after birth) (Table 1). Mother samples were defined as baseline at the time of delivery. Infant samples were collected at the first postpartum, HIV-1 PCR positive time point.

Viral Isolation

A portion of the *env* gene from V1–V5 was amplified by PCR, cloned, and sequenced in order to characterize Env genetic diversification and evolution. Sequence

analysis from each MIP showed the mother and infant viruses were epidemiologically linked. All cases were subtype C based on Env, except for 1449 (subtype A/C recombinant). All viruses were also shown to be CCR5 tropic [Zhang et al, 2005]. Between four and six representative functional and fusion-positive Env clones from each MIP baseline were used for further analysis (Table 1). The V1-V5 fragments were ultimately cloned into a proviral expression vector, pNL4-3 A/S/AV, resulting in infectious molecular clone plasmids that express Env as a chimeric protein with the patient's Env V1-V5 region and HIV-1 strain NL4.3 (Fig.1)

Cell cultures

293T and TZM-bl (NIH AIDS Research and Reference Reagent Program catalog no. 8129) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS, Gibco) and 100ug/ml penicillin-streptomycin (Invitrogen). SupT1-CCR5 (from Dr. James Hoxie, Emory University) were maintained in RPMI with 10% FBS, 100ug/ml penicillin-streptomycin, and 300ng/ml puromycin. All cells were grown at 37°C with 5% CO₂.

Proviral expression constructs

The V1-V5 region of the *env* gene was amplified by nested PCR from uncultured patient PBMC using the Gentra Kit (Purgene), cloned into the pGEM-T Easy vector and sequenced as described [Zhang et al, 2006]. The Env V1-V5 region from selected clones was amplified from the pGEM-T Easy vector and subcloned into the pSP72 NLA/S/Av shuttle vector. The chimeric Env expression construct containing patient-derived Env V1-

V5 region was generated by substituting the EcoRI-XhoI region of pSP72A/S/Av with the corresponding region of pSRH NL A/S/Av to screen for biological function as described [Zhang et al, 2010]. Functional envelope constructs were transferred into a proviral expression vector, pNL4-3 A/S/Av, by substituting EcoRI-XhoI region of pSRH NL A/S/Av with the corresponding region of pNL4-3 A/S/Av, resulting in infectious molecular clone plasmids with an NL4-3 backbone.

Viral Production

Viruses bearing patient-derived Env V1-V5 and BlaM-Vpr were produced by calcium phosphate transfection of 293T cells. Approximately 2.0×10^6 293T cells were transfected with 9ug of proviral construct, 3.0ug of pMM310 (BlaM-Vpr) (NIH AIDS Research and Reference Reagent Program catalog no. 11444), and 1.5ug of pAdVantage (from Dr. Gregory Melikyan, Emory University) in a 10cm dish. At minimum three dishes were used for each proviral construct. After 48 hours post-transfection, the infectious virus was harvested, centrifuged to clarify debris, and pooled. The viral stocks were concentrated by ultracentrifugation using the SW-41 Ti rotor (Beckman Coulter) or the SW 32 Ti rotor (Beckman Coulter) at 20,000rpm for 2 hours at 4°C. The viral pellet was resuspended at a 30x-40x final concentration and stored at -80°C. The amount of HIV-1 in the viral stocks was measured using the HIV-1 p24 ELISA kit (PerkinElmer Life Sciences, Inc.). Samples were diluted at 1:500,000 using DMEM culture media, mixed with the lysis buffer contained in the kit, added to the antibody-coated plates, and incubated at 4°C overnight. All other steps were performed according the manufacturer protocol. Viral titer was confirmed measuring the p24 levels by western blot analysis.

Protein incorporation in virions

Western blot analysis was carried out to determine the incorporation of p24, gp120, and BlaM-vpr into virions. Viral stocks were normalized by p24 (as described above) and lysed for SDS-PAGE. Viral proteins were separated on a 4-20% agarose gradient gel, transferred to a nitrocellulose membrane and then analyzed by Western blot. A polyclonal goat anti-HIV-1 gp120 antibody (Thermo Scientific Pierce) was used to detect gp120, polyclonal rabbit anti- HIV-1 Vpr (1-46) antibody (NIH AIDS Research and Reference Reagent Program catalog no. 3951) to detect vpr and BlaM-vpr, and polyclonal rabbit anti-HIV-1_{SF2} p24 antibody (NIH AIDS Research and Reference Reagent Program catalog no. 4250) to detect p24. Protein quantification was performed using the Odyssey Infrared Imager (Li-Cor).

Fusion

Viruses containing the patient Env V1-V5 and BlaM-Vpr were used to measure virus-to-cell fusion in a fluorescent resonance energy transfer (FRET) based assay as previously described [Cavrois et al, 2002]. One day prior to infection SupT1-CCR5 cells were removed from puromycin selection. On the next day, 250,000 SupT1-CCR5 cells were transferred to each well of a 96-well V-bottom plate. Based on p24 ELISA data, 50ng of p24 of each virus was added to the cells in a total volume of 100ul. Cells were incubated with virus for 2hrs at 37°C with 5% CO₂. After incubation the virus was removed from the cells and fusion was quantitated based on beta-lactamase activity using the GeneBLAzer *in vivo* Detection Kits (Invitrogen catalog no. 12578-134). A loading

solution containing a 1:4 ratio of CCF2-Am substrate, resuspended in DMSO (kit solution A), to Pluronic®-F127 surfactant in DMSO and acetic acid (kit solution B) in CO₂-Independent media, was added to the cells and incubated for 1hr at room temperature in the dark. This solution was removed and the cells were washed with the CO₂ -Independent media. A developing solution containing 2.5mM probenecid and 10% FBS in CO₂ -Independent media was added to the infected cells, and then allowed to incubate overnight (16hrs) at room temperature in the dark. After overnight incubation the developing solution was removed from the cells, the cells were then washed, and fixed in 1.2% paraformaldehyde for flow cytometry analysis.

Upon virion fusion to the plasmid membrane, the BlaM-Vpr protein can be internalized into the cell. Cells with BlaM activity will demonstrate a shift in the CCF2-AM FRET pattern from green to blue due to cleavage of CCF2-AM and are considered fusion positive. The change in emission patterns was observed by flow cytometry using a DXP 10 modified BDFacScan (Cytex Development Inc). CCF2-AM was excited at 407nm. Green emission was detected with the 545/30 filter and blue emission was detected using a 450/50 filter. Fusion was quantified by the percentage of blue fluorescing cells.

Infectivity

Viral infectivity was measured by the tissue culture dose for 50% infectivity (TCID₅₀) on TZM-bl cells. One day prior to infection, TZM-bl cells were plated at a density of 1.5×10^4 cells/100ul/well in a 96-well plate. Viral stocks were diluted 10-30-fold in culture media containing 40ug/ml DEAE-Dextran. Four fold serial dilutions (4^{-1} - 4^{-4}

⁷) were made from this stock and transferred to the cells. Each virus dilution was done in quadruplicate. After 48 hours the virus was removed from the cells, the cells were then fixed and stained for beta-galactosidase activity. Viral titer was calculated as TCID₅₀/ml using the Reed-Muench method.

Results

V1-V5 of Env is capable of influencing fusion.

Chimeric viruses bearing patient-derived Env V1-V5 in a Subtype B, NL4.3 backbone were used to assess the fusion capabilities of 5 MIPs. We used a FRET-based virus to cell fusion assay in order to quantify the percentage of cells that have undergone fusion. Infectious viruses were produced by a triple transfection of 293T cells with the proviral plasmid, the pMM310 (BlaM-Vpr) plasmid, and the pADVantage plasmid (used to boost translation). Virions produced by this method will incorporate the chimeric Env and package the BlaM-Vpr fusion protein.

We used the CCR5-tropic AYDA strain as a positive control for dye loading and BlaM activity. The AYDA V1-V5 was cloned into the NL4.3 backbone using the same strategy as the patient-derived Env variants. All fusion was plotted relative to AYDA as the reference clone. The AYDA clone consistently produced a strong positive signal with approximately 70% of the cells with a shift from green to blue fluorescence. In all MIPs tested clone-to-clone variation in fusion activities was observed (Fig. 2).

With MIPs 2617, 1449, 2873 fusion between patient-derived clones with target cells varied from 12.5-58.3%, 23.6-64.9%, and 27.9-48.7%, respectively. Relative fusion

activities of clones from MIP 2617 ranged from 43.7-104.8%, with clone M3 having the highest fusion and clone i3 the lowest (i indicates infant clones and M denotes mother clones in all designations). For MIP 1449 the mother clones had both the highest (M2) and lowest fusion (M3). Relative fusion activities of clones from MIP 1449 ranged from 28.6-81.5%. For MIP 2873, relative fusion was between 39.4-68.9%. Once again with this pair the mother clones had both the highest (M1) and lowest fusion (M3), but fusion was very similar for all viral variants from this pair. For MIP 2669 and 1084, the differences in fusion were much more dramatic. Clone i1 from 2669 fused at least 2.5 times greater than any other clone and 22 times greater than the weakest clone (mother M4). The M4 clone fused 4.3 times less than any other clone. Overall, fusion activities of clones from MIP 2669 varied from 59.4-95.5%. With MIP 1084 2 mother clones (M3 and M4) have high fusion activities, while all other clones have much weaker fusion activities compared to these clones and most of the other MIPs. The M3 and M4 fused 102.6% and 115.6% relative to AYDA while all other clones fused less than 5%. Clone i3 fused less than 1%.

There was also wide clonal variation in fusion in viruses isolated from the same patient (Fig. 2). With 1084M fusion was 100 times greater with clones M3 and M4 compared to M1. 1084i fusion varied by 8-fold amongst variants. In 2669M fusion varied over 9-fold between the lowest (M4) and highest (M2) clones. All other patients had a 2-5-fold variation with clones from the same patient.

In order to rule out any differences in fusion caused by BlaM-Vpr incorporation into virions, western blots were performed on all viral preparations. The amount of BlaM-Vpr was measured using an antibody to vpr. We did not see a difference in BlaM-

vpr incorporation between any of the MIP (data not shown). Any differences in fusion are thus attributable to Env. Since all clones also contained the same gp41 fusion domain from NL4.3, therefore the dramatic differences we see in fusion of these MIP are due to their differences in the V1-V5 region.

Fusion activities cannot predict transmission.

Overall, there was no difference in fusion activities that can differentiate between the transmitted clones and non-transmitted viral clones. None of the MIP clones, individually or as a group, displayed a pattern in fusion capabilities that could predict whether they were derived from the mothers or from the infants (Fig 3). For MIP 2617 and 1084, the mother viruses appeared to have a slight advantage in fusion over the infant viruses, but this was not significant. For MIP 2669, infant viruses have slightly higher fusion activities over mother viruses, but this is also non-significant. MIP 1449 showed similar fusion in both mother and infant viruses (Fig 3a). Overall, the mother viruses as a group had a slight advantage in fusion over infant viruses (Fig 3b). However, this advantage is not significant ($p = 0.097$). In summary, wide clone-to-clone variation among clones from all patients, but there is no distinct pattern to fusion activities that are characteristic of the transmitted infant versus the non-transmitted maternal clones.

Fusion correlates with infectivity.

We then tested the ability of the various maternal and infant viral clones to infect target cells. Viral infectivity was measured by titration on TZM-bl cells. Infectivity was found not to be predictive of transmission. Infectivity of the viral clones from mother and

infant pairs varied within a transmission pair, and among all the clones obtained from the cohort (Fig. 4). In general, clones within a pair varied up to 15 times in terms of infectivity. For example, for MIP 2669 clone i1 has much higher infectivity than the other clones, with infectivity 40 times greater than that of the least infectious clone. MIP 1084 demonstrated a more variable pattern of infectivity. Once again, infectivity of 1084 viral clones M3 and M4 were much higher than the other clones. When excluding these two viral clones, other 1084 isolates were more similar to each other (varied by less than 10 times) and were similar to other viral clones obtained from other MIPs. Overall, fusion activity correlates to infectivity (Table 2). For pairs 2669, 1084, and 2873 fusion correlated strongly with infectivity as measured by viral titer, with R^2 values of 0.899, 0.992, and 0.885, respectively. MIP 1449 showed a slightly lower correlation with an R^2 of 0.764. MIP 2617 was the only pair that did not show a strong correlation between infectivity and fusion (R^2 0.204). This pair demonstrated much less variation in fusion than other pairs, but still displayed variation in infectivity that was similar to other MIPs.

Incorporation of Env into virions does not determine fusogenicity.

In order to determine whether fusogenicity and infectivity of the various viral clones were affected by the amount of Env gp120 incorporated into the virion, we determined and compared the gp120 incorporation into each viral clone by western blot. Equal amounts of virus was lysed and loaded onto a gradient gel based on viral titer determined by p24 ELISA. Both p24 and gp120 were detected. Env incorporation was based on the amount of Env detected after normalizing to the amount of input viruses based on the p24 signal (Fig 5). Env incorporation did not significantly differ between the

mother and infant viral clones from the MIPs except 2873. Although infant viruses from MIP 2669 appear to have a higher Env incorporation, this trend was not significant (t-test, p 0.113). For MIP 2873 the mother viruses incorporated slightly more Env than infant viruses (t-test, p 0.025). For MIP 1084 clone M4 also incorporated more Env than the other clones. However, there is no correlation between fusion activities and the amount of Env incorporated by different viral clones (Table 3). Only clones from MIP 1084 showed some correlation between fusion and Env incorporation (R 0.826, R^2 0.682). For MIP 1084 clones M3 and M4 showed a dramatic increase in fusion compared to the other clones. There was correlation observed between incorporation and fusion for these two clones, the higher levels of Env incorporation may have resulted in their higher fusion activities. Whereas for the variants from 1084 that have similar fusion activities there appeared to be a slight inverse relationship between fusion and incorporation (R -0.807), but this correlation is not very strong (R^2 0.651). This is likely due to clone i3 which had very low fusion despite incorporating a relatively high amount of Env, suggesting there may be some qualitative difference among different Env in their ability to mediate fusion and infection. Such differences need to be further investigated.

Discussion

Our previous study showed that V1-V5 of *env* is capable of influencing replicative fitness of viral variants from MIP and the fitness is predictive of transmission [Kong et al, 2008]. However, envelope mediated fusion fails to explain a higher replicative fitness in infant viruses and does not predict transmission. Previously, we had

also observed that Env synthesis and processing, viral infectivity, replication kinetics, and Env incorporation into virions failed to predict transmission [Zhang et al, 2010]. The fusion results agree with the other functional data that suggests Env functions measured do not predict transmission and viral replicative fitness. It is likely that at least some of the discordance between replicative fitness and the other functional properties of Env that have been measured from clones from these MIP can be attributed to differences in viral preparations. The fitness assay represents a more heterogeneous viral population because the virus used was prepared by allowing for a recombination of the *env* as a PCR product and an *env* deficient backbone. The other properties were all measured with full-length proviral clones and represent a homogeneous viral population. The fitness assay also used a long incubation time that measured multiple rounds of replication that likely included recombination events between the mother and infant clones. Of the biological properties of Env that were measured in this study, only infectivity measures multiple rounds of replication. However, the incubation time for fitness is over twice as long as infectivity. It is likely that the very early infection events measured in the current study are different from later events and explains, at least in part, why fitness and fusion data do not correlate.

This study demonstrated a correlation between the fusion activities and infectivities of the viral clones that was expected. Fusion is a key part of the entry process and initiates infection. We would expect that viral variants with higher fusion activities are more capable of entering target cells and this is reflected in infectivity measured in the first few rounds of replication. We did not see a correlation between fusion activities of viral clones and the amount of Env incorporated into virions of the clones. The lack of

a correlation between these two Env properties of the viral clones suggests that there are some qualitative differences in Env from these clones.

This study does show that V1-V5 of *env* is capable of influencing fusion, even though all variants contained the same fusion domain (gp41). This is possibly due to the stability of the gp120-gp41 complex. Recent studies have shown potential interactions between gp120 and gp41 affect fusogenicity. Mutations in the gp41 ectodomain that produce an unstable gp120-gp41 complex correlate to a decrease in fusion function [Maerz et al, 2001]. This study also suggests that there are some biological differences found in the Env variants isolated within a patient. Within a patient (2669M) high and low fusing clones vary by seven times and this is not due to the amount of Env incorporated into the virions. Thus, fusion in general is not simply determined by the amount of Env expressed on the virion. However, there are exceptions. For example, for variants 1084 M3 and 1084 M4 the levels of Env incorporation are contributing to the dramatically higher fusion and even for these clones Env incorporation alone cannot account for the differences in fusion. It is possible that primary sequence differences between different *env* clones may influence their fusion activities. However, upon preliminary analysis there does not appear to be any common mutations, insertions, or deletions that are characteristics of the Env with either high or low fusion activities. It is likely that the Env conformation may play an important role in influencing fusion. Post-secondary modifications, such as glycosylation, that effect protein folding and stability are potential determinants of fusogenicity. Additionally, viruses that contain adaptive mutations in gp120 that allowed for usage of mutant forms of CCR5 were capable of rapid fusion kinetics due to a reduced constraint on gp41 in mediating fusion [Platt et al,

2007]. Finally mutations that accumulated in gp120 in response to fusion inhibitors were capable of enhancing fusion and this was related to the ability of these mutations to utilize both CD4 and CCR5 [Wang et al, 2011]. Env stability and CD4/CCR5 utilization should be further characterized in our clones to better understand the fusion properties and whether higher fusion activities correlate to their ability to use other coreceptors in addition to CCR5.

In summary, our data suggests that the gp120-gp41 interaction is important in determining the functional properties of Env including fusion, but fusion is not predictive of perinatal transmission. However, it is likely that the Env structure and its stability may play key roles during viral fusion and ultimately mediating entry into target cells.

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Figure Legend

Fig. 1. Organization of proviral plasmids. The V1-V5 region from each viral isolate of the MIPs was cloned into a NL4-3 backbone to produce an infectious molecular clone plasmid. Env is expressed as a chimeric protein.

Fig. 2. Relative fusion. Fusion was examined using a FRET-based assay. Virions are produced by transfection in 293T cells and incorporate the chimeric Env protein with V1-V5 from the patients and package a BlaM-Vpr fusion protein. Infections are done in SupT1-CCR5 cells and then cells are loaded with CCF2-AM and express a green fluorescence. Upon fusion, the BlaM-Vpr protein enters the cell and cleaves CCF2-AM producing a blue fluorescence. Fusion is quantified as the total percentage of blue cells. For each pair AYDA is used as a positive control and relative fusion is calculated as a percentage of the AYDA signal. Each graph represents at least four experiments, except 2873 which only represents two

Fig 3. Fusion of infant versus mother viruses. A. Comparison of infant (\diamond) and mother (o) variants with patient-derived V1-V5 for each transmission pair. B. Comparison of all infant (\diamond) and mother (o) variants. The p value was calculated using a two-tailed Student paired t-test and is shown.

Fig. 4. Infectivity of MIP-derived viruses. Viral stocks were titrated on TZM-bl cells and infectivity was measured as the TCID₅₀/ml. Four fold serial dilutions were made from viral stocks and transferred to the cells. After 48 hours the virus is removed and cells are fixed and stained for beta-galactosidase activity. The TCID₅₀/ml was measured using the Reed-Muench method.

Fig. 5. Env incorporation into virions. Western blot analysis was done to determine the incorporation of gp120 into virions. Viral stocks were normalized by p24. Viral proteins were separated on a 4-20% agarose gradient gel, transferred to a nitrocellulose membrane and analyzed by Western blot. A polyclonal goat anti-HIV-1 gp120 antibody (Thermo Scientific Pierce) was used to detect gp120 and polyclonal rabbit anti-HIV-1_{SF2} p24 antibody (NIH AIDS Research and Reference Reagent Program catalog no. 4250) to detect p24. Protein quantification was performed using the Odyssey Infrared Imager (Licor). Env incorporation into virions was calculated as the gp120/p24 ratio; the NL4-3 ratio was set at 1 and all values calculated relative to this value.

Table 1. Summary of mother and infant transmission pairs.

MIP		Time point analyzed^a	Number of clones	Co-receptor	Disease progression	Cause of death
2669	Infant	2 mo.	3	CCR5	Rapid	Bronchitis
	Mother	Delivery	3	CCR5		
2617	Infant	2 mo.	3	CCR5	Rapid	Pyrexia
	Mother	Delivery	3	CCR5		
1084	Infant	6 mo.	3	CCR5	Slow	Survived
	Mother	Delivery	3	CCR5		
1449	Infant	2 mo.	2	CCR5	Rapid	Pneumonia
	Mother	Delivery	2	CCR5		
2873	Infant	2 mo.	3	CCR5	Rapid	Tuberculosis
	Mother	Delivery	3	CCR5		

^a Months after birth.

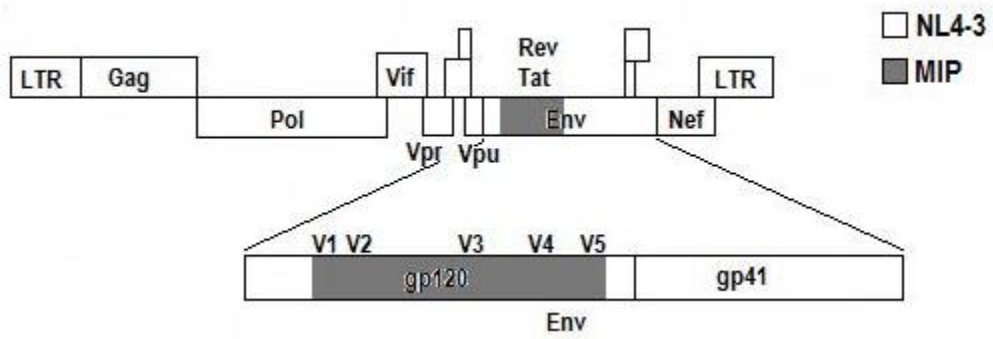
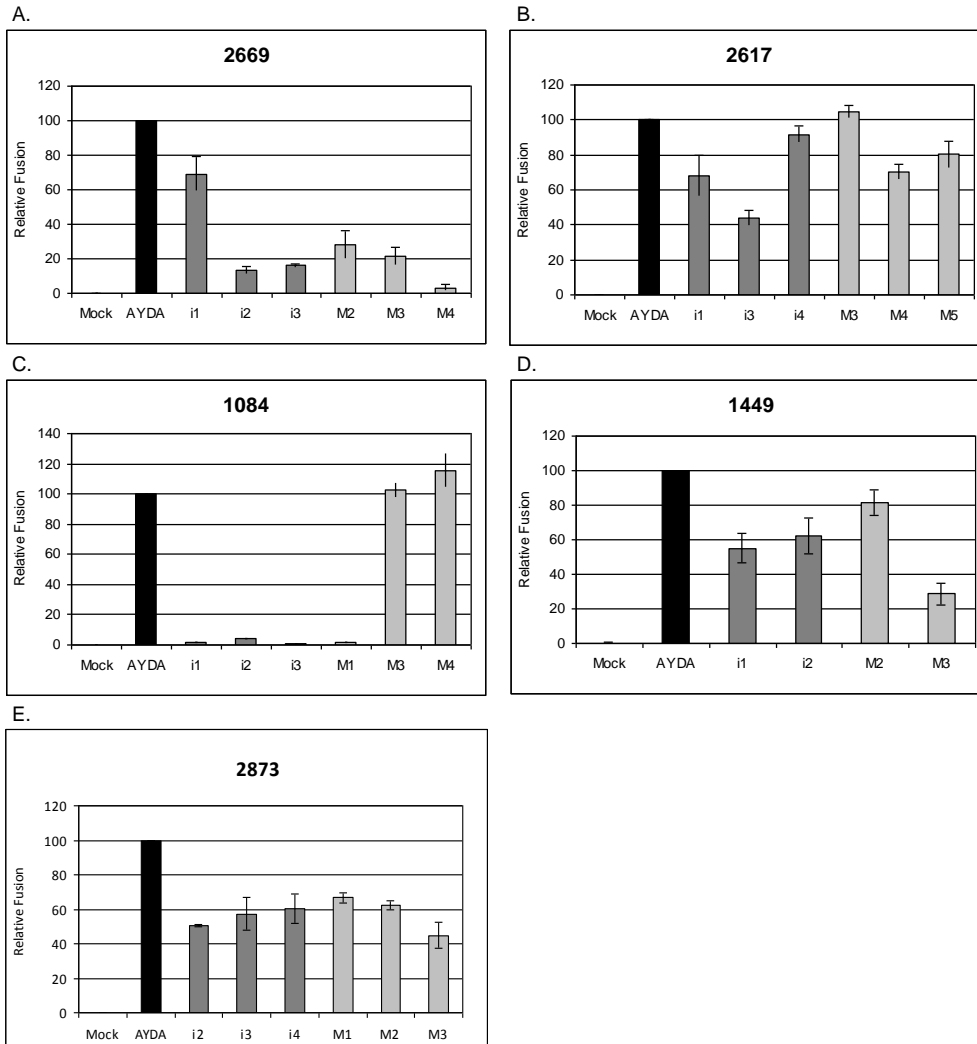
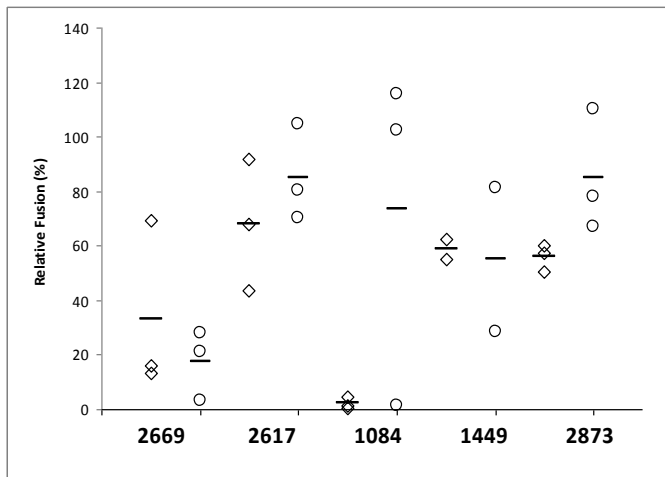


Figure 1

**Figure 2**

A.



B.

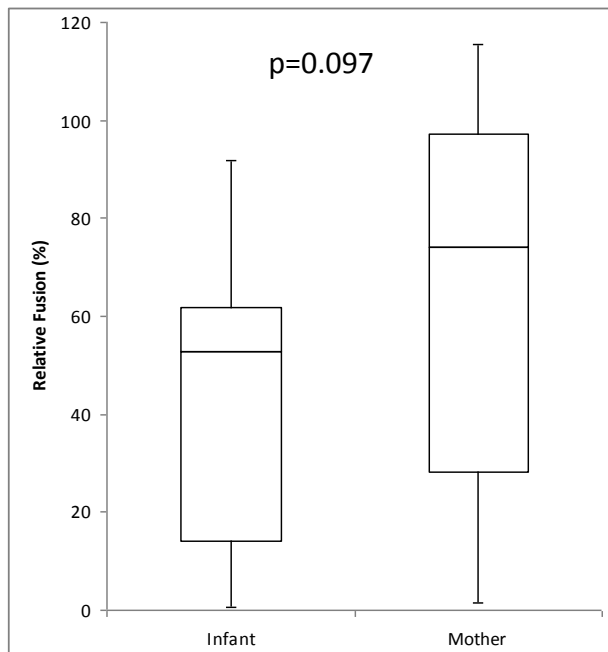


Figure 3

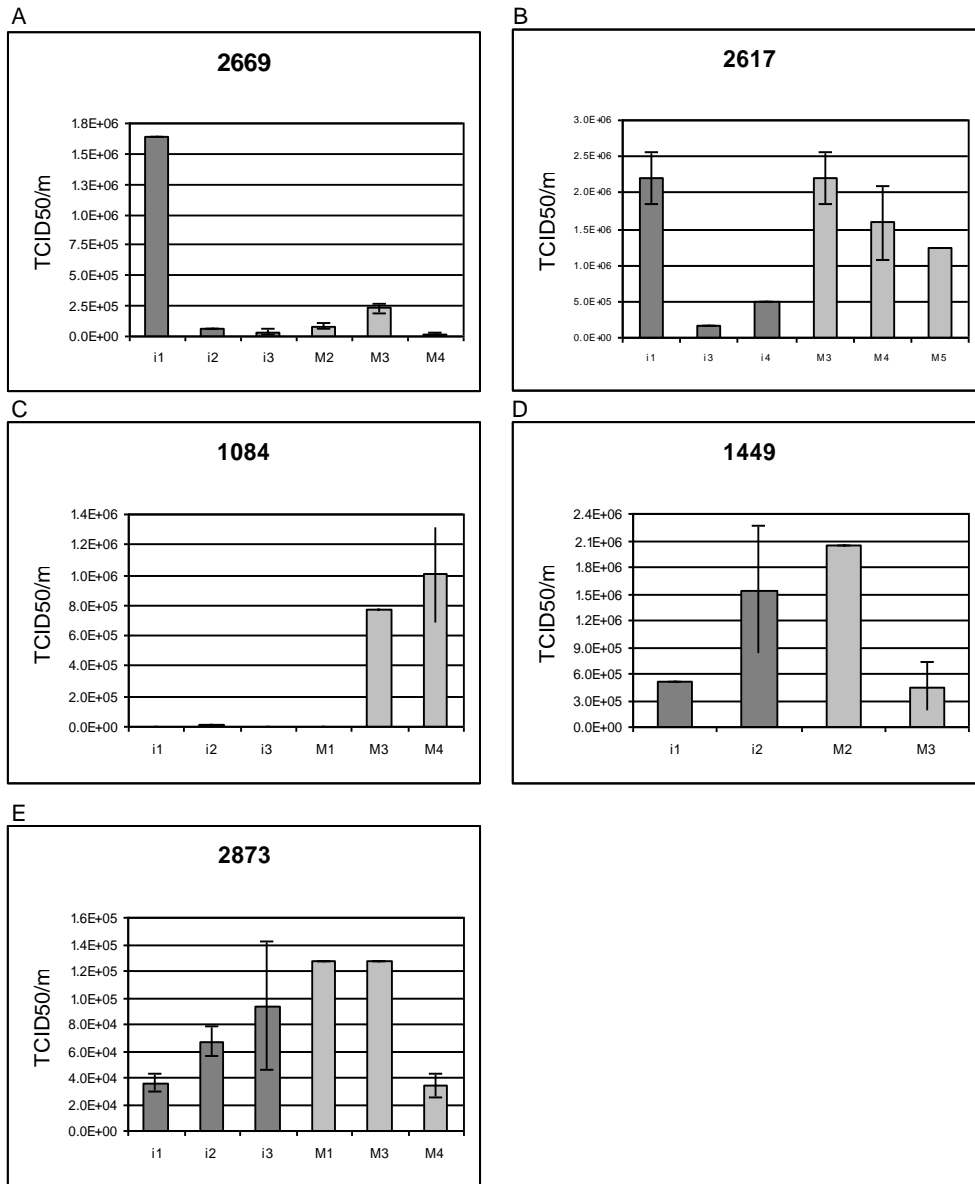


Figure 4

Table 2. Correlation between infectivity and fusion.

MIP	R value	R²
2669	0.948	0.899
2617	0.452	0.204
1084	0.996	0.992
1449	0.874	0.764
2873	0.941	0.885

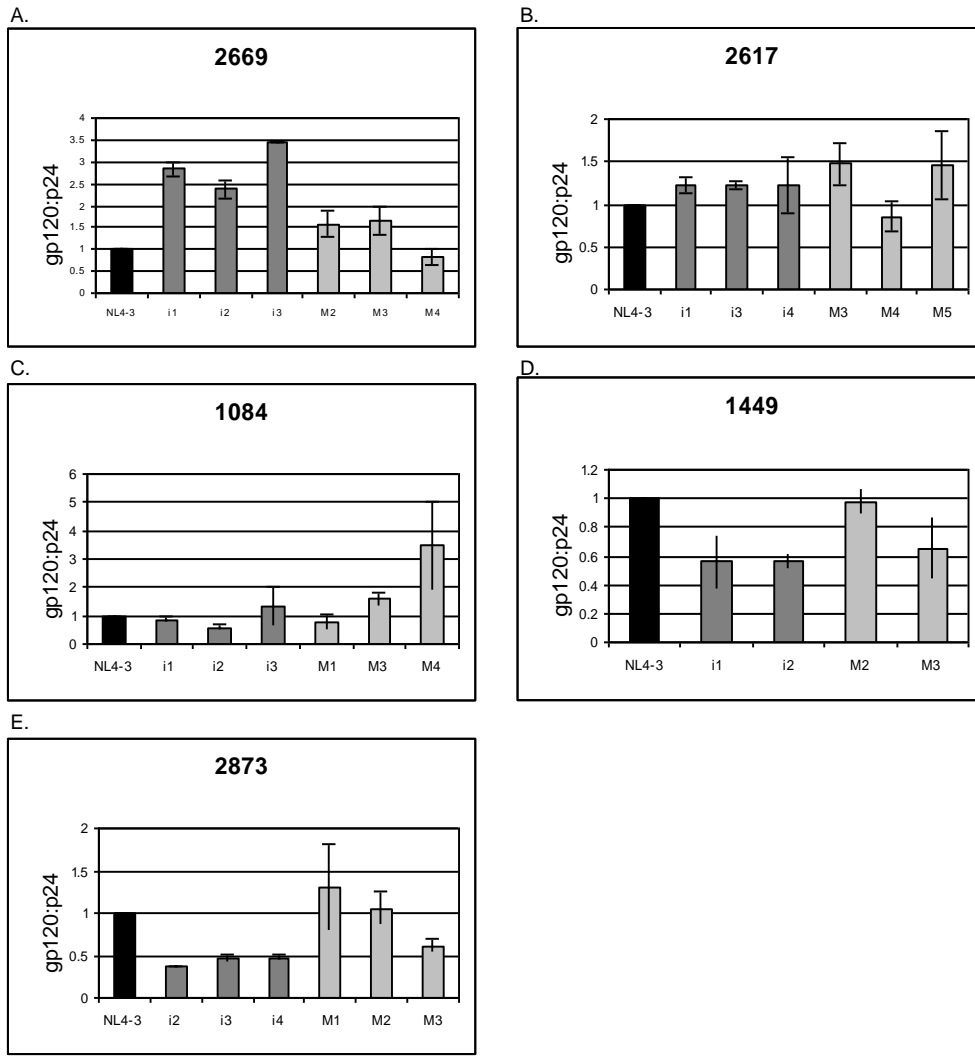
**Figure 5**

Table 3. Correlation between Env incorporation and fusion.

MIP	R value	R ²
2669	0.404	0.164
2617	0.429	0.184
1084	0.826	0.682
*i1, i2, i3, M1	-0.807	0.651
*M3, M4	1	1
1449	0.587	0.344
2873	0.578	0.335

Chapter 3: Virus to cell binding of HIV-1 is influenced by envelope V1-V5, but does not predict transmission.

Introduction

Viral entry into target cells is a multi-step process that is initiated by the binding of virions to the viral receptor, CD4. Upon CD4 engagement, a conformational change is induced in Env that exposes the V3 loop and the coreceptor binding site. Engagement of the coreceptor, CCR5 or CXCR4, another conformational change occurs that exposes the fusion domain. Fusion then occurs subsequent to binding. Both binding and fusion are the primary functions of Env.

The major role of gp120 is in binding the virion to the target cell. There are multiple CD4 binding sites within gp120 that are brought into the proper conformation by the gp120 tertiary structure to allow for CD4 binding [Lasky et al, 1987; Kowalski et al, 1987; Olshevsky et al, 1987]. The coreceptor, both CCR5 and CXCR4, binding site is located in the V3 loop, but other domains within the V1-V5 region can also be involved to strengthen or weaken binding [Cann et al, 1992; Chesebro et al, 1991; Hwang et al, 1991; O'Brien et al, 1990; Shioda et al, 1991]. Coreceptor usage is determined by the gp120 sequence; specific mutations within V3 facilitate the switch from CCR5 (R5) to CXCR4 (X4) usage [Fouchier et al, 1992; Pollakis et al, 2004]. V3 also plays a role in

membrane fusion [Freed et al, 1991] and contains epitopes for neutralizing antibodies [Goudsmit et al, 1988; Palker et al, 1988; Rusche et al, 1988].

As the main function of gp120 of env is viral binding, we sought to compare the binding efficiencies of the V1-V5 region of the gp120 derived from viruses isolated from subtype C infected mother-infant-pairs (MIP) and determine whether their binding abilities can predict transmitted viruses during perinatal transmission, and if it is contributing to higher replicative fitness in infant viruses from our cohort. We also sought to examine the relationship between binding and fusion as we examined fusion using the FRET-based assay (previously described) in parallel with binding.

We examined binding in three of the MIPs (2669, 2617, and 1084) described previously. All these pairs represent chronically infected mothers with their infants classified as rapid (2617, 2669) versus slow (1084) progressors. It is also important to note that all the viral variants examined for binding use CCR5 for entry. We sought to examine binding using the EGFP/DsRed2 expressing viruses that were used for the growth competition assay [Kong et al, 2008]. We also used viruses without any marker to examine binding. In addition, we decided to examine fusion and binding in parallel and used the SupT1-CCR5 cell line and the BlaM-Vpr containing viruses.

Materials and Methods

Patient information

The three mother infant pairs (2617, 2669, 1084) in this study have been described previously [Zhang et al, 2006]. All mothers were HIV-1 positive at delivery, asymptomatic, and not undergoing ART. The infants were all breast-fed and drug naïve as well. Mother samples were defined as baseline at the time of delivery. Infant samples were collected at the first postpartum, HIV-1 PCR positive time point.

Cell cultures

293T and TZM-bl (NIH AIDS Research and Reference Reagent Program catalog no. 8129) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS, Gibco) and 100ug/ml penicillin-streptomycin (Invitrogen). SupT1-CCR5 (from Dr. James Hoxie, Emory University) were maintained in RPMI with 10% FBS, 100ug/ml penicillin-streptomycin (pen/strep), and 300ng/ml puromycin. U87 (NIH AIDS Research and Reference Reagent Program catalog no. 2188) cells were maintained in DMEM with 10% FBS and 100ug/ml pen/strep. U87.CD4.CCR5 cells (from Dr. Lee Ratner, Washington University) were maintained in DMEM with 10% FBS, 4mM Glutamine, 1mM Sodium Pyruvate, 200 µg/ml G418, and 1 µg/ml Puromycin. All cells were grown at 37°C with 5% CO₂.

Proviral expression constructs

The V1-V5 region of *env* gene was amplified by nested PCR from uncultured patient PBMC using the Gentra Kit (Purgene), cloned into the pGEM-T Easy vector and sequenced as described [Zhang et al, 2006]. Generation of infectious molecular clone plasmids with MIP-derived Env V1-V5 and an NL4-3 backbone was described

previously. The chimeric env region was also substituted into the 4-3 EGFP/DsRed2 using EcoRI and BamHI, resulting in molecular clone plasmids that express either EGFP or DsRed2.

Viral Production

Viruses bearing patient-derived Env V1-V5 and BlaM-Vpr were produced as described previously. (NIH A with or without EGFP/DsRed2 were produced by transfection of 293T cells using the FuGENE 6 (Roche). 1.5×10^5 293T cells were transfected with 1.5ug of proviral DNA and 5ul of FuGENE in 1 well of a 6-well plate. The total volume in each well was 2ml and multiple wells were combined for each virus. Viral stocks were filtered through 0.45um filters and stored at -80°C. All viral stocks were measured using a HIV-1 p24 ELISA kit (PerkinElmer Life Sciences, Inc.). Samples were diluted 1:500,000 using DMEM culture media, mixed with the lysis buffer contained in the kit, added to the antibody-coated plates, and placed at 4°C overnight. All other steps were performed according the manufacturer protocol.

Virus-to-cell Binding

Binding of viruses containing the patient Env V1-V5 with or without EGFP/DsRed2 was done in U87-derived cells. One day prior to the assay U87 and U87.CD4.CCR5 cells were plated at 4×10^5 /well in 1 well of a 6-well plate. The next day 40ng of p24 of each virus was added to the cells and incubated for 1hr at 4°C. Cells were vigorously washed with PBS. A lysis buffer containing 0.5% NP40 was added to the plates and cells were collected in Eppendorf tubes. Cultures were lysed at 4°C for 1hr

with constant agitation. Lysates were clarified by centrifuging at 10,000xg for 10min and assayed for bound p24 by p24 ELISA as described above. Relative binding is determined using the U87 cell line to measure background signal (non-specific binding) and expressed as a fold increase over this level.

Binding of viruses containing the patient Env V1-V5 and BlaM-Vpr was carried out with SupT1-CCR5 cells. One day prior to infection SupT1-CCR5 cells were removed from puromycin selection. On the next day, 250,000 SupT1-CCR5 cells were transferred to each well of a 96-well V-bottom plate. Based on p24 ELISA data, 50ng of p24 of each virus was added to the cells in a total volume of 100ul of CO₂-independent media. Cells were incubated with virus for 2hrs at 4⁰ C. After 2hrs cells were washed with PBS to remove unbound virus. Cells were lysed in a solution containing 0.5% Triton X-100 for 1 hr at 4⁰ C. Lysates were clarified by centrifugation at 10,000xg for 10min. Bound p24 in the lysate was measured by p24 ELISA as described above. Percent binding was calculated as the bound p24/input p24x100.

Virus-to-cell Fusion

Viruses containing the patient Env V1-V5 and BlaM-Vpr were used to measure virus-to-cell fusion in a fluorescent resonance energy transfer (FRET) based assay as previously described [Cavrois et al, 2002]. Virus-to-cell fusion was performed as described previously using SupT1-CCR5 cells.

Results

Env V1-V5 influences viral binding in adherent cells

Initially, we sought to determine virus-to-cell binding in adherent cell lines using the U87 and U87.CD4.CCR5 cell lines. To determine binding we incubated U87 and U87.CD4.CCR5 cells with viral preps at 4°C to prevent fusion. Next we attempted to wash away any unbound virus, collected and lysed the cells, and assayed bound p24 using an ELISA. The U87 cell line was used to determine the background signal of the assay (a combination of unbound virus and non-specific binding). Relative binding was then measured as fold-increase over the background. First, we tested binding using MIP 1084 and viruses with either an EGFP (infants) or DsRed2 (mothers) fluorescent tag. We saw a small clone-to-clone variation in viral isolates from MIP 1084 (Figure 1a). Clones i3, M2, and M3 all showed similar levels of relative binding at 1.92, 1.98, and 1.72, respectively. Clone i1 showed a slightly elevated binding at 2.60. However, when we attempted to test relative binding in non-tagged virus this pattern did not remain consistent. A representative experiment is shown in Figure 2a. All clones in this experiment had similar levels of binding, ranging from 1.42-2.29. In contrast to the previous experiment, clone i1 showed a lower binding (1.76) than other infant clones, but it was still higher than the mother clones (M1 1.44, M3 1.42).

Binding does not predict transmission in adherent cells.

Overall, the relative binding is not predictive of transmission. When using the non-tagged viral preparations; we observed a trend towards higher binding in the infants

($p=0.056$, Fig. 2b). However, we did not observe the same trend using the fluorescently-tagged viral stock ($p=0.303$, Fig. 1b). Ultimately we were unable to resolve these discrepancies. While there might be a trend towards higher binding in infant viruses; the role of binding as an indicator of transmission remains unclear.

Env V1-V5 influences viral binding in suspension cells

Next, we observed fusion in suspension cells. We used the same SupT1-CCR5 that we had previously used to determine fusion. As we assayed for both fusion and binding concurrently in this set of experiments, we used the viral stocks with the BlaM marker. To determine binding we incubated SupT1-CCR5 cells with viral preps at 4°C, washed away any unbound virus, lysed the cells, and assayed bound p24 using an ELISA. In this assay binding is measured as the percentage of bound virus and calculated as bound virus/input virus x 100. We tested three MIPs (2669, 2617, 1084). Overall, we saw very little binding in this assay (Fig. 3a-c). The highest percent binding observed was 3.34% (2669 M4). A majority of the clones bound less than 1% of the initial virus available. In the case of 1084, less than 0.1% of viruses were capable of binding. In the case of MIPs 2669 and 2617, differences in binding between clones could be quite significant. For 2669, binding varied up to 10-fold between the highest (M2 3.33%, M4 3.34%) and lowest (i3 0.30%) clones. In 2617 binding varied over 30-fold between M5 (2.50%) and i1 (0.075%). MIP 1084 showed much less variation in binding among the clones, varying less than 2% between all clones.

Binding does not correlate with fusion and does not predict transmission

We examined binding and fusion concurrently in the SupT1-CCR5 cells. Unlike with the binding assay, cells were incubated with virus at 37°C to allow binding to progress through fusion and entry to be completed. Fusion was assayed as described above. We see a difference in fusion among viral variants from each MIP (Fig. 3d-f). However, this difference in fusion does not correlate to the differences in binding (Table 1). In fact, both MIP 2617 and 1084 showed an inverse relationship. This relationship is not significant as the correlation was extremely weak at $R = -0.192$ and $R = -0.027$ for MIP 2617 and 1084 respectively. MIP 2669 showed a positive and stronger correlation at $R = 0.424$, but again this is not significant.

Neither binding nor fusion were capable of predicting transmission in any of the MIPs we assayed (Fig. 4). Despite dramatic differences among clones in binding and fusion, there was no pattern between mother and infant clones that could distinguish them from each other (Fig. 4a). MIP 2669 was the closest to approaching a significant trend ($p = 0.192$), with mother clones having a higher average binding. For 2669, mother clones averaged 2.366% binding and infants averaged 0.535% binding. However, variation in binding was high among the mother clones with M3 binding similar levels to the infant clones and preventing a clear trend for transmission from emerging. MIP 2617 had a similar trend as 2669, where mother clones had a higher average binding (0.928%) than infants (0.175%). This trend was also not significant ($p = 0.417$). The difference in binding between mothers and infants can be attributed to one clone (M4) that bound much more than any other clone. MIP 1084 had similar levels of binding among all clones and the average binding for mothers was 0.055% and for infants was 0.064%.

Discussion

Our previous studies showed that V1-V5 of *env* is capable of influencing replicative fitness of viral variants from MIP and the fitness is predictive of transmission [Kong et al, 2008]; however, Env synthesis and processing, viral infectivity, replication kinetics, and Env incorporation into virions failed to predict transmission [Zhang et al, 2010]. We have also shown that Env fusion does not predict transmission. In this study we have also shown that viral binding does not predict transmission and cannot account for the differences that were observed in replicative fitness. As with fusion we can hypothesize we failed to find a correlation between binding and replicative due in part to measuring a single round versus multiple rounds of infection.

In all cell types and with all viral preparations we observed variation in binding abilities from the clones derived from the MIPs. However, we did not see a correlation between binding activities and fusion activities in any of the clones derived from the MIP. It is possible that a correlation does not exist, because bound virus is inactivated due to non-productive endocytosis, endocytosis that does not lead to fusion [Miyachi et al, 2009]. Our binding assay may be measuring virus that is bound to CD4, but is incapable of binding to CCR5 and thus will not complete fusion. Another study found that monoclonal antibody (MAb) inhibition of HIV-1 entry and fusion did not correlate with inhibition of binding of a gp120-sCD4 complex to CCR5 positive cells [Olson et al, 1999]. It has been hypothesized that entry has three distinct stages, receptor binding, coreceptor binding, and fusion. There may be common factors that are capable of

influencing all three stages, but it is possible that these stages are semi-independent of each other.

There was a major limitation in our study due to the methodology. There was a high level of variation between experiments and within experiments; producing a large standard deviation that remained above the mock infection, but close to these levels. The major drawback of this methodology appears to be an inability to distinguish a true positive signal from background. This problem could be due to an over representation of background (unbound virus that is not washed away), an under representation of bound virus (cells or virus that is washed away), or a combination of both. These factors could also account for differences we see between the different viral preparations. We would expect the same trends to be seen in the EGFP/DsRed2 viruses, the BlaM viruses, and the viruses without any markers. However, we do not see a repeatable trend. There is a slight possibility that the addition of the marker to the proviral clones affects the other biological properties of the viruses and is producing the differences that are observed between the different viral preparations. For example, we do see a lower viral titer of viruses containing the BlaM-Vpr fusion protein compared to viruses produced from proviral constructs without a marker. Another method must be applied to measure viral binding to confirm our results and draw conclusions about the role viral binding plays during transmission.

In summary, our data suggests that the Env V1-V5 region derived from subtype C transmitting MIP is capable of influencing viral binding. However, binding is not predictive of perinatal transmission, fusion, or replicative fitness. Viral entry into target

cells is a complicated process that is influenced by multiple factors and it is likely that no single biological function of Env determines entry or transmission.

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Figure Legend

Figure 1. Relative binding of EGFP/DsRed2 labeled viruses. Binding of viruses containing the patient Env V1-V5 with EGFP/DsRed2 was done in U87-derived cells. One day prior to the assay U87 and U87.CD4.CCR5 cells were plated, virus was added the next day next day and incubated for 1hr at 4°C. Cells were vigorously washed with PBS, lysed, and collected. Lysates were clarified by centrifuging at 10,000xg for 10min and assayed for bound p24 by p24 ELISA as described above. Relative binding is determined using the U87 cell line to measure background signal (non-specific binding) and expressed as a fold increase over this level. A. Relative binding of infant (EGFP) and mother (DsRed2) clones from MIP 1084. B. Average binding of all infant and mother clones.

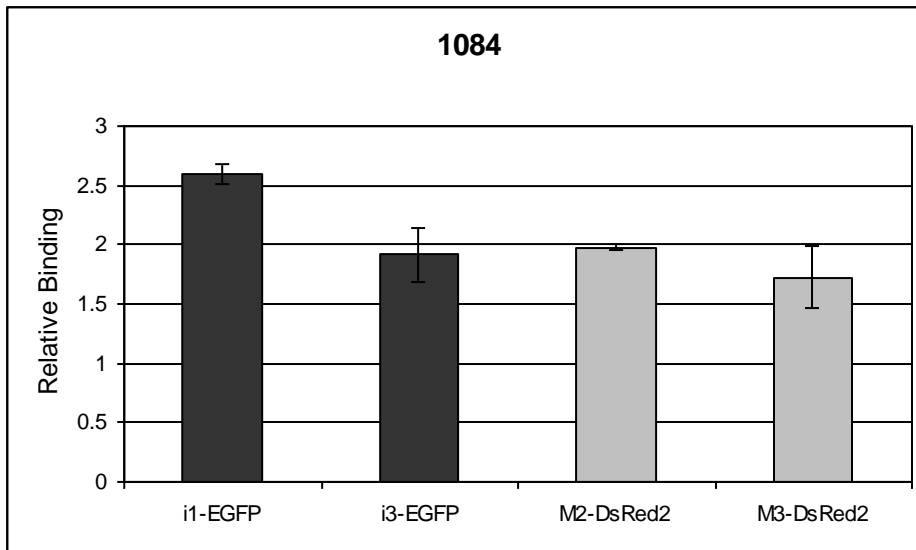
Figure 2. Relative binding of viruses from MIP 1084. Binding of viruses containing the patient Env V1-V5 was done in U87-derived cells. One day prior to the assay U87 and U87.CD4.CCR5 cells were plated, virus was added the next day next day and incubated for 1hr at 4°C. Cells were vigorously washed with PBS, lysed, and collected. Lysates were clarified by centrifuging at 10,000xg for 10min and assayed for bound p24 by p24 ELISA as described above. Relative binding is determined using the U87 cell line to measure background signal (non-specific binding) and expressed as a fold increase over this level. A. Relative binding of infant (EGFP) and mother (DsRed2) clones from MIP 1084. B. Average binding of all infant and mother clones.

Figure 3. Percent binding and fusion of MIP-derived viruses. Both binding and fusion were done in SupT1-CCR5 cells as described previously. A-C. Percent Binding. Binding

is calculated as the percentage of input virus that was bound to the cells. D-F. Percent Fusion. Fusion is calculated as the percent of cells with BlaM activity.

Figure 4. Binding and fusion of infant versus mother viruses. A. Comparison of binding in infant (◆) and mother (●) variants with patient-derived V1-V5 for each transmission pair. Average binding is noted with the black bar. B. Comparison of fusion in infant (◆) and mother (●) variants with patient-derived V1-V5 for each transmission pair. Average fusion is noted with the black bar

A.



B.

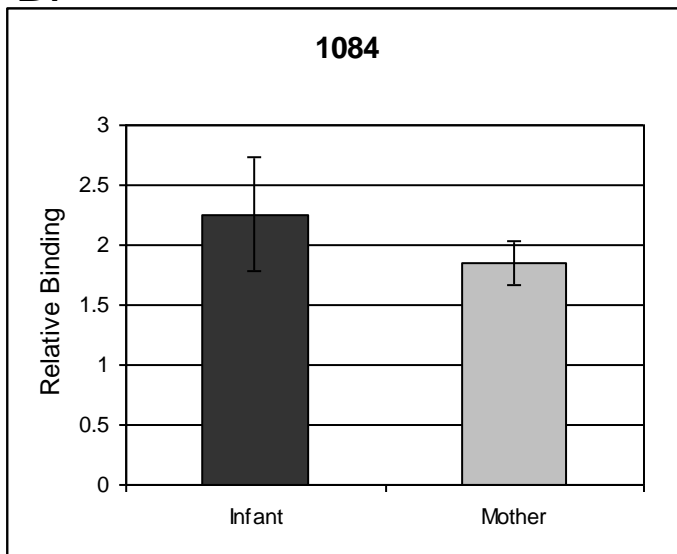
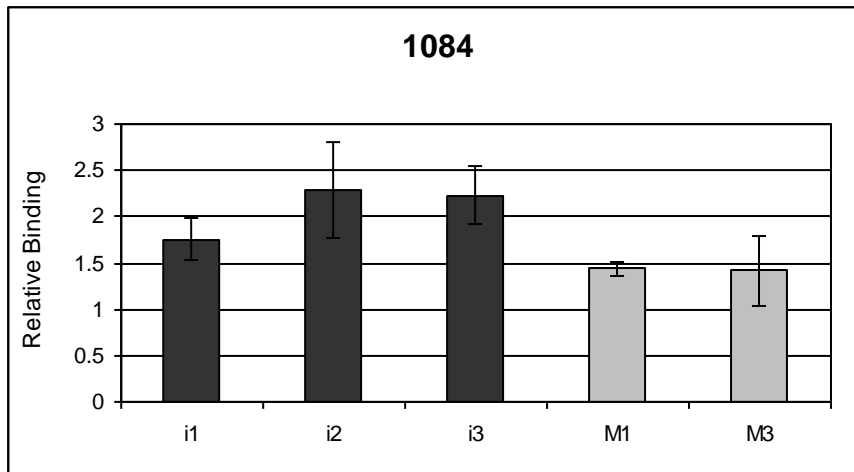


Figure 1

A.



B.

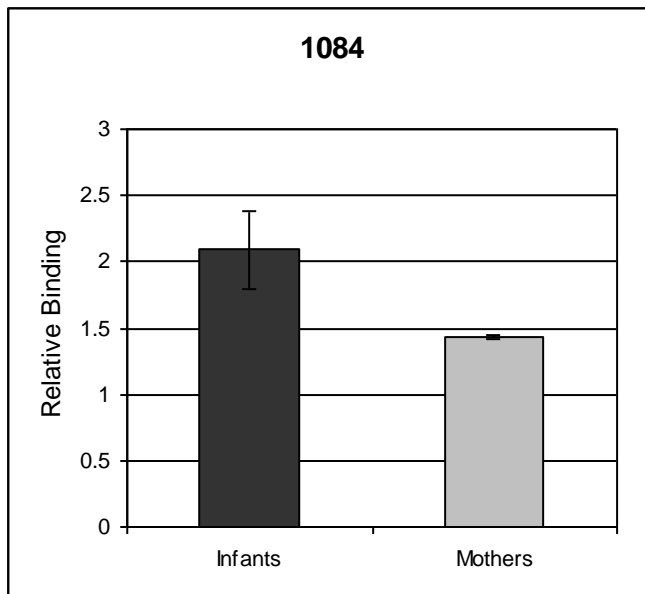
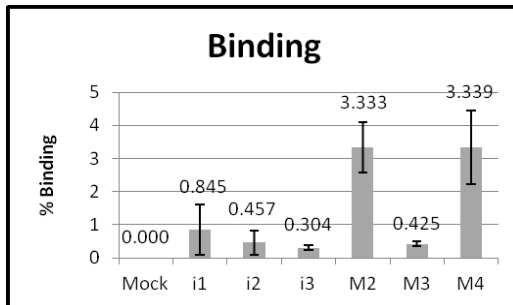
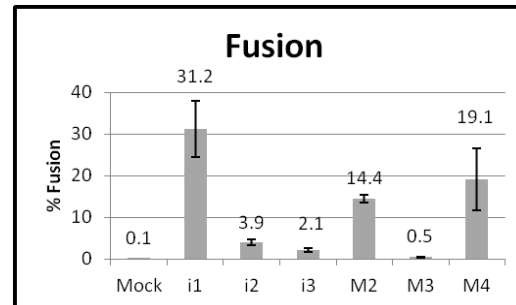


Figure 2

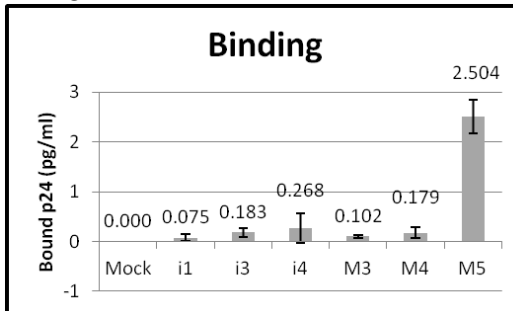
A. 2669



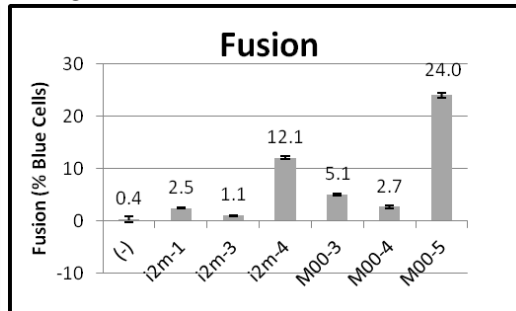
D. 2669



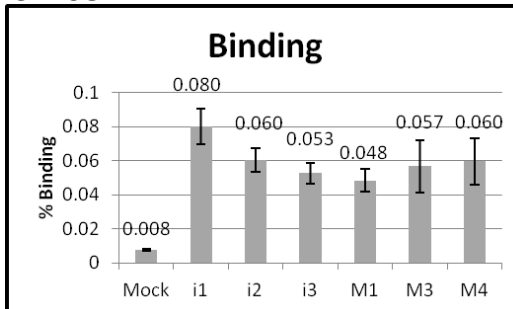
B. 2617



E. 2617



C. 1084



F. 1084

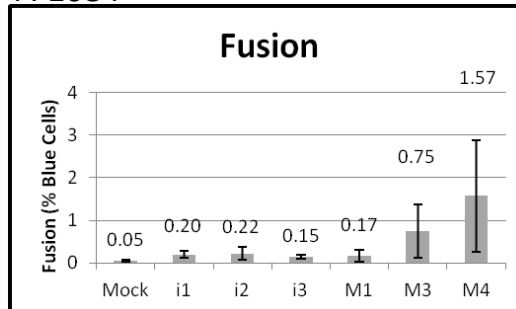


Figure 3

Table 1. Correlation
between binding and fusion.

MIP	R	R²
2669	0.424	0.180
2617	0.192	0.037
1084	0.027	0.0007

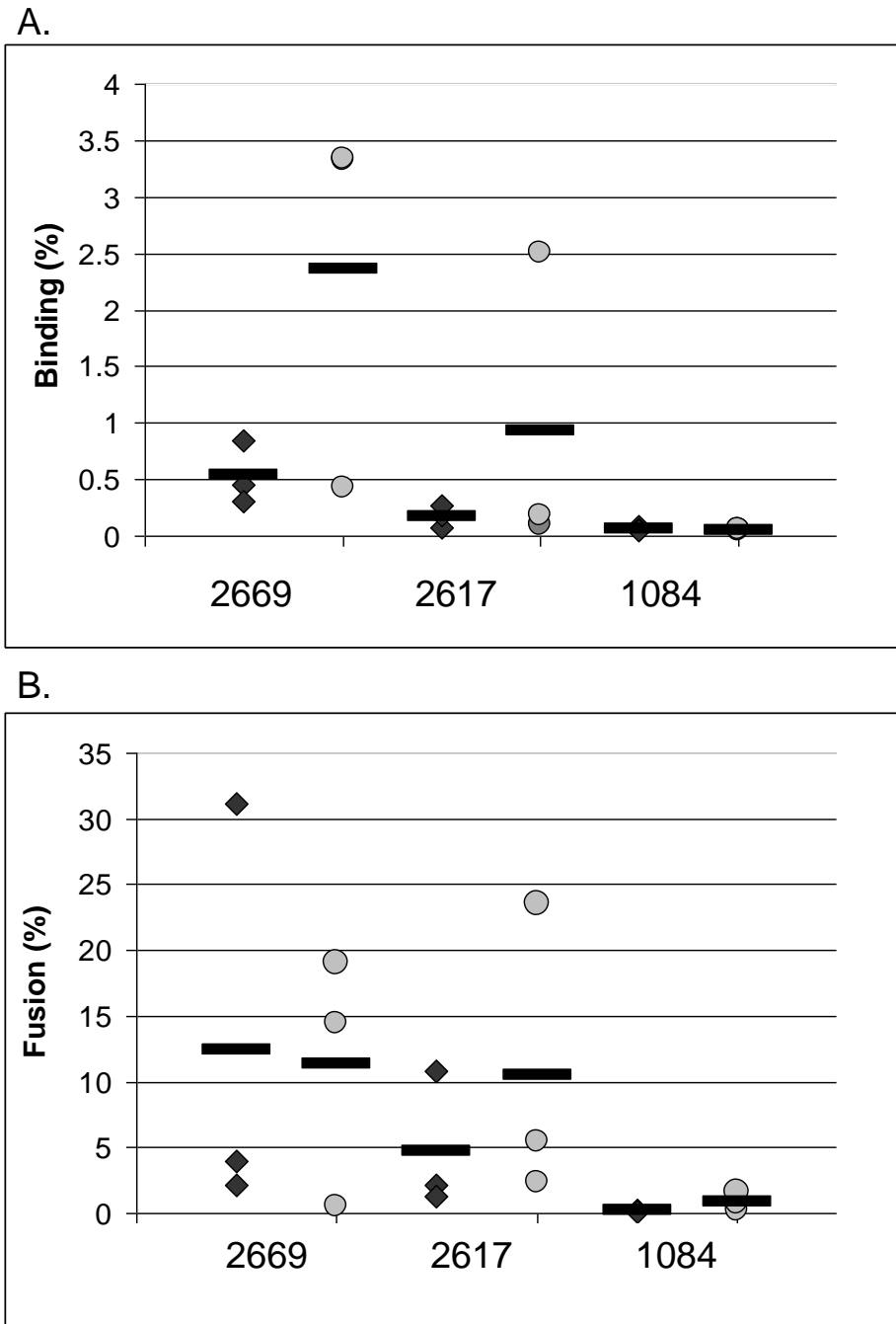


Figure 4

Conclusion

Our previous study demonstrated that replicative fitness, influenced by the Env V1-V5 region derived from subtype C transmitting MIP, is predictive of transmission. Replicative fitness was higher in transmitted infant-derived viruses than non-transmitted mother-derived viruses. However, no other biological properties of Env that have been measured are predictive of replicative fitness or perinatal transmission. The current study sought to determine if fusion and binding activities are influenced by Env V1-V5 and if any differences correlate to replicative fitness and transmission. We found that neither fusion nor binding is predictive of transmission. However, we did see clonal variation in both fusion and binding activities of all MIP Clones studied, demonstrating that Env V1-V5 is capable of influencing both activities.

Differences that we observed in fusion of the viral variants from the MIP did not correlate with transmission, but did correlate with infectivity. We also saw that the amount of Env incorporated into virions cannot account for differences in fusion activities. It is likely that the primary genetic sequence of the Env from the MIP, the structure/stability of the Env from the MIP, or a combination of both factors may play a role in fusion mediated viral entry into target cells. We also found that fusion and binding did not correlate, which provides further evidence that entry occurs in distinct stages that are influenced by different viral and cellular factors.

There are some limitations with our study. The assay we used to measure viral binding was not yet optimized and additional binding assays should be employed to confirm these results. There are also a number of follow-up analyses that need to be

carried out. First, in depth analysis of the Env sequences from the MIP clones should be performed. Mutagenesis analysis should also be done to determine if any mutations are capable of influencing either fusion or binding or both. Second, the location of PNGS should also be analyzed to determine if they are influencing fusion or binding. Third, the structural analysis should be done to determine the stability of the gp120-gp41 complex of the Env derived from the MIPs. Nevertheless, the data from this study have provided several avenues of new inquiry to better understand the functional properties of Env and its role in perinatal transmission.