Role of Vpu in HIV-1 pathogenesis

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by

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Certificate

This is to certify that the research work embodied in this thesis entitled **"Role of Vpu in HIV-1 Pathogenesis"** has been carried out by Mr. Amjad Husain under my direct supervision at the Virology Laboratory, International Centre for Genetic Engineering and Biotechnology, New Delhi. This work is original and no part of this thesis has been submitted for the award of any other degree or diploma to any other university.

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Declaration

I hereby declare that the research work described in this thesis entitled ***"Role of Vpu in HIV-1 pathogenesis"*** has been carried out by me under the supervision of Dr. Shahid Jameel in the Virology Laboratory, International Centre for Genetic Engineering and Biotechnology, New Delhi.

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"A journey is easier when you travel with people and interdependence is certainly more valuable than independence."

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Abbreviations and Symbols

Ab	Antibody
AIDS	Acquired Immunodeficiency Syndrome
ATP	Adenosine Triphosphate
bp	base pair
BSA	Bovine Serum Albumin
Ca++	Calcium
CCR5	C-C Chemokine Receptor 5
CD	Cluster of Differentiation
CDK	Cyclin Dependent Kinase
cDNA	complementary Deoxyribonucleic Acid
CRF	Circulating Recombinant Form
CMV	Cytomegalovirus
CXCR4	CXC Chemokine Receptor 4
DMSO	Dimethyl Sulphoxide
DMEM	Dulbecco's Modified Eagle Medium
ECFP	Enhanced Cyan Fluorescent Protein
EDTA	Ethylene Diamine Tetra acetic Acid
EGFP	Enhanced Green Fluorescent Protein
FBS	Fetal Bovine Serum
GST	Glutathione-S-Transferase
HIV	Human Immunodeficiency Virus
HIV-1	Human Immunodeficiency Virus Type 1
hr (s)	hour(s)
HRP	Horse Radish Peroxidase
IFN	Interferon
IL	Interleukin
Ig	Immunoglobulin
IPTG	Isopropyl-Thio Galactoside
kDa	Kilodalton
kb	Kilobases
LTR	Long Terminal Repeat
MAPK	Mitogen Activated Protein Kinase
MHCI	Major Histocompatibility Complex Class I
MHCII	Major Histocompatibility Complex Class II
mg	milligram
ml	millilitre
µg	microgram
µl	microlitre
µM	micromolar
min	minute(s)
nM	nanomolar

nm	nanometer
NSI	Non-Syncytium Inducing
nt	Nucleotide
NRE	Negative Regulatory Element
ORF	Open Reading Frame
PBS	Phosphate Buffer Saline
PKC	Protein Kinase C
PMSF	Phenyl Methyl Sulphonyl Fluoride
PTK	Protein tyrosine Kinase
PCR	Polymerase Chain Reaction
p-TEFb	positive Transcription Elongation Factor b
PTK	Protein Tyrosine Kinase
RNA	Ribonucleic Acid
RTK	Receptor Tyrosine Kinase
SI	Syncytium inducing
SH2	Src Homology 2 Domain
SH3	Src Homology 3 Domain
SDS PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TBP	TATA Binding Protein
TGN	Trans Golgi Network
TNF	Tumor Necrosis Factor
X-gal	5-bromo-4-chloro-3-indoylyl-galactoside

Contents

Chapter 1

Introduction and Review of Literature

1. Introduction and Review of Literature

1.1 Historical Background

Human immunodeficiency virus (HIV) is a retrovirus that causes Acquired Immuno Deficiency Syndrome (AIDS), a condition in humans in which the immune system fails, leading to life-threatening opportunistic infections. This virus has been the subject of intense investigations for more than two decades, and a great deal has been learnt about how the virus infects cells, multiplies and causes disease. In the past few years, substantial progress has been made towards understanding the detailed biochemical function of each viral component.

The disease AIDS was originally recognized based upon a report in the Morbidity and Mortality Weekly Report, from the United States Centers for Disease Control and Prevention (CDC) in 1981. That report described five cases of *Pneumocystis carinii* pneumonia occurring in otherwise healthy males. Subsequent cases showed a common feature of impaired T cell function to mitogen and antigen stimulation in AIDS patients. This T cell dysfunction stemmed from a viral infection of CD4 positive (CD4+) T cells. At that time, AIDS had already appeared as a long-lasting disease, with an extremely long lag time between exposure to the agent (through blood or sexual activity) and the profound state of immune suppression characterized by generalized and persistent lympadenopathy, and opportunistic infections such as *Pneumocystis carinii* pneumonia, *Toxoplasma gondaii* encephalitis, cytomegalovirus-associated retinitis, cryptococcal meningitis, and a variety of unusual cancers like non-Hodgkin's lymphoma and Kaposi's sarcoma (Barre-Sinoussi 1983). A laboratory finding common to all such individuals was a marked depletion of the CD4+ T-lymphocyte population in their peripheral blood (Moss 1989). The findings regarding the T-cell subgroup suggested an agent that specifically targeted CD4+ T cells, and HTLV was known to be one such agent.

The human T-cell leukemia virus type-I (HTLV-I) was suspected to be the causative agent either because it was isolated from patients with lymphadenopathy, or due to the presence of antibody cross-reactivity among these patients (Essex 1983; Gallo 1983).

Moreover, there were animal models in which lymphotropic retroviruses caused not only leukemias or lymphomas, but also an AIDS-like wasting syndrome. Furthermore, HTLV was transmitted through blood and sexual activity, as well as from mother to infant. Finally, the CDC reported cases of AIDS in patients with hemophilia who had received only filtered clotting factors, which seemed to eliminate the possibility that the agent was a microorganism larger than a virus. This set of arguments convinced to initiate the search for an HTLV-like virus in patients with AIDS.

In early 1983, an isolate was obtained in Paris, from cultured T lymphocytes derived from a lymph-node biopsy specimen from a patient with lymphadenopathy, a syndrome that was considered to be a precursor of AIDS. This virus proved to be different from HTLV in terms of antigenicity and morphology, but it could be propagated only in fresh cultures of T lymphocytes and not in permanent T-cell lines, which impeded its full characterization. This technical breakthrough was first achieved in late 1983. The growth of the putative virus in T-cell lines was an enormous step, facilitating the development of a blood test for HIV, which became available in blood-transfusion centers in 1985 and produced convincing evidence of the association between HIV infection and AIDS. The causative virus was identified as a retrovirus in 1983 (Barre-Sinoussi 1983). It was initially called as lymphadenopathy associated virus (LAV) and subsequently named as human immunodeficiency virus type 1 (HIV-1). A second similar but antigenically distinct retrovirus was isolated from AIDS patients in West Africa in 1986 and was named as human immunodeficiency virus type 2 (HIV-2). While HIV-2 is prevalent in West Africa, HIV-1 is prevalent worldwide.

1.2 Classification of HIV

HIV was classified as a member of the genus Lentivirus of the family Retroviridae. Retroviral genomes are composed of ribonucleic acid (RNA) and contain three basic polyprotein coding genes called group specific antigen (*gag*), polymerase (*pol*) and envelope (*env*) which were also found in HIV. The HIV was further placed into genus Lentivirus, which differ from other retroviruses by their long latent period before the

manifestation of clinical illness and carry a complex combination of genes besides *gag*, *pol* and *env*. Lentiviruses have many common morphologies and biological properties and are transmitted as single-stranded, positive-sense, enveloped RNA viruses. Two species of HIV that infect humans are HIV-1 and HIV-2. Of these, HIV-1 is more virulent, is easily transmitted and causes a majority of HIV infections globally; while HIV-2 is less transmittable and is largely confined to West Africa. HIV-1 is thought to have originated in southern Cameroon after jumping from wild chimpanzees (*Pan troglodytes troglodytes*) to humans during the twentieth century. HIV-2 may have originated from the Sooty Mangabey (*Cercocebus atys*), an Old World monkey of Guinea-Bissau, Gabon, and Cameroon. Three groups of HIV-1 have been identified based on phylogenetic analysis of the nucleotide sequences of the *env* and *gag* gene, the 'major' M-group, 'minor' N-group and a genetically distant 'outlier' O-group. The M group includes 95% of all HIV-1 isolates, and is subdivided into at least ten clades A to J. The distribution of these clades is geographically distinct as shown in Figure 1.1.

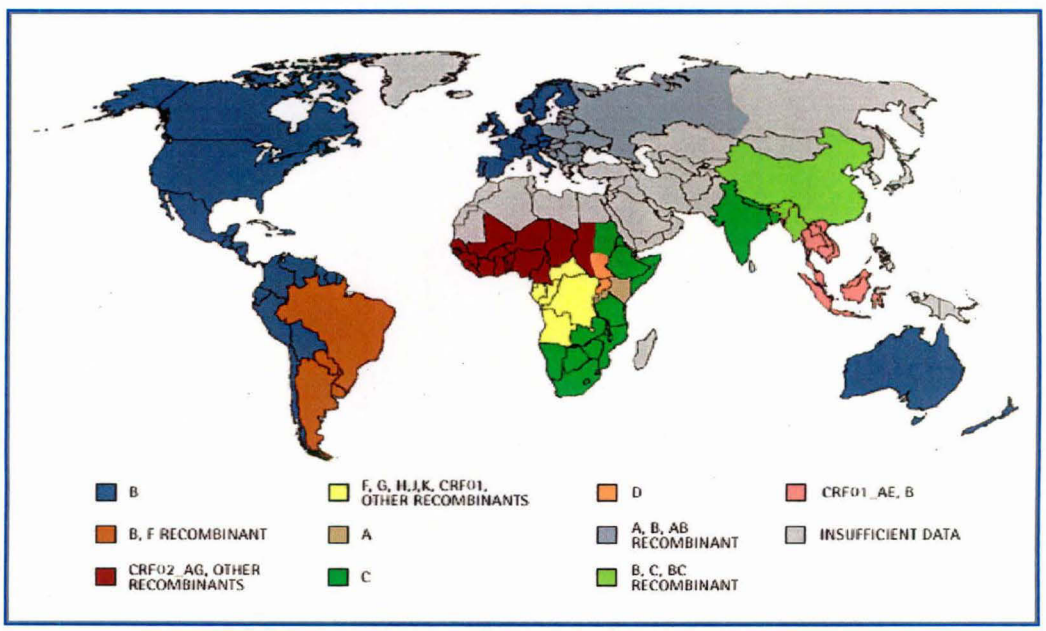


Figure 1.1: Global distribution of different subtypes of HIV-1 infection (Source: UNAIDS/WHO report, 2005)

Subtype C is the most prevalent and is estimated to account for about 50% of global HIV-infections. It is largely predominant in southern and eastern Africa, India and Nepal.

Historically, subtype B has been the most common subtype in Europe, the Americas, Japan and Australia. Although this remains the case, other subtypes are becoming more frequent and now account for at least 25% of new infections in Europe (Kanki 1999). Occasionally, two viruses of different subtypes can meet in the cell of an infected person and create a new hybrid virus. Many of these new strains do not survive for long, but those that infect more than one person are known as "circulating recombinant forms" or CRFs based on the complete sequence of the viral genome (Louwagie 1993). The HIV-1 subtypes and CRFs are unevenly distributed throughout the world, with the most widespread being subtypes B and C. Subtype A and CRF A/G predominate in West and Central Africa, with subtype A possibly also causing much of the Russian epidemic (Bobkov 2004). Subtype D is generally limited to East and Central Africa; A/E is prevalent in South-East Asia, F has been found in Central Africa, South America and Eastern Europe; G and A/G have been observed in western and eastern Africa and Central Europe. Subtype H has only been found in Central Africa; J only in Central America; and K only in the Democratic Republic of Congo and Cameroon.

Within each subtype of HIV-1 and HIV-2 there is a high degree of variation even when samples are recovered from a single patient. These variations are not only at the nucleotide level but also result in amino acid substitutions (non-synonymous), whereas some variants show nucleotide change but do not result in a different protein sequence (synonymous). Among all the genes of HIV the maximum variability is found in the *env* gene making five hypervariable regions (V1-V5) in Env protein. The variants of virus within an individual are called as quasi-species which can vary up to 10% in terms of nucleotide sequences within an infected individual (Myers 1994). At the nucleotide level, viral subtypes differ from each other by about 12-14% in the *gag* coding sequence also but this variation is up to 30% in their *env* coding sequences (Louwagie 1993; Myers 1994). This sequence diversity and different isolates at times show variable phenotypes such as infectivity, transmission and neutralization. It is almost certain that new HIV genetic subtypes and CRFs will be discovered in the future, and indeed that new ones will develop as virus recombination and mutations continue to occur. There are many cases of people co-infected with two or more strains documented till date. However, it is now thought that "superinfection" can also occur. In these cases, the second infection would

have occurred several months after the first. It would appear that the body's immune response to the first virus would sometimes not be enough to prevent infection with a second strain, especially with a virus belonging to a different subtype.

1.3 Origins of HIV

HIV is a lentivirus which literally means 'slow virus' as they take such a long time to produce any adverse effects in the host. They have been found in a number of different animals, including cats, sheep, horses and cattle. However, the most interesting lentivirus in terms of the investigation into the origins of HIV is the Simian Immunodeficiency Virus (SIV) that affects monkeys. It is now generally accepted that HIV is a descendant of SIV because certain SIV strains bear a very close resemblance to HIV-1 and HIV-2. The HIV-2 corresponds to *SIVsm*, a strain found in the sooty mangabey (also known as the green monkey), which is indigenous to western Africa. The more virulent, pandemic strain of HIV, namely HIV-1, was until recently more difficult to place. Until 1999, the closest counterpart that had been identified was *SIVcpz*, found in chimpanzees. Subsequently, Gao, et al (1999), had reported a type of *SIVcpz* that was almost identical to HIV-1. This particular strain was identified in a frozen sample taken from a captive member of the sub-group of chimpanzees known as *Pan troglodytes troglodytes* (*P. t. troglodytes*), which were once common in west-central Africa, suggesting that the virus had at some point crossed species from chimps to humans. Researchers have concluded that wild chimps had been infected simultaneously with two different SIVs which had shown recombination to form a third virus that could be passed on to other chimps and, more significantly, was capable of infecting humans and causing AIDS. It was also proposed that all three 'groups' of HIV-1 - namely Group M, N and O came from the SIV found in *P. t. troglodytes*, and that each group represented a separate crossover 'event' from chimps to humans. It has been known for a long time that certain viruses can pass between species. Indeed, the very fact that chimpanzees obtained SIV from two other species of ape shows just how easily this crossover can occur. When a viral transfer between animals and humans takes place, it is known as zoonosis. Some of the most

common theories about how this 'zoonosis' took place, and how SIV became HIV in humans are stated below.

1.3.1 The 'Hunter' theory

This is the most commonly accepted theory, which states that *SIVcpz* was transferred to humans as a result of chimpanzees being killed and eaten, or their blood getting into cuts or wounds on the hunter. Normally the hunter's body would have fought off SIV, but on a few occasions it adapted itself within its new human host and became HIV-1. Wolfe et al (2004) showed how retroviral transfer from primates to hunters is still occurring. In a sample of 1099 individuals in Cameroon, they discovered ten (1%) that were infected with SFV (Simian Foamy Virus), a virus which, like SIV, was previously thought only to infect primates. All these infections were believed to have been acquired through the butchering and consumption of monkey and ape meat.

1.3.2 The Contaminated Needle theory

This is an extension of the original 'hunter' theory. In the 1950s, the use of disposable plastic syringes became common around the world as a cheap, sterile way to administer medicines. However, to African healthcare professionals, the huge quantities of syringes needed would have been very expensive. It is therefore likely that one syringe would have been used to inject multiple patients without any sterilization in between. This would have rapidly transferred viruses from one person to another. This would also create an opportunity for the virus to mutate and replicate in each new individual it entered, even if the SIV within the original person infected had not yet converted to HIV.

1.3.3 The Colonialism theory

It is one of the more recent theories, which explains how this original infection could have led to an epidemic (Chitnis et al 2000). During the late 19th and early 20th century, much of Africa was ruled by colonial forces. In areas such as French Equatorial Africa and the Belgian Congo, the colonial rule was particularly harsh and many Africans were forced into labour camps where sanitation was poor, food was scare and physical

demands were extreme. These factors alone would have been sufficient to create poor health in anyone, so SIV could easily have infiltrated the labour force and taken advantage of their weakened immune systems to become HIV. A stray and perhaps sick chimpanzee infected with SIV would have made a welcome extra source of food for the workers.

This theory also suggests that many of the labourers would have been inoculated with unsterile needles against diseases such as smallpox to keep them alive and working, creating numerous possibilities for onward transmission. A large number of labourers would have died before they even developed the first symptoms of AIDS. One final factor, which supports this theory, is the fact that the labour camps were set up around the time that HIV was first believed to have passed into humans - the early part of the 20th century.

1.4 Global Summary of HIV Infection

The global AIDS epidemic continues to grow and there is concern that some countries are seeing resurgence in new HIV infection rates, which were previously stable or declining. Since the first cases of AIDS were reported in 1981, infection with HIV has grown to pandemic proportions, resulting in an estimated 65 million infections and 25 million deaths. During 2005 alone, an estimated 2.8 million persons died from AIDS, 4.1 million were newly infected, and 38.6 million were living with HIV (Figure 1.2). HIV continues to disproportionately affect certain geographic regions such as sub-Saharan Africa and the Caribbean, and certain subpopulations such as women in sub-Saharan Africa, men who have sex with men (MSM), injection-drug users (IDUs), and sex workers. Effective prevention and treatment of HIV infection with antiretroviral therapy (ART) is now available, even in countries with limited resources.

In sub-Saharan Africa, HIV prevalence has remained constantly high for the past several years. Approximately 10% of the world population lives in sub-Saharan Africa, but the region is home to approximately 64% of all HIV infected people. Some 2.8 million adults and children became infected with HIV in 2006, more than in all other regions of the

world combined. The 2.1 million AIDS-related deaths in sub-Saharan Africa represent 72% of global AIDS deaths. Transmission is primarily through heterosexual contact, and more women are HIV infected than men. Southern Africa is the epicenter of the AIDS epidemic; all countries in the region except Angola have an estimated adult (i.e., aged 15 to 49 years) HIV prevalence exceeding 10%. In Botswana, Lesotho, Swaziland, and Zimbabwe, the estimated adult HIV prevalence exceeds 20%. South Africa, with an HIV prevalence of 18.8% and 5.5 million persons living with HIV, has the largest number of persons living with HIV in the world. Recently, declines in adult HIV prevalence have been observed in Kenya, Uganda, Zimbabwe, and urban areas of Burkina Faso. Although in these countries, HIV-related sexual risk behaviors and HIV incidence have decreased, AIDS death rates continue to rise. In sub-Saharan Africa, only an estimated 17% of those in need of ART received it in 2005.

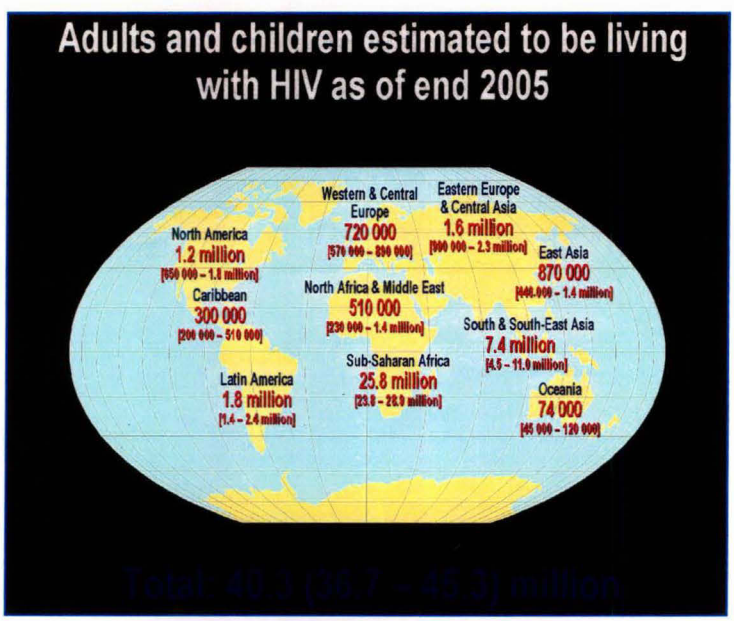


Figure 1.2: Global figures of HIV infection by the end of 2005 (Source: UNAIDS/WHO AIDS Report 2005).

In Asia, adult HIV prevalence is lower than countries in sub-Saharan Africa. The epidemic in most Asian countries is mainly attributed to various high-risk behaviors such as unprotected commercial sex, IDUs and MSM. Of the 8 million HIV-infected persons in Asia, 2.5 million live in India, where the prevalence varies by state. Approximately 80% of HIV infection s in India are acquired heterosexually. In China, about 650,000

IDUs account for approximately half of persons living with HIV infection. In contrast, the epidemics in Thailand and Cambodia have been driven largely by commercial sex. However, HIV prevalence among MSM in Bangkok increased from 17% in 2003 to 28% in 2005. Only 16% of persons in need of ART in Asia received it in 2005.

In Americas, HIV infection is reported mostly among MSM, IDUs, and sex workers. Brazil, the second most populous country in the Americas, has an adult HIV prevalence of 0.5% and has approximately 30% of the population living with HIV in South and Central America and the Caribbean. High-risk behavior among Brazilians aged 15 to 24 years remains high; one in three report initiating sexual activity before 15 years of age, and one in five report having had more than 10 sex partners. Brazil provides free ART to all in need of treatment, and approximately 83% of HIV-infected persons receive therapy.

After sub-Saharan Africa, the Caribbean is the second most HIV-affected region of the world. Like sub-Saharan Africa, HIV transmission in the Caribbean is largely heterosexual. HIV prevalence has declined in urban areas of Haiti but has remained constant in other areas of the Caribbean. Overall in South and Central America and the Caribbean, approximately 68% of persons in need of ART received it in 2005.

From 2003 to 2005, estimates of adult HIV prevalence were lowered in many countries. Some of these reductions might be attributable to the addition of new surveillance sites and population-based surveys that provide better estimates in rural populations, which usually have lower HIV prevalence. However, some countries (including Kenya, Uganda, Zimbabwe, and urban parts of Burkina Faso and Haiti) have reported evidence of actual declines in HIV prevalence. Changes in sexual behavior appear at least partly responsible for these declines, although increasing mortality might have been a contributing factor (Hallett 2006).

During 2003-2005, substantial gains were made in the number of persons receiving ART in resource-limited countries. The "3 by 5" initiative, a strategy of the World Health Organization and UNAIDS, sought to provide treatment to 3 million persons (50% of

those in need of treatment worldwide) in low- and middle-income countries by 2005. By December 2005, 18 countries had met their "3 by 5" target, and the number of persons receiving ART had increased from 0.4 million in December 2003 to 1.3 million. Despite the gains in ART, only 20% of persons in need of treatment in low- and middle-income countries were receiving it in December 2005. Despite a 5-year scale-up of interventions to prevent mother-to-child transmission (PMTCT) of HIV, approximately one in 10 pregnant women were offered PMTCT services. As more HIV-infected persons receive ART, the number of persons living with HIV infection will increase, requiring that prevention programs scale up to prevent HIV transmission from those living with HIV infection and for those at risk of infection.

1.5 AIDS in India

With over one billion inhabitants in India, at least 2.5 million are currently living with HIV. The crisis continues to deepen, with the epidemic affecting all sectors of Indian society, not just groups such as sex workers, that were originally associated with it.

At the beginning of 1986, despite over 20,000 AIDS cases worldwide, India had no reported cases of HIV or AIDS. Later that year, the first cases of HIV infection were diagnosed among sex workers in Chennai, Tamil Nadu. It was noted that contact with foreign visitors had played a role in initial infections among sex workers, and as HIV screening centers were set up across the country there were calls for visitors to be screened for HIV. Gradually, these calls subsided as more attention was paid to ensuring that HIV screening was carried out in blood banks.

In 1987 a National AIDS Control Programme was launched to co-ordinate national responses for AIDS. Its activities covered surveillance, blood screening, and health education (NACO 2006, UNGASS India report). By the end of 1987, out of 52,907 who had been tested, around 135 people were found to be HIV positive and 14 had AIDS. Most of these initial cases had occurred through heterosexual sex, but a rapid spread of HIV was also observed among injecting drug users in Manipur, Mizoram and Nagaland - three north-eastern states of India bordering Myanmar.

At the beginning of the 1990s, as infection rates continued to rise, responses were strengthened. In 1992 the government set up the National AIDS Control Organization (NACO), to oversee the formulation of policies, prevention and control programmes relating to HIV and AIDS. Increasingly, cases of infection were observed among people that had previously been seen as *'low-risk'*, such as housewives and more affluent members of society.

HIV had now spread extensively throughout the country. A 2004 NACO report revealed that the total number of people living with HIV had risen from 0.2 million in 1990 to 3.86 million in 2000. By 2003, 5.1 million infections had been reported. There is disagreement over how many people are currently living with HIV in India. The UNAIDS estimates that there were 5.7 million people in India living with HIV by the end of 2005, suggesting that India has a higher number of people living with HIV than any other country in the world (UNAIDS, 2006 Report on the global AIDS epidemic). On the other hand, NACO has established an estimate of 2.5 million HIV-infected people (NACO June 2007, HIV/AIDS epidemiological Surveillance & Estimation report for the year 2006). These estimates are more accurate than those of previous years, as they are based on an expanded surveillance system and a revised and enhanced methodology. Overall, around 0.36% of India's population is living with HIV. The national HIV prevalence has risen dramatically since the start of the epidemic, but a study released at the beginning of 2006 suggests that the HIV infection rates have fallen in southern India, the region that has been hit hardest by AIDS. In addition, NACO has released figures suggesting that the overall rate of new HIV infections in the country is slowing down as a result of successful prevention campaigns.

1.6 HIV Pathogenesis and Prophylaxis

1.6.1 Modes of HIV Transmission

The course of HIV infection can vary considerably among individuals. The mean interval from infection to the development of AIDS is about 10 years. The spectrum of HIV infection ranges from an asymptomatic state to severe immunodeficiency with associated

opportunistic infections, neoplasms, and other conditions. Initial infection can be followed by an acute flu-like illness with fever, lymphadenopathy, sweats, myalgia, arthralgia, rash, malaise, sore throat, and headache.

The risk of disease progression increases with the duration of infection. Less than 5% of HIV-infected adults develop AIDS within 2 years of infection; without therapy, approximately 20-25% develop AIDS within 6 years after infection, and 50% within 10 years (Alban 2007). Although many diseases/conditions have been identified as AIDS indicators, *P. carinii* pneumonia, HIV wasting syndrome, and candidiasis of the oesophagus are the most common (Mercante 2006). HIV can be transmitted from person to person through infected blood, semen or vaginal secretions, which have the highest concentrations of the virus, by sharing needles and/or syringes (primarily for drug injection) with someone who is infected, or less commonly through transfusions of infected blood or blood clotting factors. Babies born to HIV-infected women may become infected before or during birth or through breast-feeding after birth. Approximately one-quarter to one-third of all untreated pregnant women infected with HIV pass the infection to their babies. A study sponsored by the National Institute of Allergy and Infectious Diseases (NIAID) in Uganda found a highly effective and safe drug for preventing transmission of HIV from an infected mother to her newborn. Results from this study show that a single oral dose of the antiretroviral drug nevirapine (NVP) given to an HIV-infected woman in labor and another to her baby within 3 days of birth reduces the transmission rate of HIV by half compared with a similar short course of AZT (Azidothymidine).

In the health care setting, workers have been infected with HIV after being stuck with needles containing HIV-infected blood or, less frequently, after infected blood gets into a worker's open cut or a mucous membrane (for example, the eyes or inside of the nose). All reported cases suggesting new or potentially unknown routes of transmission are thoroughly investigated by CDC and no additional routes of transmission have been recorded. The HIV does not survive well in the environment, making the possibility of environmental transmission remote. The CDC studies have shown that drying of even high concentrations of HIV reduces the amount of infectious virus by 90-99% within

several hours. No one has been identified to be infected due to environmental contact. Additionally, HIV is unable to reproduce outside its living host. A theoretical HIV transmission risk does exist if the unhealed or abraded tissues come into contact with an infected person's blood or other infectious body fluid. CDC has investigated only one case of HIV infection that may be attributed to contact with blood during open-mouth contact. Contact with saliva, tears, or sweat has never been shown to result in transmission of HIV.

From the onset of the HIV epidemic, there has been concern about transmission of the virus by biting and blood sucking insects. However, studies have shown no evidence of HIV transmission through insects (Gershon et al., 1990), even in areas where there are many cases of infection and large populations of insects such as mosquitoes. HIV survives for only a short time inside an insect and, unlike organisms that are transmitted via insect bites, it does not multiply in insects. There is also no reason to fear that a biting or bloodsucking insect, such as a mosquito, could transmit HIV from one person to another through HIV-infected blood left on its mouthparts. Two factors serve to explain why this is so. First, infected people do not have constant, high levels of HIV in their bloodstreams and, second, insect mouthparts do not retain large amounts of blood on their surfaces. Further, biting insects normally do not travel from one person to the next immediately after ingesting a blood meal.

In case of HIV infection, a person may have a flu-like illness including fever, headache, tiredness and enlarged lymph nodes within a month or two after exposure to the virus. These symptoms usually disappear within a week to a month and are often mistaken for those of another viral infection. During this period, people are very infectious, and HIV is present in large quantities in genital fluids. More persistent or severe symptoms may not appear for 10 years or more after HIV first enters the body in adults, or within 2 years in children born with HIV infection. This period of "asymptomatic" infection varies greatly in each individual. Some people may begin to have symptoms within a few months, while others may be symptom-free for more than 10 years.

Even during the asymptomatic period, the virus is actively multiplying, infecting, and killing cells of the immune system. The most obvious effect of HIV infection is a decline in the number of CD4+ T cells (Capon 1991). As the immune system worsens, a variety of complications start to take over. For many people, the first signs of infection are large lymph nodes or "swollen glands" that may be enlarged for more than 3 months. Other symptoms often experienced months to years before the onset of AIDS include lack of energy, weight loss, frequent fevers and sweats, persistent or frequent yeast infections (oral or vaginal), persistent skin rashes or flaky skin, pelvic inflammatory disease in women that does not respond to treatment and short-term memory loss. Some people develop frequent and severe herpes infections that cause mouth, genital, or anal sores, or a painful nerve disease called shingles.

The CDC's definition of AIDS includes all HIV-infected people who have fewer than 200 CD4+ T cells per cubic millimeter of blood (healthy adults usually have CD4+ T-cell counts of 1,000 or more). In addition, the definition includes 26 clinical conditions that affect people with advanced HIV disease (Crowe 1991). Most of these conditions are opportunistic infections that generally do not affect healthy people. Symptoms of opportunistic infections common in people with AIDS include coughing and shortness of breath, seizures and lack of coordination, difficult or painful swallowing, mental symptoms such as confusion and forgetfulness, severe and persistent diarrhea, fever, vision loss, nausea, abdominal cramps, and vomiting, weight loss and extreme fatigue, severe headaches and coma. Children with AIDS may get the same opportunistic infections, as do adults with the disease. In addition, they also have severe forms of the typically common childhood bacterial infections, such as conjunctivitis (pink eye), ear infections, and tonsillitis. People with AIDS are also particularly prone to developing various cancers, especially those caused by viruses such as Kaposi's sarcoma and cervical cancer, or cancers of the immune system known as lymphomas.

1.6.2 Course of infection

Although CD4+ T cells are usually considered to be the most important cells in the course of HIV infection and it is their loss that leads to immune suppression, other cells

do become infected. Macrophages and dendritic cells (DCs) are very important as they form a reservoir outside the blood and carry the virus into extravascular tissues. Non-proliferating mature macrophages can support HIV production for a long time without being killed. There is no latency in these cells, the virus just buds.

HIV can also bind to CD4 negative cells *in vivo* and referred as cell bound virus (Olinger 2000). Some CD4 negative cells become infected; for example, epithelial cells of the vagina and rectum, endothelial cells of brain capillaries and other cells of the CNS such as astrocytes and glial cells. These may take up HIV via a galactocerebroside receptor. Dendritic cells may also be very important. These cells appear to trap virions and carry them to the lymph nodes. If acquired by sexual activity, the virus enters the body in infected macrophages in semen or vaginal secretions. Dendritic cells in the mucosal linings bind the virus shed by macrophages and carry it to the lymph nodes where CD4+ T cells become infected. During the course of the disease, the virus migrates to other cell types. Initially, HIV infection produces a mild disease that is self-limiting. This is not seen in all patients and about half remain asymptomatic during the initial period of infection. In the period immediately after infection, virus titres rise (about 4 to 11 days after infection) and continue at a high level over a period of a few weeks. There is a "window period" of seronegativity (Figure 1.3) during which an infected person does not

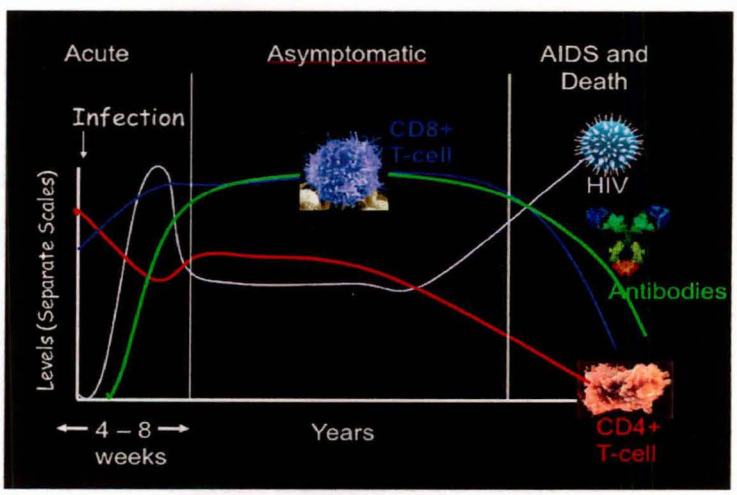


Figure 1.3: Course of HIV infection showing different stages of HIV infection up to the onset of disease. Loss of CD4+ T-cells compromises the ability of CTL to work in late stage of HIV infection (Source: www.studentreader.com)

give a positive western blot or ELISA test for HIV, even though the viral load is high and the patient may exhibit some symptoms. This seronegative period can last for six months before seroconversion.

The period of clinical latency varies, from as little as 1 to 2 years to more than 15 years. Onset of AIDS is rare in less than 3 years except in children. But, eventually, the virus can no longer be controlled as helper CD4+ cells are destroyed (Figure 1.3). Ironically, the killer cells needed to control HIV also damage the helper T cells that they need to function efficiently. With the lack of CD4+ cells, new cytotoxic T cell responses cannot occur as helper cells are lacking and such new responses are required as the virus mutates. As the CD4+ cells fall below 200/mm^3, virus titers rise rapidly and immune activity drops precipitously. It is the loss of immune competence that enables normally benign opportunistic parasites such as other viruses, fungi or protozoa to cause infections. Once AIDS develops, there is considerable variability. Some patients with clinical AIDS do survive for several years while others who appear relatively healthy can suddenly succumb to a major opportunistic infection. At this stage, syncytium-inducing HIV strains also appear in about half of the AIDS patients. These are more CD4+ cell tropic than the initially infecting HIV and this contributes to the rapid loss of CD4+ cells in later stages of the disease.

1.6.3 Treatment of HIV infection

Antibodies against HIV generally do not reach noticeable levels in the blood for 1 to 3 months following infection. It may take the antibodies as long as 6 months to be produced in quantities large enough to show up in standard blood tests. Hence, to determine whether a person has been infected recently (acute infection), the HIV genetic material is screened, typically in a polymerase chain reaction (PCR) test.

For the prevention or treatment of HIV there is no successful vaccine yet. The United States Food and Drug Administration (FDA) has approved a number of drugs for treating HIV infection. The first group of drugs include reverse transcriptase (RT) inhibitors, interrupts an early stage of the virus replication (Figure 1.4). These drugs, nucleoside

reverse transcriptase (RT) inhibitors are mainly nucleoside analogs, include AZT, ddC (dideoxy-3'-hydroxymethyl cytidine), ddI (dideoxyinosine), Stavudine, Lamivudine, Abacavir, and Tenofovir etc. There are some non-nucleoside reverse transcriptase inhibitors (NNRTIs), such as, Delavridin, Nevirapine and Efravirenz.

A second class of drugs has also been approved for treating HIV infection. These drugs, are protease inhibitors, include Ritonavir, Saquinivir, Indinavir, Amprenivir, Nelfinavir, Lopinavir, Atazanavir, and Fosamprenavir. Protease inhibitors are used in combinations with reverse transcriptase inhibitors. This makes it more difficult for the virus to develop multiple mutations simultaneously to escape the effects of multiple drugs.

A third and new class of drugs, known as fusion or entry inhibitors, Fuzeon (enfuvirtide or T-20), the first approved fusion inhibitor, works by interfering with the ability of HIV-1 to enter into cells by blocking the merging of the virus with the cell membranes (Figure 1.4). Fuzeon is designed for use in combination with other anti-HIV treatment.

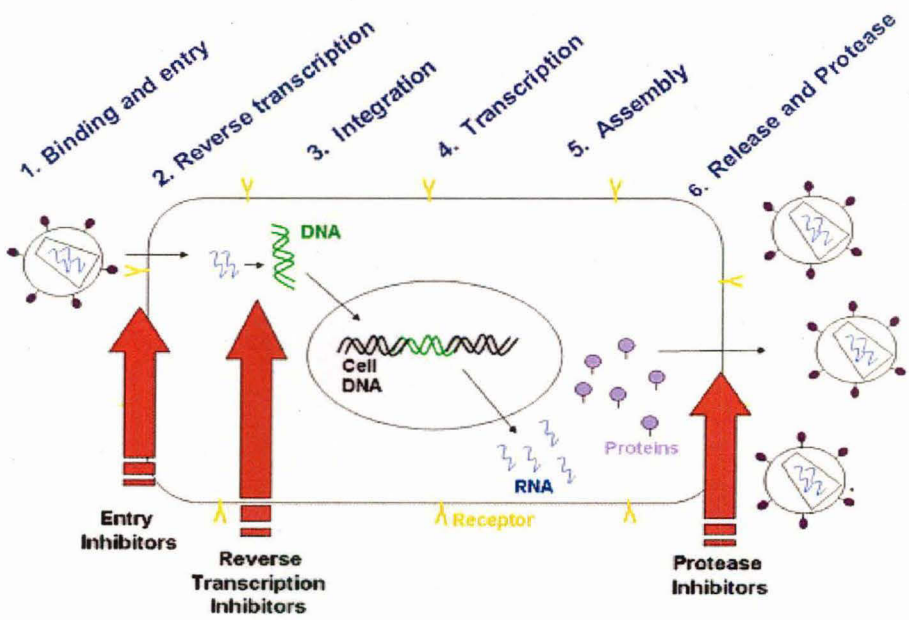


Figure 1.4: Different drug targets for various stages of HIV life cycle. Important steps in the replication of HIV are blocked by anti-HIV drugs including reverse transcriptase inhibitors, protease inhibitors and fusion inhibitors (Source:www.bcm.edu)

Because HIV can become resistant to any of these drugs, health care providers must use a combination treatment to effectively suppress the virus. When multiple drugs (three or more) are used in combination, it is referred as highly active antiretroviral therapy, or HAART, and can be used by people who are newly infected with HIV as well as those with AIDS. While HAART is not a cure for HIV infection, it has greatly improved the health of many people with HIV and it reduces the amount of virus circulating in the blood to nearly undetectable levels. Despite the beneficial effects of HAART, there are side effects associated with the use of antiviral drugs that can be severe. Some of the nucleoside RT inhibitors may cause a decrease of red or white blood cells, especially when taken in the later stages of the disease. The most common side effects associated with protease inhibitors include nausea, diarrhoea, and other gastrointestinal symptoms. Fuzeon may also cause severe allergic reactions such as pneumonia, breathing trouble, chills and fever, skin rash, haematuria, vomiting, and low blood pressure. Local skin reactions are also possible since it is given as an injection underneath the skin.

1.7 Genomic organisation of HIV and life cycle

The virion of HIV-1 has a spherically shaped structure of approximately 110 nm in diameter. The icosahedral core of the virion is composed of two single-stranded RNA molecules tightly bound to nucleocapsid proteins, p7 and enzymes needed for the development of the virion such as reverse transcriptase, proteases, ribonuclease and integrase. A matrix composed of the viral protein p17 surrounds the capsid ensuring the integrity of the virion particle. This is, in turn, surrounded by the viral envelope which is composed of lipid bilayer taken from the cell membrane when a newly formed virus particle buds from the cell. Embedded in the viral envelope are proteins from the host cell and copies of Env protein, consisting of gp120 and gp41 molecules that anchor the structure into the viral envelope (Figure 1.5). This glycoprotein complex enables the virus to attach and fuse with target cells.

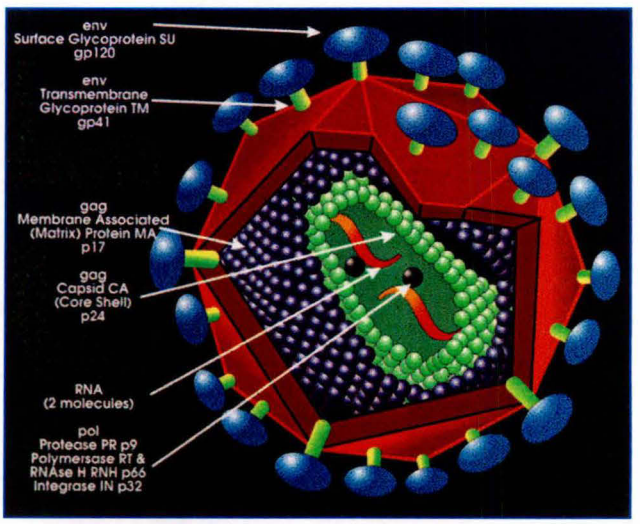


Figure 1.5: Structure of HIV particle: About 110 nm in diameter, with a cone-shaped nucleocapsid surrounded by a lipid bilayer membrane, which contains envelope glycoprotein spikes. (Source: biology.kenyon.edu)

The size of HIV genome is 9.8 kb containing nine open reading frames, that produce a total of 15 proteins as a result of cleavage of three of the primary products Gag, Pol and Env as shown in Figure 1.6. The end of each strand of HIV RNA contains a long terminal repeat (LTR). Regions in the LTR act as switches to control production of new viruses.

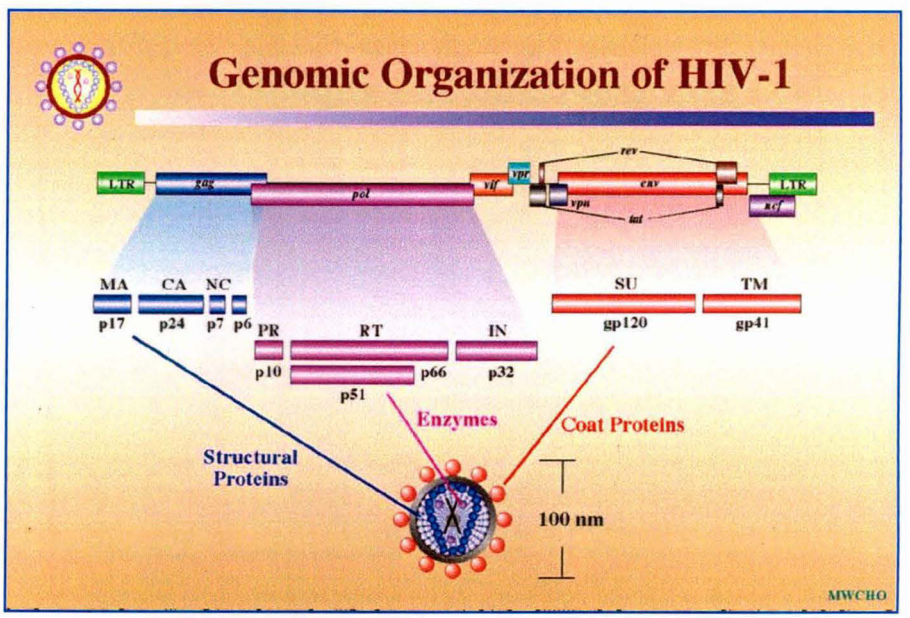


Figure 1.6: Organization of the HIV Proviral Genome. An overview of the organization of the approximately 9-kilobase genome of the HIV provirus and a summary of its gene products (Source: www.stanford.edu)

The full length mRNA of virus encodes Gag, Pol and Env precursor polyproteins, which are subsequently cleaved into individual proteins. The four Gag proteins, MA (matrix), CA (capsid), NC (nucleocapsid), and p6, and the two Env proteins, SU (surface or gp120) and TM (or gp41), are structural components that make up the core of the virion and the outer membrane envelope respectively. The three Pol proteins, PR (protease), RT (reverse transcriptase), and IN (integrase), provide essential enzymatic functions and are also encapsulated within the particle. The six remaining genes, *nef, vif, vpr, tat, rev* and *vpu* (or *vpx* in the case of HIV-2), encode six additional proteins. The Tat and Rev are called as regulatory proteins while Nef, Vif, Vpr and Vpu are often called accessory proteins, of which Nef, Vif and Vpr, are also found associated with the viral particle.

The virus enters the cell as a ribonucleocapsid. The HIV RNA undergoes reverse transcription by using the virus-encoded reverse transcriptase and RNaseH activities and

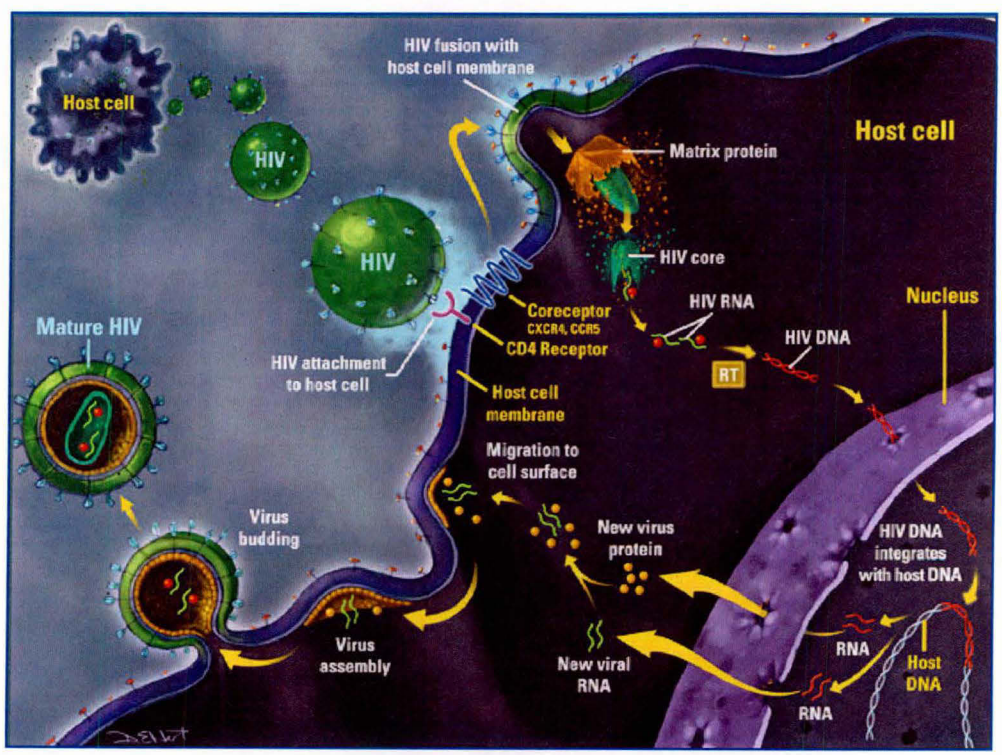


Figure 1.7: The replicative cycle of HIV. The viral envelope protein (Env) of HIV binds CD4 and one of two chemokine receptors CCR5 (R5 strains) or CXCR4 (X4 strains); enters cells by fusion of the viral and cellular membranes. Uncoating of the viral capsid releases the Pre- integration complex, which is reverse transcribed to give ds DNA that integrates into the host genome. Because of the large number of nuclear-localization signals on integrase (IN), matrix (MA) and viral protein r (Vpr) proteins, and with the help of the cDNA flap, the pre- integration complex can enter the nucleus (Source: depts.washington.edu)

eventually forms a double-stranded DNA copy of its genome (cDNA) (Peterlin 2003). The cDNA is a part of the pre-integration complex containing the viral MA, Vpr, and integrase proteins. This complex is transported to the nucleus, as a circular but non-covalently bound molecule, which integrates into the cellular chromosome (Figure 1.7). This karyophilic transport depends on an interaction of MA and Vpr with a cellular karyopherin complex (Gallay 1996). The integration of the HIV provirus appears to be random and is essential for the cells to produce progeny virions.

In several studies HIV integration was found to be important for efficient gene expression (Engelman 1995). Recent studies suggest that the integration is a bit specific and directed to certain susceptible regions of the genome. In permissive activated T cells, HIV undergoes activation and replication within 24 hours. In macrophages the process is similar, but progeny production may take 36 to 48 hours. However, under conditions of stimulation by macrophage colony stimulating factor (M-CSF), replication in macrophages is more rapid and very efficient. Following virus integration, the earliest mRNA species made in the infected cell are doubly spliced transcripts encoding the major regulatory proteins, particularly Tat, Rev and accessory protein Nef. Viral assembly takes place at the cell membrane, where viral RNA is incorporated into capsids that bud from the cell surface, together with the viral envelope containing the envelope glycoproteins gp120 and gp41. Final maturation of the virion protein is mediated by the viral protease and takes place within the budding particle.

1.7.1 The Gag polyprotein

The *gag* and *gag-pol* genes together are translated into large polyproteins which are then cleaved (Figure 1.8) by a virus-encoded protease that is part of the POL polyprotein. In fact, both Gag and Pol are encoded by the same mRNA but are translated into different proteins through the usage of alternative reading frames. Thus for both proteins to be produced from the same RNA, a -1 frame shift needs to occur after ribosomes have scanned the mRNA to translate the Gag protein (Luciw 1996). This frame shifting is facilitated by the nucleotides that span the overlapping coding region for Gag and Pol.

The actual sequence responsible for inducing the shift is a short tract of homopolymeric uracil followed by an RNA hairpin structure. The efficiency of this frame-shifting is about 5% and therefore the virus always produces large amounts of Pr55-Gag and comparatively smaller amounts of Pr160-Gag-Pol. Like all other retroviruses the Gag is essential and sufficient to form non-infectious virus-like particle (VLP). When Gag is synthesized on cytosolic ribosomes, its N-terminus is cotranslationally myristoylated by

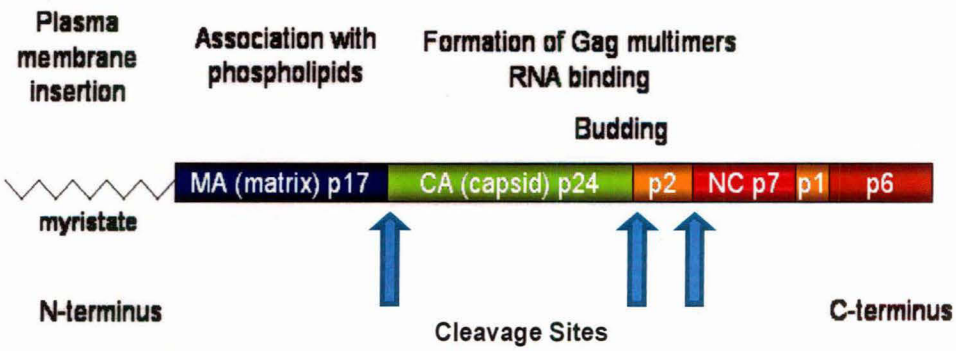


Figure 1.8: Structure of Gag polyprotein showing various cleavage sites. After cleavage Gag polyprotein produce MA, CA, NC and p6 proteins and two spacer peptides p1 and p6. (Source: pathmicro.med.sc.edu)

cellular N-myristoyl transferase, a modification necessary for association of Gag to the cytoplasmic side of the cell membrane. The membrane associated Pr55 Gag recruits two copies of viral RNA and triggers the budding of viral particles from the surface of infected cells. After budding it is cleaved by a viral protease PR (p4) to produce matrix protein MA (p17), capsid protein CA (p24), nucleocapsid protein NC (p7), p6 and two spacer peptides P1 between NC and p6, and P2 between MA and CA (Figure 1.8).

1.7.1.1 Matrix (MA)

The Matrix protein (p17) is the N-terminal component of the Gag polyprotein and is important for targeting Gag and Gag-Pol precursor polyproteins to the plasma membrane prior to viral assembly. In the mature viral particle, the 132 amino acid MA protein lines the inner surface of the virion membrane. Two discrete features of MA are involved in membrane targeting: an N-terminal myristoyl group and basic residues located within the first 50 amino acids. The trimer of MA has three myristoyl molecules which embed in the membrane while the lysine-containing region associates with the negatively charged

phospholipid head groups. The trimeric form of MA is presumed to be biologically relevant because mutation of residues involved in trimerization (aa 42–77) abolishes viral assembly. However, the N-terminal basic region is not strictly required for the formation of virus particles because non-infectious virus particles that lack MA can be produced if a myristoyl group is placed directly upstream of CA. In addition to targeting Gag and Gag-Pol to the membrane, MA also appears to help incorporate Env glycoproteins with long cytoplasmic tails into viral particles (Mammano 1995). A direct interaction has been reported between the cytoplasmic domain of the HIV-1 Env protein and the viral matrix protein. Deletions in the C-terminal region of the Env cytoplasmic domain abrogate the interaction, as well as some mutations in the N-terminal portion of the matrix protein. This interaction with the matrix protein directs the Env protein to budding HIV-1 virions. In addition to its function in viral assembly, MA facilitates infection of non-dividing cell types, principally macrophages. Its precise role in viral entry is controversial. Some studies have shown that a subset of phosphorylated MA proteins are associated with viral pre-integration complexes and that MA contains a nuclear localization signal (NLS) that interacts with Rch1, a member of the karyopherin family, to facilitate rapid nuclear transport (Gallay 1995a, 1995b; Bukrinskaya 1996; Bukrinsky 1993a, 1993b). Phosphorylation of Tyr131 was shown to mediate association with IN, thereby linking MA to the pre-integration complex (Gallay 1995a, 1995b). Other studies, however, have found no evidence for NLS in the MA protein (Freed 1997). Instead, mutation of the putative NLS in a macrophage-tropic HIV-1 isolate decreased infectivity in both non-dividing and dividing cells and resulted in delayed proteolytic processing of the Gag polyprotein, presumably because the mutations affect association of MA with the membrane (Fouchier 1997). Additional studies are needed to clarify the role of MA in infection of non-dividing cells.

1.7.1.2 Capsid (CA)

The Capsid protein (p24) is the second component of the Gag polyprotein and forms the core of the virus particle. Like MA, CA also functions in the early and late stage of virus replication. A 24-27 kDa CA protein is released from the central portion of the viral Gag

polyprotein. The C-terminal domain (aa 152–231) functions primarily in assembly and is important for CA dimerization (Gamble 1997). Although mutations in the N-terminal domain (aa 1–151) do not prevent assembly or budding, this domain is important for infectivity, apparently by participating in viral uncoating through its association with a putative cellular chaperone, cyclophilin A (CypA) (Luban 1996). The structures of two dimeric forms of the N-terminal domain, one complexed to an antibody fragment and the other complexed to CypA, show the same monomeric CA structure but different subunit interfaces. Both CA and Pr55Gag bind to cyclophilin A and B. These interactions are specific to HIV-1 and not HIV-2 or SIV, and incorporate cyclophilin A and B into the virus particle. Cyclophilins are ubiquitous propyl isomerases and mediate the correct assembly of proteins. The treatment of HIV-1 infected cells with cyclosporin A or its analogs, or mutation of CA residues that participate in the CA-cyclophilin interaction, block cyclophilin recruitment into the virion and reduce virus infectivity and replication (Franke 1994; Thali 1994).

1.7.1.3 Nucleocapsid (NC)

The Nucleocapsid protein (p7) is the third component of the Gag polyprotein and coats the genomic RNA inside the virion core. The primary function of the NC protein is to bind specifically to the packaging signal and deliver full-length viral RNAs into the assembling virion. The packaging signal consists of three hairpins in the RNA. HIV-1 p7 has two copies of a cysteine-histidine motif (CCHC) that is similar to the metal-binding finger domains of several proteins that interact with nucleic acids. Virion p7 enters cells with the viral genomic RNA and accumulates in the nucleus about 8 hours after entry. The early functions of NC in HIV-1 infection are reported as enhancing the first-and second-strand viral DNA synthesis and enhancing HIV-1 integrase activity *in vitro*. The NC is a basic protein that also binds single-stranded nucleic acids non-specifically, leading to coating of the genomic RNA that presumably protects it from nucleases and compacts it within the core. Nonspecific binding also provides chaperone-like functions that enhance other nucleic acid dependent steps in the life cycle, such as promoting annealing of the tRNA primer, melting of RNA secondary structures, or DNA strand

exchange reactions during reverse transcription (Huang 1997) or stimulating integration (Carteau 1997). The NC protein is 55 residues long and contains two zinc finger domains flanked by basic amino acids. Its structure has been determined by NMR and shows two well-ordered zinc domains with a relatively flexible linker in the absence of RNA (Summers 1992). Mutations in the zinc finger domains impaired p7 nuclear localization and also retarded HIV-1 early spliced mRNA expression. Basic residues Arg7, Arg32, and Lys33 are shown to be particularly important for *in vitro* RNA binding and Lys11 and Lys14 are for viral replication, though mutation of Arg32 or Lys33 seems to have little effect on RNA packaging *in vivo* (Poon 1996). Disulfide-substituted benzamide compounds specifically remove zinc from the NC domains and inhibit viral replication (Rice 1995), providing additional evidence for the importance of these structures.

1.7.1.4 The p6 protein

The Gag polyprotein also produces a proline-rich, 6 kDa protein known as p6. Mutations in p6, specifically within a highly conserved Pro-Thr-Ala-Pro (PTAP) motif called the viral late domain block a late step in virus assembly such that virions accumulate at the plasma membrane but fail to release efficiently. Interestingly, mutations in PR largely reverse this defect suggesting a functional interplay between p6 and the protease (PR) (Huang 1995). The p6 polypeptide region also mediates interactions between Gag and the accessory proteins Vpr and Vpx, leading to the incorporation of Vpr and Vpx into newly assembled virions.

1.7.2 Pol polyprotein

The POL polyprotein contains multiple domains as shown in Figure 1.9.

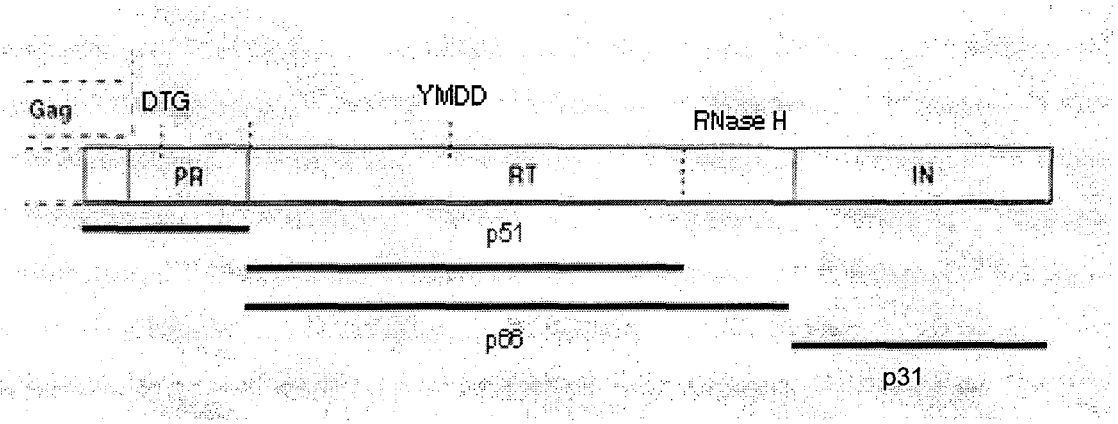


Figure 1.9: Structure of Pol polyprotein showing different regions before cleavage including precursors for protease, reverse transcriptase (p51), reverse transcriptase and RNaseH (p66) and integrase (p31) (Source: bioafrica.mrc.ac.za).

1.7.2.1 Protease (PR)

During assembly, the virion core includes Gag, Gag-Pol, Vif, Vpr, Nef proteins, genomic RNA and membrane coat containing SU and TM that surrounds the particle. After assembly the virus buds from the membrane surface and immature particles thus formed are non-infectious. The Gag and Gag-Pol polyproteins must be cleaved by PR, and conformational rearrangements must occur within the particle to produce mature infectious viruses. The PR protein cleaves at several polyprotein sites to produce the final MA, CA, NC, and p6 proteins from Gag and PR, RT, and IN proteins from Pol. Because assembly and maturation must be highly coordinated, factors that influence PR activity can have dramatic effects on virus production. PR functions as a dimer and is part of Pol, so PR activity initially depends on the concentration of Gag-Pol and the rate of auto processing, which may be influenced by adjacent p6 sequences (Zybarth 1995). The first cleavage events catalyzed by the retroviral PRs during or immediately after virion release from the cell serves to liberate PR from the Gag-Pol precursor. The PR protein appears to be most active during the budding process just prior to the release of the virus particle from the cell, leading to virus maturation and formation of a tightly packed cone-shaped core. During viral maturation, the PR protein cleaves the Pol polypeptide away from Gag and further digests it to separate itself (p10), RT (p51), RNaseH (p15) and integrase (p31)

activities. Cleavage efficiencies can vary substantially among sites, thereby influencing the order of appearance of different processed proteins (Dunn 1994). Overexpression of PR can lead to aberrant rates of processing and decreased infectivity (Luukkonen 1995). The mature form of PR monomer is 99 amino acids in length with a molecular mass of 10 kDa and the active protein is a homodimer. PR has been a prime target for drug design, and crystal structures of many PR-inhibitor complexes have been solved, including peptidomimetic and nonpeptide inhibitors (Wlodawer 1993). The active site resembles that of other aspartyl proteases and contains a conserved triad sequence, Asp-Thr-Gly. Knowledge of this structure has led to a class of drugs directed towards inhibiting the HIV protease function. These antiviral compounds (e.g., Indinavir, Saquinavir) together with RT inhibitors (e.g., AZT and 3TC) are used for highly active antiretroviral therapy or HAART, and have greatly improved the treatment regimen for HIV infection. Several PR inhibitors are in wide clinical use, and mutants resistant to multiple inhibitors have been observed. Resistance mutations are located both within the inhibitor binding pocket and at distant sites, and some mutants show increased catalytic activities. An alternative approach to inhibitor design involves the use of inactive subunits that act as dominant negative inhibitors (McPhee 1996).

1.7.2.2 Reverse Transcriptase (RT)

Before the viral genome can be integrated into the host chromosome, it must first be reverse transcribed into duplex DNA. RT catalyzes both RNA-dependent and DNA-dependent DNA polymerization reactions and contains an RNaseH domain that cleaves the RNA portion of RNA-DNA hybrids generated during the reaction. Reverse transcription initiates from the 3' end of a tRNA3Lys primer annealed to the primer binding site near the 5' end of the genomic RNA. RT can use other tRNAs if complementary binding sites are provided, but reverse transcription is most efficient with tRNA3Lys (Essink 1996). The tRNA3Lys is incorporated into virions during assembly and is often extended by several nucleotides inside the particle. The remainder of the reaction probably occurs after uncoating in the cytoplasm. Following RNA-primed initiation, reverse transcription involves two DNA strand transfer reactions that are

catalyzed by RT and are important for priming the synthesis of both minus and plus strands (Katz 1994). The RT protein has also been a major target for drug design, and crystal structures of unliganded RT, a RT-DNA complex, and RT-inhibitor complexes have been solved (Hsiou 1996). It is a heterodimer containing a 560-residue subunit (p66) and a 440-residue subunit (p51) both derived from the Pol polyprotein. Each subunit contains a polymerase domain composed of four subdomains called fingers, palm, thumb, and connection, and p66 contains an additional RNaseH domain (Figure 1.10). Even though their amino acid sequences are identical, the polymerase subdomains are arranged differently in the two subunits, with p66 forming a large active-site cleft and p51 forming

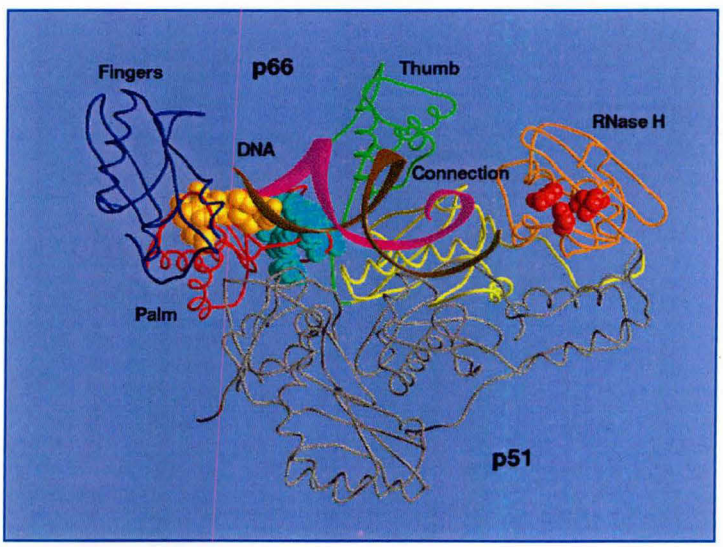


Figure 1.10: Heterodimeric structure of reverse transcriptase containing two subunits, larger active p66 and smaller inactive p51. Each subunit contains a polymerase domain composed of four subdomains called fingers, palm, thumb, and connection, and p66 contains an additional RNaseH domain. (Source: www.stanford.edu)

an inactive closed structure (Wang 1994). The p66 polymerase active site contains a catalytic triad (Asp110, Asp185, and Asp186) conserved in many polymerases, and the 3'OH group of the primer strand in a RT-DNA complex is positioned close to the active site for nucleophilic attack on the incoming nucleoside triphosphate. The DNA in this complex has primer and template strands clamped between the palm, thumb, and finger subdomains of p66 and is bent. Portions of the DNA near the active site adopt an A-form geometry expected of RNA-DNA hybrids or RNA duplexes bound during reverse transcription.

The reverse transcriptase has been the target for mainly two classes of drugs. One class of RT inhibitors is the nucleoside analogs like AZT, ddI, ddC, and d4T. These dideoxy compounds lack a 3' oxygen, causing DNA chain termination when they are incorporated into a growing DNA strand. Another class of compounds that inhibit HIV-RT is the non-nucleoside inhibitors (NNIs). These inhibitors (e.g., APA) have been shown to bind in a pocket formed away from the polymerase active site (Esnouf 1995; Ren 1995a, 1995b). Unfortunately, resistance to these compounds develops rapidly in patients, perhaps reflecting the fact that resistant variants exist even before the initiation of therapy. Mutations that confer resistance to nucleoside or non-nucleoside inhibitors map to different parts of RT, including regions in and around the active site and DNA binding cleft, suggesting that some mutations directly alter the drug-binding site while others have more indirect effects (Tantillo 1994). Structures of the unliganded RT show substantial variability in the positioning of the p66 thumb subdomain (Rodgers 1995) indicating that large-scale conformational rearrangements occur upon nucleic acid or drug binding. Such conformational changes may be important during reverse transcription; for example, to allow translocation of RT along the nucleic acid or to correctly position the RNaseH and polymerase active sites.

1.7.2.3 Integrase (IN)

The HIV-1 integrase is a multidomain enzyme that is required for the integration of viral DNA into the host genome. The full length HIV-1 integrase is a 288 amino acid protein consisting of three domains: the catalytic core, the C-terminal, and the N-terminal domains. Although all three domains are required for integration, the catalytic core domain contains the active site responsible for catalysis of all the reactions of integration. The catalytic core domain contains the invariant triad of acidic residues, comprising residues Asp64, Asp116 and Glu152 in the case of HIV-1 integrase. Following reverse transcription, IN catalyzes a series of reactions to integrate the viral genome into host chromosome. In the first step, IN removes two 3' nucleotides from each strand of the linear viral DNA, leaving overhanging CA_{OH} ends (Katz 1994). The CA dinucleotide is found at the ends of many retrotransposons, and mutation of these nucleotides

substantially reduces the efficiency of 3' end processing. In the second step, the processed 3' ends are covalently joined to the 5' ends of the target DNA. In the third step, which probably involves additional cellular enzymes, unpaired nucleotides at the viral 5' ends are removed and the ends are joined to the target site 3' ends, generating an integrated provirus flanked by five base-pair direct repeats of the target site DNA. The viral substrate used for integration is a linear DNA molecule containing a complete minus strand and a discontinuous plus strand, which is presumably completed by cellular enzymes following integration (Miller 1995b). The enzymatic mechanism involves two sequential trans-esterification reactions and requires no exogenous energy source, but an appropriate metal cofactor (either Mn^{2+} or Mg^{2+}) is needed (Katz 1994). Integration can occur at many target sites within the genome. The accessibility of the chromosomal DNA within chromatin, rather than specific DNA sequences, seems to influence the choice of integration sites. Kinks in the DNA within chromatin are thus hotspots for integration, at least *in vitro*. Preferential integration into regions of open and transcriptionally active chromatin may facilitate expression of the provirus. Viral genes are not efficiently expressed from non-integrated proviral DNA (Pruss 1994a; Wiskerchen 1995). It has been suggested that interactions with other DNA-binding proteins might target IN to specific sites, and a yeast two-hybrid screen has identified a human Snf5-related protein, Ini1, as a possible partner (Miller 1995a). Ini1 influences the efficiency of integration, but its effect on target site selection *in vivo* is unknown. Another cellular factor, HMGI(Y), is associated with pre-integration complexes and plays a crucial role in integration (Farnet 1997).

1.7.3 The Envelope protein

Enveloped viruses enter cells by a two-step process. The first step involves the binding of a viral surface protein to receptors on the plasma membrane of the host cell. After receptor binding, a membrane fusion reaction takes place between the lipid bilayer of the viral envelope and host cell membranes. This fusion reaction can occur either at the plasma membrane or in acidic endosomes following receptor-mediated endocytosis. In either case, the membrane fusion reaction delivers the viral nucleocapsid into the host cytoplasm, allowing the infection to proceed. Viral proteins embedded in the lipid bilayer

of the viral envelope catalyze receptor binding and membrane fusion reactions.

1.7.3.1 Env Precursor Biosynthesis and Processing

The Env glycoprotein of HIV-1, like those of other retroviruses, is synthesized as a polyprotein precursor molecule, which is proteolytically processed by a host protease to generate the surface (SU) and transmembrane (TM) subunits of the mature Env glycoprotein complex. The unprocessed Env precursor has been designated, based on its apparent molecular mass, gp160 (Figure 1.11). The mature SU and TM Env glycoprotein subunits are designated as gp120 and gp41, respectively. Sequence comparison of a number of HIV-1 isolates indicated that gp120 is highly variable between virus isolates and this variability is non-uniform, leading to the designation of conserved (C) and hypervariable (V) domains within gp120 (Figure 1.11A and C).

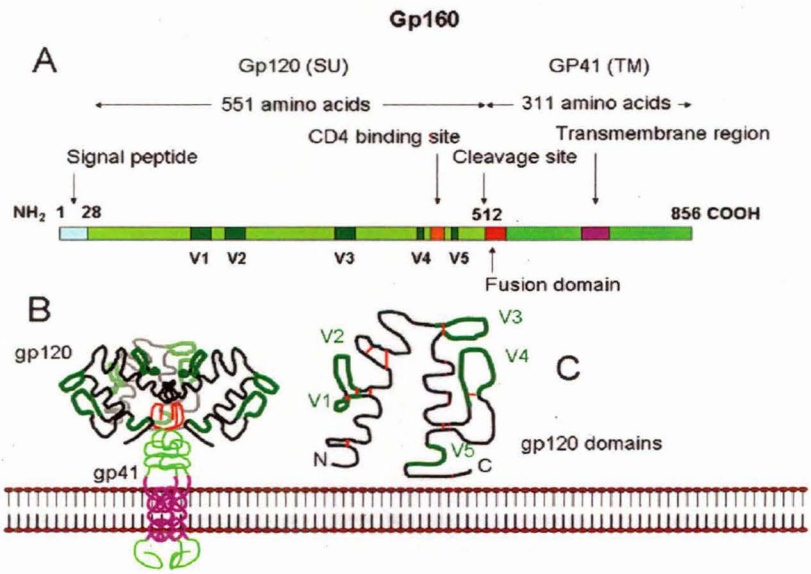


Figure 1.11: The structure of gp160. (A) The linear domain structure of gp160 is shown at the top. Gp160 is cleaved into gp120 (the surface protein) and gp41 (the transmembrane fusion protein). (B) A trimer of gp120/gp41 is associated with the viral membrane. (C) Gp120 has a number of hyper variable domains (V1-V5). The red bars show disulfide bridges (Source: pathmicro.med.sc.edu)

Similar to other glycoproteins destined for the plasma membrane, gp160 is synthesized on the rough endoplasmic reticulum (ER) and is co-translationally inserted into the lumen of the ER where it is glycosylated. Shortly after synthesis, gp160 monomers oligomerize,

a process that is thought to be required for transport from the ER to the Golgi complex. Once in the Golgi, some of the high mannose, ER-acquired *N*-linked oligosaccharide side chains are modified to more complex forms, and gp160 is proteolytically cleaved to gp120 and gp41. Proteolytic cleavage of gp160 is catalyzed by a host protease at a Lys/Arg-*X*-Lys/Arg-Arg motif that is highly conserved among viral Env glycoprotein precursors. Following gp160 cleavage, the oligomeric, noncovalently associated gp120-gp41 complexes are transported to the cell surface, where they are incorporated into budding virions.

1.7.3.2 The gp120 surface protein

The SU or surface protein subunit also known as gp120, is derived from gp160 (encoded in the *env* gene) along with TM (gp41). It is the fusion protein and therefore participates directly in virus entry. The predicted amino acid sequence analysis of HIV-1 gp120 reveals about 25 potential sites for N-linked glycosylation (Asn-X-Ser/Thr) with about 13 of these motifs being conserved in different viral isolates. *In silico* models suggest that very few regions of the gp120 peptide backbone protrude from the carbohydrate mass due to this extensive glycosylation (Nara et al.1991). Further, the predicted sequence of HIV-1 gp120 shows 18 cysteine residues which are highly conserved in the glycoproteins of diverse HIV-1 and HIV-2/SIV strains, suggesting that disulphide bonds are presumed to be critical for the structure and function of these viral proteins (Hoxie, 1991). Based on biochemical analysis, the model for the gp120 subunit shows nine intra-chain disulphide bonds (Figure 1.11B). This disulphide bonding is also important for recognition of the CD4 receptor (Leonard 1990).

The initial step in HIV-1 infection involves the binding of virion-associated gp120 to the CD4 protein expressed on the surface of a subset of T cells and primary macrophages, and serves as the major receptor for HIV-1 and the related HIV-2 and SIV (Klatzmann 1984). The CD4 binding to gp120 induces conformational changes in both gp120 and gp41 that result in the exposure of Env domains that are thought to be involved directly in the membrane fusion reaction (Sattentau 1991). The 515-aa gp120 binds CD4 with high affinity, and amino acids important for binding have been mapped primarily to four

separate conserved regions of gp120. The gp120-CD4 interaction is not sufficient for HIV-1 entry. Instead, a group of chemokine receptors, a family of seven transmembrane G-coupled proteins that mobilize intracellular calcium and induce leukocyte chemotaxis serve as essential viral coreceptors (Clapham 1997). There are two major classes of HIV-1: those that are macrophage (M)-tropic and non-syncytium inducing (NSI) and those that are T-cell (T)-tropic and syncytium inducing (SI). The CXCR4/fusin protein was the first coreceptor identified which permits entry of T-tropic but not M-tropic viruses while the CCR5 protein is a major coreceptor for M-tropic but not T-tropic viruses. Other surface proteins, including CCR3, CCR2b, Bonzo/STRL33, and BOB/GPR15, serve as coreceptors for some HIV-1 isolates (Clapham 1997). The physiological ligands for CXCR4, CCR5, and CCR3 are able to inhibit viral entry by competing with the cognate coreceptor. Binding of CD4 to gp120 appears to cause structural changes in Env that facilitate coreceptor binding and subsequent viral entry (Clapham 1997).

1.7.3.3 The transmembrane protein gp41

The primary function of gp41, a 345–amino acid protein located in the viral membrane is to mediate fusion between the viral and cellular membranes following receptor binding. An N-terminal hydrophobic glycine-rich "fusion" peptide has been predicted to initiate fusion, and a transmembrane region is important both for fusion and for anchoring Env in the viral membrane (Hernandez 1996). The gp41 sequence contains four potential glycosylation sites and three Cys residues (Freed 1990). About 20 amino acids at the N-terminus of gp41 are hydrophobic and form the fusion peptide (aa 512-527 of gp160) that is required for fusion of the virion membrane with the plasma membrane of the cell during the entry step in viral replication (Horth 1991; Kowalski 1987). A second hydrophobic domain spans virions and cell membranes and thereby enables gp41 to serve as an anchor for the Env glycoprotein heterodimer. The region between the two hydrophobic domains contains an external highly conserved sequence (Leu553 to Leu590 of gp160), similar to the leucine zipper motif and is required for protein-protein interactions. Mutation in this Leu motif of gp41 blocks viral infectivity and cell fusion without affecting synthesis, oligomer formation, transport, and proteolytic processing of

the Env glycoprotein (Chen 1993; Ivanoff 1992; Cao 1993). During viral entry, a portion of the gp41 ectodomain, including the leucine zipper, has been proposed to adopt a coiled-coil conformation, which facilitates insertion of the gp41 fusion peptide into the target cell membrane.

1.7.3.4 CD4 Binding

The initial step in HIV-1 infection involves the binding of virion-associated gp120 to the cell surface molecule CD4, which serves as the major receptor for the virus. This induces conformational changes in both gp120 and gp41 that result in the exposure of Env domains that are thought to be involved directly in the membrane fusion reaction. In addition to binding CD4 on the cell surface during the early phase of virus infection, HIV-1 Env associates with CD4 intracellularly soon after gp160 synthesis in the ER. Pulse-chase and transport inhibition studies suggested that within approximately 30 min of synthesis, gp160 adopts a conformation suitable for CD4 binding. The association of Env and CD4 early in the transport pathway leads to the downregulation of CD4 expression from the surface of Env-expressing cells (Jabbar 1990). This decrease in the level of cell surface CD4 may reduce the ability of Env-expressing cells to become infected with additional virions, a phenomenon that is described for other retroviruses as well and known as superinfection interference. Env expression in an infected cell can also lead to cell-to-cell fusion, or syncytium formation with neighboring CD4 cells, a process that contributes to HIV cytopathicity in culture and possibly *in vivo* as well (Sodroski 1986). The first domain recognized as being directly involved in HIV-1 Env-induced membrane fusion was the highly hydrophobic sequence at the amino terminus of gp41. Analysis of lentiviral Env glycoproteins indicated that single amino acid changes in the highly hydrophobic amino-termini of the HIV-1, HIV-2, and SIV TM glycoproteins blocked Env-induced syncytium formation (Freed 1990). The HIV-1 Env glycoprotein also undergoes a series of conformational changes following CD4 binding, one outcome of which is the exposure of the gp41 fusion peptide. In addition to the amino-terminal fusion peptide, other domains within gp41 have also been reported to play a role in the fusion process. Mutations in a putative leucine zipper motif in the gp41 ectodomain

(Delwart 1990) blocked syncytium formation and virus infectivity without affecting Env oligomerization, transport, processing, or CD4 binding (Chen 1993).

In gp120, two regions are primarily involved in membrane fusion. A number of studies determined that antibodies to V3 were capable of neutralizing virus infectivity (Goudsmit 1988) without affecting virus binding to CD4. Mutational analysis demonstrated that single amino acid substitutions within the HIV-1 V3 loop, and the analogous domain of HIV-2, blocked Env-induced syncytium formation and virus infectivity (Freed 1992). More recent studies have also implicated the importance of V1/V2 region in membrane fusion. Mutations within V1/V2 were reported to block syncytium formation without affecting the gp120-gp41 interaction or CD4 binding (Sullivan 1993) and the transfer of V2 sequences from syncytium-inducing Env glycoproteins conferred the ability to induce fusion on non-syncytium-inducing Env glycoproteins. Consistent with a role for V1/V2 in membrane fusion, antibodies to this region are capable of neutralizing virus infectivity.

1.7.4 Tat protein

The Tat protein exerts its transcriptional effect by binding the trans activation-responsive region (TAR) and enhancing transcriptional elongation. Transcription of the HIV-1 provirus is characterized by an early, Tat-independent and a late, Tat-dependent phase. In the absence of Tat, polymerases generally do not transcribe beyond a few hundred nucleotides. Transcription from the HIV-1 LTR is increased several hundred-fold in the presence of Tat. The Tat protein is an unusual transcription factor because it interacts with a cis-acting RNA enhancer element, TAR, present at the 5' end of all viral transcripts (Berkhout 1990; Pumfery 2003). In fact, TAR was the first demonstration of a RNA enhancer element. Unlike other eukaryotic enhancers, however, the TAR element was only functional when it was placed 3' to the HIV promoter and in the correct orientation and position (Selby 1989). Kao et al (1987) reported that in the absence of Tat the majority of RNA polymerases initiating transcription stall near the promoter, and later Laspia et al (1989) reported a small effect of Tat on transcription initiation but a large effect on transcription elongation. The observation that Tat binds specifically to the TAR

RNA (Garcia 1989) and could function as an RNA binding protein (Dingwall 1989) gave further support for the elongation model. It is now quite well accepted that through interaction with TAR, the Tat protein promotes the assembly of an active transcription elongation complex. The more recent finding that Tat promotes the binding of P-TEFb, a transcription elongation factor composed of cyclin T1 and cdk9 (Marshall 1992) and, more recently, Brd4 in the active nuclear complex (Jang 2005), is consistent with the elongation model. Along this it has also been reported that Tat physically interacts with the pre-initiation complex including transcription factors such as Sp1, TATA binding protein (TBP), cylinE/cdk2, TFIIH, Tip60, RNA Pol II, as well as coactivators such as CBP/p300 and p/CAF (Herrmann 1993, 1955; Marshall 1992, 1995).

1.7.5 Rev protein

When viral mRNAs are first produced, most are doubly spliced and encode the Tat, Rev, and Nef proteins. Later, when other viral components are needed to assemble infectious virions, singly spliced and unspliced transcripts are transported to the cytoplasm, where they are translated and where genomic RNAs are packaged. Rev is important in this switch because it overcomes the default pathway in which viral mRNAs are spliced prior to nuclear export and functions by binding to the Rev-response element (RRE) located in the *env* coding region. Whether Rev directly enhances the export of unspliced mRNAs or inhibits splicing has been unclear, but recent studies lend strong support to a role in export (Hope 1997). Microinjection experiments in *Xenopus* oocytes have shown that Rev is required to export unspliced RNAs that contain a RRE (Fischer 1995). The Rev protein contains a leucine-rich nuclear export signal (NES) that allows it to shuttle between the nucleus and cytoplasm (Meyer 1994). It interacts with a nucleoporin-like protein (hRip/Rab) located at the nuclear pore (Fritz 1995; Bogerd 1995). This interaction may be bridged by CRM1, a nuclear export receptor that is important for Rev export (Ullman 1997). Thus, Rev binding to the RRE is believed to target the attached RNA to the nuclear export machinery. There is evidence that entry into the splicing pathway may also be important for Rev function because mutating 5' splice sites on RRE-containing RNAs eliminates Rev activity but compensatory mutations in U1 snRNA, which binds at

5' splice sites, can restore activity. Furthermore, Rev can directly inhibit splicing by preventing entry of additional snRNPs during the later stages of spliceosome assembly (Kjems 1993). Possible relationships between the splicing and transport pathways and the precise mechanism of Rev function remain to be clarified. The RRE contains several hairpins and binds several Rev monomers, nucleated by the interaction of a single monomer with a high-affinity site, hairpin IIB (Zemmel 1996).

1.7.6 Nef protein

The *nef* gene is highly conserved in all primate lentiviruses e.g. HIV-1, HIV-2 and SIV and the encoded Nef protein appears to be a virulence factor critical for the development of AIDS. This protein resides both in the cytoplasm and in association with the cytosolic face of cellular membranes. The *nef* was first identified as an open reading frame (ORF) that partially overlaps with the 3'-long terminal repeat of HIV-1 and the protein encoded was reported to have a negative effect on viral replication, hence the name 'negative factor' or Nef (Terwilliger 1986; Ahmad 1988; Cheng-Mayer 1989). Since then, extensive studies of Nef biology have shown this to be a misnomer and have characterized multiple cellular pathways that are regulated by expression of the Nef protein. Consequently, Nef has been hypothesized to function as a molecular adaptor, altering cellular pathways via multiple protein-protein interactions. Indeed, Nef is able to modulate diverse cellular functions such as protein trafficking events, signal transduction cascades, and apoptotic pathways.

The Nef protein is expressed in abundance during the early phase of HIV infection together with Tat and Rev. The mRNA encoding Nef is estimated to represent three quarters of the early viral mRNA load of the cell (Guy 1987; Klotman 1991). Nef induces high viral titers both in cell culture and *in vivo*. Rhesus monkeys infected with an engineered strain of SIV that lacked a functional Nef protein also showed slow progress to clinical disease (Kestler 1991). In fact, *nef* deleted SIV strains have been proposed as candidates for vaccination trials with live attenuated vaccine in the simian model system. A similar requirement for the maintenance of high virus loads has been demonstrated for HIV-1 in the SCID-Hu model (Aldrovandi 1996; Duus 2001; Jamieson 1998; Su 1995).

In this system, viruses with an intact *nef* ORF replicate faster, achieve higher titers, and deplete thymocytes better than their *nef*-deleted counterparts (Daniel 1992). Moreover, long-term survivors of HIV infection who show lack of disease progression are commonly associated with either a deletion in the *nef* gene or defective *nef* alleles (Deacon 1995; Kirchhoff 1995; Learmont 1999; Mariani 1996; Switzer 1998). In transgenic mouse models, Nef expression in CD4+ cells caused an AIDS-like disease (Hanna 1998a, 1998b; Skowronski 1993). Of note, the Nef protein of SIV and HIV are functionally interchangeable (Sinclair 1997). Enhanced virus replication and infectivity are associated with *nef* expression upon propagation of HIV-1 in peripheral blood mononuclear cells (PBMCs) cultures *in vitro* (Jamieson 1994; Spina 1994). Increased inocula of *nef*-deleted viruses can overcome the decreased infectivity of the viral progeny but still does not induce illness in infected animals, suggesting a role for Nef in HIV pathogenesis as well. Nef was originally characterized as a negative regulator of HIV infection and, it was found that *nef*-defective viruses replicate slightly faster than the wild type viruses in CD4+ cell lines. In addition, it was shown that Nef could downregulate HIV-1 LTR activity. However, these initial findings were later refuted by other groups. It has become clear that Nef positively affects viral replication and infectivity. Further, it has also been shown that Nef does not inhibit transcription of the HIV-1 LTR in a variety of cell types.

1.7.6.1 Effect of Nef on virion infectivity and replication

The *nef* gene of HIV is critical for development of AIDS and pathogenesis in humans as well as in animal models. Using the Hela-CD4-LTR-β-galactosidase indicator cell line, *nef*+ HIV-1 was found to productively infect 5 to 20-fold more cells than equal amounts of *nef*-defective HIV-1 (Kimpton 1992). The replication rates of *nef*+ and *nef*-defective HIV-1 clones in activated primary blood lymphocytes (PBL) indicated that Nef directly promotes HIV-1 replication by enhancing the infectivity of virions (Chowers 1994). The infectivity of *nef* defective HIV-1 can be rescued by expressing Nef in trans in the virus-producing cell. Viruses produced from proviral DNAs containing mutated *nef* are 4 to 40 times less infectious than the wild type virus in single-round infection assays. A

significant role for Nef in viral replication has been found in post-infection-stimulated PBMCs or lymphoid cultures, immature dendritic cell-T cell co-cultures and in the *ex vivo* tonsillar culture system. On the other hand, Nef generally has no effect on viral replication in activated peripheral blood mononuclear cells (PBMCs), activated CD4+ T cells and mature dendritic cell-T cell co-cultures.

The inclusion of Nef in the virion may facilitate the incorporation of Nef-associated cellular kinases that phosphorylate various substrates, including the viral matrix protein, important for the production of fully infectious viral particles (Freed 1990). Productive HIV-1 infection is also regulated by the ability of Nef to induce the release of a lymphocyte-stimulating factor by macrophages. This leads to an environment in which Nef promotes viral replication in the host by increasing the pool of substrate lymphocytes without additional stimuli (Swingler 1999).

1.7.6.2 CD4 Downmodulation by Nef

The CD4 protein is a coreceptor required for HIV infection. However after HIV infection, the presence of CD4 on the cell surface reduces the ability of the newly formed particle to properly bud and escape the infected cell, and thereby reducing viral infectivity. One of the major benefits of Nef-induced CD4 downregulation could be the enhancement of viral particle release by preventing the sequestration of viral envelope by the CD4 receptor or its influence on the assembly and entry of viral particles (Arganaraz 2003). It could also prevent superinfection, which would lead to premature death of the host cell (Arganaraz 2003). The downmodulation of steady-state levels of CD4 on the cell surface is one of the most extensively studied functions of Nef (Aiken 1994). Almost all Nef isolates downmodulate cell surface CD4 in all mammalian cell types tested, and under nearly all experimental conditions. To decrease the half-life of CD4 on the cell surface, it is proposed that Nef binds the cytoplasmic tail of CD4. Indeed, Nef has been shown to bind to the CD4 cytoplasmic domain in yeast two-hybrid assay systems (Rossi 1996), in purified protein assay systems (Grzesiek 1996), and in living cells (Galzi 2005). An NMR structural analysis demonstrated that the direct physical interaction occurred

between a hydrophobic pocket in the Nef core domain and a 13-amino-acid peptide (QIKRLLSEKKT) from the CD4 cytoplasmic tail (Grzesiek 1996). This *in vitro* interaction was quite weak but highly specific, as the disruption of the dileucine motif in this peptide abrogated the association (Grzesiek 1996). Two other HIV-1 proteins Env and Vpu, also downregulate CD4. The Nef-meditated CD4 downregulation occurs early after infection. In contrast to the other two HIV proteins, Nef acts on CD4 molecules that have already reached the cell surface. Nef-induced CD4 downregulation involves accelerating endocytosis of CD4 followed by its degradation through the endo-lysosomal pathway (Aiken 1994). It has no detectable effect on either CD4 synthesis or transport through the exocytotic machinery (Schwartz 1995).

1.7.6.3 Downmodulation of costimulatory molecules by Nef

The outcome of T cell receptor (TCR) engagement is normally decided by the presence or absence of costimulatory signals delivered via accessory molecules such as CD28 (Arora 2002). In addition to surface downmodulation of CD4, Nef also increases internalization of the CD28 costimulatory molecule, which is required for maximal T-cell activation. The Leu-Leu165/166 and His197 residues of Nef are critical for CD28 downmodulation and this is a phenomenon commonly observed in different isolates of HIV-1 and SIV (Bell 2001; Swigut 2001). The benefit of CD28 surface downmodulation to the virus is not clear, however it has been hypothesized that by downmodulating CD28, the virus can block T-cell activation. The interactions between CD28 and B7 molecules and between TCR/CD4 and MHCII molecules are thought to be important for maintenance of a tight and prolonged interaction between T cells and antigen presenting cells (APCs). Indeed, Nef has also been shown to downregulate the surface expression of B7 family of costimulatory molecules, CD80 and CD86 on APCs (Chaudhary 2005). This was recently shown to occur through a novel endocytic pathway triggered by Nef. The Nef-mediated downmodulation of CD28, CD80 and CD86 in addition to CD4, probably limits the strong interaction of Nef-expressing T-cells with APCs. This would increase subsequent movement of infected T-cells into the circulation and to other APCs to enhance the spread of the virus.

1.7.6.4 MHC Class I Downmodulation by Nef

Antigen presentation by MHCI provides a mechanism by which an infected cell can present foreign antigens to signal the presence of a viral infection. If a foreign antigen-MHCI complex is detected, antigen specific CTL are triggered that lead to the lysis of the infected cell. To counteract this host defense mechanism, several persistent viruses such as poxviruses, herpes viruses, and retroviruses, have developed strategies that target the assembly and trafficking of newly synthesized MHCI molecules in infected cells (Yewdell 2002).

HIV-1 Nef reduces the surface expression of MHCI (Schwartz 1996), thus preventing the exposure of viral antigens on the surface of HIV-infected cells. This function allows HIV-infected cells to escape recognition and lysis by anti-HIV CTLs *in vitro* (Collins 1998), and there is evidence that the ability of Nef to disrupt MHCI antigen presentation is very important for viral disease pathogenesis *in vivo* (Carl 2001). However, MHCI also provides inhibitory signals for natural killer (NK) cells. Thus, infected cells that lack sufficient surface MHCI expression are susceptible to lysis by NK cells. To perhaps avoid this problem, HIV-1 Nef preferentially disrupts HLA-A and HLA-B expression but not that of HLA-C and HLA-E (Cohen 1999). The preservation of these molecules on the cell surface may provide the proper inhibitory signal to avoid detection of virus-infected cells by NK cells. This differential modulation of MHCI expression can be mapped to a tyrosine based sequence (YSQAASS) in the cytoplasmic domains of HLA-A and HLA-B allotypes (Williams 2002), that is absent in HLA-C and HLA-E. However, it should be noted that Nef does not protect completely from immune surveillance, as there is a strong CTL response to HIV infection.

In presence of Nef, the synthesis of MHCI molecule is unaltered and these molecules transport normally through the ER/Golgi route. However, MHCI molecules are rapidly internalized from the cell surface through the endosomes, transported back to accumulate in the trans-golgi network (TGN), and misdirected into post-Golgi clathrin-containing vesicles in Nef expressing cells (Bell 2001). Mutagenesis studies have revealed

functional domains in Nef that are required for its effects on MHCI trafficking. Two additional Nef sorting motifs which are critically important for MHCI downregulation include the SH3-domain binding site (PXXP) and the Met20 residue within the amphipathic helix. Disruptions in these domains also abrogate the ability of Nef to associate with the MHCI cytoplasmic tail domain (Williams 2005), highlighting the importance of this interaction.

1.7.6.5 MHC Class II Downmodulation by Nef

In addition to regulating MHC class I and reducing MHCI-restricted lysis of HIV-infected cells by CTLs, Nef might also affect the stimulation of CD4+ T helper cells by APCs that requires interaction between the T-cell receptor and antigen presented in the context of MHCII on the surface of infected cells. It is well established that Nef impairs T-cell receptor signaling in infected CD4+ T cells by down-modulating the cell surface expression of CD4 and CD28 and the expression or signaling of CD3 (Howe 1998). Recently, it has been shown that Nef also affects MHCII antigen presentation by down-regulation of surface expression of mature MHCII and upregulation of the MHCII-associated invariant chain (Ii, CD74) (Schindler 2003). MHCII associated with Ii represents a maturation intermediate of mature, peptide-loaded MHCII molecules and is inactive in stimulating CD4+ T cells.

Modulation of MHCII-restricted antigen presentation by Nef might contribute to persistent viral replication in HIV-1-infected individuals. It has been demonstrated that the ability of Nef to downregulate surface expression of MHCII and upregulate Ii are well conserved among patient-derived HIV-1 *nef* alleles. Similar observations were made with HIV-2 and SIV Nef proteins (Schindler 2003). Mutational analysis suggests that both these functions are genetically separable. The acidic domain of Nef is involved in downregulation of MHCII but dispensable for upregulation of Ii chain. Mutations in the C-proximal flexible loop consistently abolish the ability of Nef to modulate Ii surface expression but has little effect on downregulation of MHCII. Recent work done by Schindler (2003) also showed that the functional activity of Nef in downmodulation of

MHCII did not decrease during late stages of infection in contrast to MHCI downmodulation, which decreases late in infection.

1.7.7 Vpr protein

The viral protein R (Vpr) of HIV-1 is a small basic protein of 96 amino acids (14 kDa), and is well conserved in HIV-1, HIV-2 and SIV (Tristem 1992). Despite its small size, Vpr has been shown to play multiple functions during virus replication, including an effect on the accuracy of the reverse-transcription process, the nuclear import of the viral DNA as a component of the pre-integration complex, cell cycle progression, regulation of apoptosis, and the transactivation of HIV-LTR as well as host cell genes. Furthermore, Vpr is found in virions, in cells, and exists as free molecules found in the sera and the cerebrospinal fluid of AIDS patients, indicating that it may exert its biological functions through different targets and mechanisms. Vpr is expressed at a late stage of the virus life cycle, but it is present during the early steps of infection of target cells since it is packaged into virions released from the producing cells. The incorporation of Vpr occurs through a direct interaction with the carboxy-terminal p6 region of the gag-encoded Pr55Gag precursor (Accola 1999; Bachand 1999). After assembly and proteolytic cleavage of Pr55Gag in matrix, capsid, nucleocapsid and p6 mature proteins, Vpr is recruited into the conical core of the virus particle (Accola 2000) where it is tightly associated with the viral RNA (Zhang 1998). In addition to a potential role in the initiation step of the reverse transcription process (Stark 1998), it has been shown that Vpr modulates the *in vivo* mutation rate of HIV-1 by influencing the accuracy of reverse transcription. Quantification of the *in vivo* rate of forward virus mutation per replication cycle revealed that the mutation rate was as much as four-fold higher in the absence of Vpr expression when measured in actively dividing cells using a genetically engineered system (Mansky 1996). This activity correlates with the interaction of Vpr with the nuclear form of uracil DNA glycosylase (UNG2) (Mansky 2000), an enzyme involved in the base excision repair pathway that specifically removes the RNA base uracil from DNA. Initially identified from a yeast two-hybrid screening using Vpr as bait, the interaction with UNG was confirmed both *in vitro* and *ex vivo* in Vpr-expressing cells.

The association of Vpr with UNG2 in virus-producing cells allows the incorporation of a catalytically active enzyme into HIV-1 particles where UNG2 may directly influence the reverse transcription accuracy, and this plays a specific role in the modulation of the virus mutation rate. Vpr has also been reported to enhance the transport of viral DNA into the nucleus of non-dividing cells (Connor 1995; Heinzinger 1994) by promoting direct or indirect interactions with the cellular machinery regulating the nucleo-cytoplasmic shuttling. Vpr localizes in the nucleus, but a significant fraction is anchored at the nuclear envelope and can be visualized as a nuclear rim after staining (Kamata 2000; Vodicka 1998).

In addition to its nuclear uptake function, Vpr can also induce G2 cell cycle arrest prior to nuclear envelope breakdown and chromosome condensation, and sustained expression can kill T cells by apoptosis (Emerman 1996). Vpr acts before dephosphorylation of the p34Cdc2 cyclin-dependent kinase by Cdc25, which is required to initiate mitosis (Emerman 1996). Amino acids important for G2 arrest are located in the C-terminal region of Vpr, and cellular proteins have been identified that bind Vpr, including the 65-kDa regulatory subunit of protein phosphatase 2A (PP2A), a serine/threonine phosphatase that regulates the transition from G2 to mitosis (Emerman 1996). In addition to roles in nuclear localization and cell cycle arrest, Vpr can also influence mutation rates during viral DNA synthesis (Mansky 1996) and has been proposed to form an ion channel (Lamb 1997).

1.7.8 Vif protein

The Vif is a 192-residue protein that is important for the production of highly infectious mature virions. Vif mutant viruses show markedly reduced levels of viral DNA synthesis and produce highly unstable replication intermediates (Cohen 1996), suggesting that Vif functions before or during DNA synthesis. It is intriguing that Vif mutants show defects in infectivity only when produced in certain cell types, designated non-permissive or semipermissive, but not when produced in permissive cells. By fusing non-permissive and permissive cells to form heterokaryons, it was demonstrated that the non-permissive

phenotype is dominant. It was hypothesized that permissive cells produced factor(s) that compensate for a lack of Vif or that expression of Vif in permissive cells blocked an inhibitor of viral infectivity (Cohen 1996). Differential expression screening of non-permissive over permissive cells identified a protein, first named as CEM15, which specifically inhibited the replication of *vif*-minus HIV-1 (Spiegel 2000). The CEM15 protein is now known as APOBEC3G and is a cytidine deaminase. The main function of Vif has now been shown to block the antiviral activity of APOBEC3G, that attacks the viral DNA as it is synthesized in the infected cell. The APOBEC3G is incorporated in substantial amounts into the viral particles of Vif-mutants. When the Vif-mutant virus produced in a non-permissive cell infects a naive cell, this encapsidated enzyme gets activated to deaminate cytosine residues in the first (minus polarity) strand of the DNA as it is synthesized by the reverse transcriptase. This deamination converts 1-2% of all cytosines to uracils. As a result, C-U mutations are observed both in pre-integration and post-integration proviral DNAs. Following deamination by APOBEC3G, the heavily modified viral DNA is recognized by the host uracil-DNA glycosidase, which removes dU residues in DNA. The resulting DNA is in turn recognized and degraded by the DNA base excision repair enzymes. The viral cDNA is highly sensitive to this mechanism of attack because the transient DNA intermediates of reverse transcription are single-stranded following RNaseH action and cannot be repaired like double-stranded DNA, using the complementary strand. The presence of many dU residues in the minus strand DNA specifically has an impact on reverse transcription. Alternatively, if reverse transcription is successfully completed at low efficiency and the resulting double-stranded DNA is integrated into the host genome, the C to U conversion on the minus strand leads to corresponding G to A hypermutation on the plus strand. The resulting provirus is so heavily mutated that it is unable to encode functional viral proteins (Conticello 2003; Lecossier 2003; Mangeat 2003; Mariani 2003; Yang 2003). Vif activity may be regulated by post-translational modification because mutation of one of the three serine phosphorylation sites (Ser144) causes a defect in viral infectivity (Yang 1996). Compared with mature wild-type virions, Vif mutant viruses have similar protein and RNA contents but grossly altered core structures, suggesting that Vif may also play a role in viral assembly and/or maturation (Cohen 1996). Consistent with this role, the

infectivity defect can be complemented by supplying *vif* in *trans* in virus-producing cells but not in target, nonpermissive cells, as also seen with Nef. It has been estimated that 10 to 100 molecules of Vif are packaged into the virion (Camaur 1996), suggesting that Vif may function directly within the particle. Incorporation of Vif is probably nonspecific because there is no apparent requirement for any viral protein or RNA and, like Nef, Vif can be incorporated into Mo-MLV particles (Camaur 1996).

1.7.9 The Vpu protein

1.7.9.1 Structural Considerations

Viral protein u (Vpu) is a small integral membrane protein which is cotranslationally inserted into membranes of infected cells (Strebel 1989). This protein consists of a short extracellular N-terminus followed by a transmembrane (TM) domain and a cytoplasmic domain, the latter with two prominent alpha helices (Cohen 1988) (Figure 1.12). The first cytoplasmic helix is reasonably well conserved and shows an amphipathic character with hydrophobic and polar sides supporting a model in which a part of the former is buried in the plasma membrane and the latter exposed to the cytoplasm (Bour 2003). The second cytoplasmic helix is poorly conserved but is prominently made up of acidic amino acids. The region between the two helices is highly conserved with acidic amino acids and two serine residues (S52 and S56) (Figure 1.12) that are phosphorylated by the ubiquitous protein kinase CK-2 (Schubert 1992; Schubert 1994; Friborg 1995). Vpu forms homo-oligomeric structures (Maldarelli 1993), a feature that may be critical for a proposed ion channel function of Vpu. Based on 2D proton NMR spectroscopy of a peptide corresponding to the cytoplasmic domain of Vpu (Wray 1995; Federau 1996), it was proposed that the cytoplasmic domain of Vpu contains two α-helices, helix-1 and helix-2, of Vpu, which could play an important role in the formation of ion channels (Schubert 1996b).

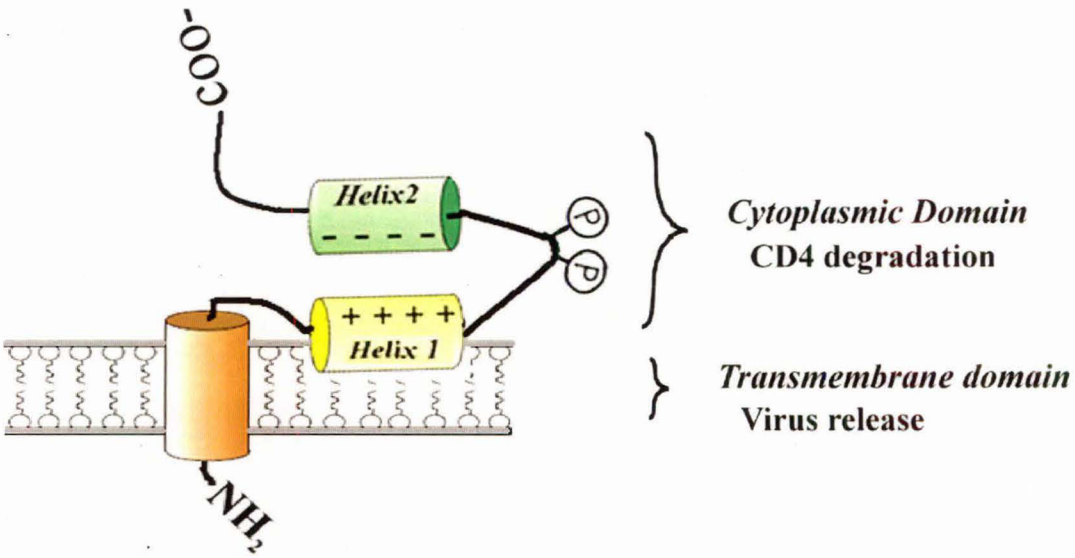


Figure 1.12: Structural domains of Vpu. Vpu consists of an N-terminal hydrophobic domain, that functions as membrane anchor, and a hydrophilic cytoplasmic domain. The cytoplasmic domain contains two amphipathic κ-helical domains of opposite polarity. They are separated by an unstructured region containing two conserved seryl residues which are phosphorylated by protein kinase CK-2. The cytoplasmic domain contains sequences critical for CD4 degradation while the membrane anchor domain has a critical function in regulating virus release and plays an important role in the formation of cation selective ion channels. Vpu forms homo-oligomeric complexes. Only the monomeric form is shown. A putative interaction between helix-1 and helix-2 as shown in the figure suggests only one of many possible conformations of Vpu.

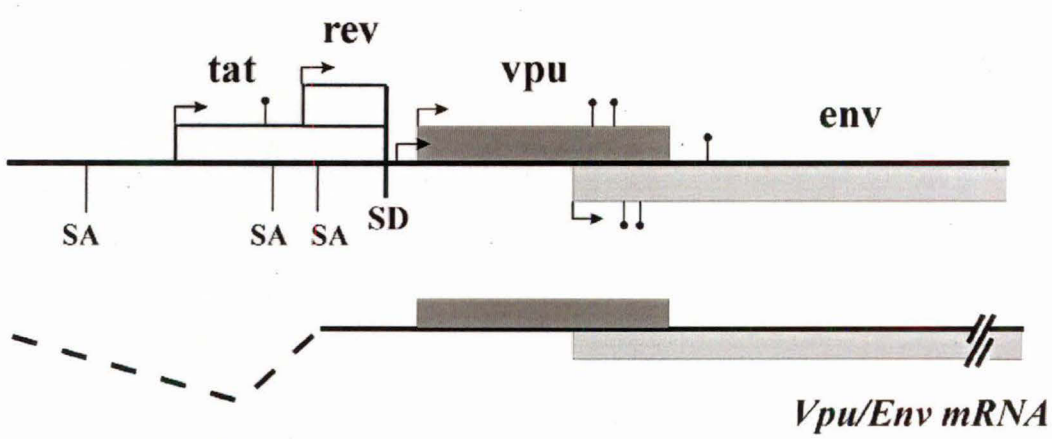


Figure 1.13: Structure of the vpu gene. The vpu gene overlaps at its 3'end with the env gene. Both Vpu and Env are expressed from the same bicistronic mRNA at roughly equimolar levels. The positions of AUG codons at the beginning of open reading frames are marked by arrows. Internal AUG codons are denoted by filled circles. SA = splice acceptor sites; SD = splice donor site (Source: Strebel 1996).

The *vpu* gene overlaps at its 3'-end with the *env* gene (Figure 1.13). Indeed, Vpu and Env are expressed from the same bicistronic mRNA in a Rev-dependent manner, presumably by leaky scanning of ribosomes through the *vpu* initiation codon. In tissue culture, this arrangement results in the synthesis of roughly equimolar levels of Vpu and Env proteins and it is possible that this unusual utilization of viral transcripts might reflect a requirement for the coordinate action of the two viral gene products. Several HIV-1 isolates were found to carry point mutations in the Vpu translation initiation codon but have otherwise intact *vpu* genes. Since removal of the Vpu initiation codon results in increased expression of the downstream *env* gene, it is possible that HIV-1 actually uses this mechanism as a molecular switch to regulate the relative expression of Vpu or Env in infected cells. The possible benefits of such a regulation are unclear. However, it is conceivable that under certain circumstances, *vpu*-defective isolates expressing increased levels of Env protein have a selective advantage over "wild-type" viruses that express Vpu.

1.7.9.2 Functions of Vpu

While Vpu has been shown to be dispensable for HIV-1 replication in culture, *in vitro* studies have shown that Vpu has two primary biological activities. These include the augmentation of virus release from the plasma membrane and degradation of CD4 in the endoplasmic reticulum. In addition, expression of Vpu has been associated with a reduction in syncytia formation of infected cells (Strebel 1989; Klimkait 1990; Yao 1993; Terwilliger 1989; Schubert 1994). This latter phenomenon may be a consequence of the reduced presence of viral Env protein at the cell surface (Yao 1993) due to the more efficient shedding of viral particles in the presence of Vpu. Aside from that, several other less well-defined functions have been associated with Vpu activity. These include the regulation of ER-to-Golgi transport of proteins (Vincent 1995) or the modulation of MHCI antigen complexes.

a) Vpu facilitates the release of virus particles

The original biological phenotype associated with viruses lacking a functional *vpu* gene was the impairment of virus particle secretion from infected cells (Figure 1.14) (Terwilliger 1989). This defect is manifested by the increased budding of viruses from internal membranes and the accumulation of budding particles at the cell surface where they remain loosely attached to the plasma membrane (Klimkait 1990). Electron microscopic data suggest that the inefficient release of virions in absence of Vpu is due to defect in detachment process (Klimkait 1990). This could be due to altered membrane fluidity or activity of other unknown host factors. Such particles can be released however, by vigorously shaking the cultures and are fully infectious (Klimkait 1990). This suggests that Vpu regulates one of the final steps in virus release that is required for the efficient detachment of virions from the plasma membrane of HIV-1 infected cells. Though distinct from its CD4 degradation function, it is not clear whether Vpu enhances virus release through modification of the cellular environment or specific interactions with cellular or viral factors. The formation of conducting ion channels by Vpu (Ewart 1996) and its interaction with a novel tetratricopeptide repeat containing protein (Callahan 1998) favour both possibilities. The TM domain of Vpu has been shown to be important for enhancement of virus release (Paul 1998) and pathogenicity (Hout 2005). This domain is also critical for its ion channel activity (Ewart 1996). An earlier study has used chemical cross-linking to show that Vpu can form oligomers (Maldarelli 1993). *In silico* modeling studies have predicted the same for the TM domain of Vpu (Moore 1998). Although it has generally been accepted that Vpu augments virus secretion from a variety of human cells, including PBMCs, macrophages or CD4+ T cell lines, as well as non-T cell lines such as HeLa or SW480 cells (Terwilliger 1989; Klimkait 1990; Westervelt 1992; Yao 1992; Göttlinger 1993; Balliet 1994; Kawamura 1994; Schubert 1994; Sakai 1995; Theodore 1996), the enhancing effect of Vpu varies between different cell types and has been reported to range from a mere 2-to 3-fold in PBLs to up to 1000-fold in primary macrophages. Despite these cell type specific variations, the principal function of Vpu is not restricted to certain human cell types. In contrast Vpu mediated enhancement of virus secretion was not observed in simian cell lines such as COS-1 and COS-7 cells

(Geraghty 1994; Gottlinger 1991). It is possible that the efficient and Vpu-independent release of viruses from these cells is due to the expression of a cellular equivalent of Vpu in simian cells. Further studies suggest that Vpu has the ability to form cation selective ion channels (Ewart 1996; Schubert 1996b). This activity of Vpu correlates with its ability to regulate virus release.

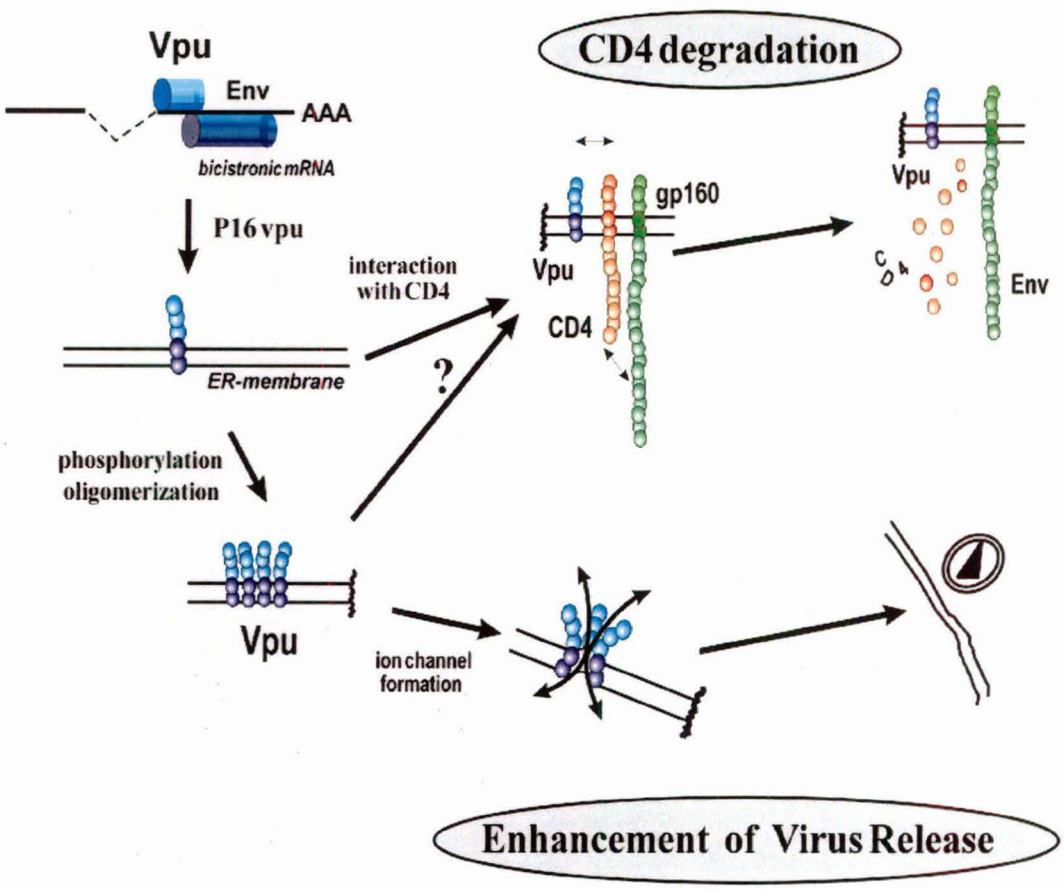


Figure 1.14: Vpu enhances virus particle secretion and induces degradation of CD4. The 81 aa Vpu protein (P16vpu) is synthesized from a bicistronic mRNA and cotranslationally inserted into membranes of the endoplasmic reticulum. Vpu is phosphorylated by CK-2 and forms homo-oligomeric structures. Newly synthesized CD4 is retained in the ER due to the formation of stable complexes with Env protein. Vpu physically associates with CD4 thereby triggering a mechanism that results in the destruction of CD4 and in the release of Env protein from the ER blockage. It is unclear whether this function of Vpu involves monomeric or oligomeric Vpu (? in the cartoon). Vpu exits the ER and accumulates at or near the Golgi. In cells overexpressing Vpu, small amounts of Vpu reach the plasma membrane. Oligomeric forms of Vpu have the capacity to form ion channels which presumably are involved in the regulation of virus particle release (Source: Strebel 1996).

The ability of Vpu to form ion channels requires the integrity of the TM domain, and alterations in the primary structure of the TM domain not only abolish the ability of Vpu to form ion conductive pores but also negate the capacity to regulate virus release (Schubert 1996a, 1996b). The observations that mutations in Vpu that inhibited ion channel activity also affected the enhancement of virus release suggested that these two activities are interrelated. However the question that how an ion channel activity of Vpu could affect virus release is still not very clear. Recent work done by Varthakavi (2003) shows that the requirement of Vpu for efficient viral release is host cell dependent and involves cellular factors. The effect of Vpu on virus release varied significantly among human cell lines. In HeLa cells, Vpu stimulated both HIV-1 and MLV release by about 10- to 20-fold (Gottlinger 1993). Recent studies show that HIV-1 and MLV Gag accumulate in endosomal compartments in HeLa cells and this accumulation is prevented by Vpu through a mechanism that does not involve inhibition of endocytosis (Neil 2006). A recent study showed that expression of dominant negative Rab11a or MyosinVb can inhibit HIV-1 release in the presence of Vpu suggesting that a functioning recycling endosomal compartment is required (Varthakavi 2006). The effect of Vpu on virus release varied significantly between human cell lines, with HeLa cells proving particularly useful for studying this property. Vpu stimulated both HIV-1 and MLV release by about 10- to 20-fold from these cells while in human osteosarcoma cells efficient virus release was completely independent of Vpu. Recent work by Neil (2006) showed that Vpu prevented accumulation of HIV-1 and MLV Gag in endosomal compartment of Hela cells and instead constrained Gag accumulation to the plasma membrane. The accumulation of Gag at intracellular sites in the absence of Vpu required a functional L-domain and was blocked by inhibitors of clathrin-dependent endocytosis, such as DN-dynamin and EPS-15. While Vpu was shown to prevent endocytosis of nascent retrovirus particles, it did not appear to inhibit endocytosis in a general sense, as observed in transferrin uptake assays. Moreover, generalized inhibition of endocytosis prevented intracellular accumulation of HIV-1 and MLV Gag but could not substitute for Vpu function (Neil 2006).

b) Vpu induces CD4 degradation

A second function of Vpu that has been extensively investigated is its ability to induce CD4 degradation in the endoplasmic reticulum (ER) via its cytoplasmic domain (aa 28-81). Vpu exerts a positive effect on HIV-1 infectivity by down-modulating CD4 receptor molecules at the surface of HIV-1-producing cells. During HIV replication in CD4+ cells there is formation of stable complexes between cellular CD4 and the HIV Env protein. These complexes are retained in the ER (Bour 1991; Crise 1990; Kawamura 1989; Jabbar 1990) and therefore prevent transport of both CD4 and Env to the cell surface. In the presence of Vpu however, Env was found to be liberated from CD4/Env complexes (Willey 1992a) concomitant with a significant reduction in the detectable steady state levels of CD4. The work done by Willey (1992) showed that Vpu-induced degradation reduced the half-life of CD4 from 4 to 6 hours to only about 10 minutes in Hela cells. Efficient degradation of CD4 requires its retention in the endoplasmic reticulum (ER). This is normally accomplished by the formation of stable complexes with HIV Env. However, Env protein per se is not involved in CD4 degradation since it is not required when CD4 is artificially retained in the ER (Willey 1992a). Vpu mediated CD4 degradation requires phosphorylation of the serine residues at positions 52 and 56 of the Vpu cytoplasmic domain by casein kinase II (Scubert and Strebel 1994) and the binding of Vpu to CD4 trapped in the ER via the formation of a complex with the HIV-1 envelope precursor gp160 (Bour 1995). The Vpu protein binds CD4 in the ER (Bour 1995), and through its phosphoserine residues binds the beta transducin-repeat containing protein (β-TrCP) in the cytoplasm (Margottin1998). The β-TrCP protein recruits other proteins such as Skp1, Cul-1 and the Cdc34 E2 ubiquitin ligase (Bai 1996). This results in ubiquitination of CD4, its dislocation from the ER and degradation by the proteosome (Schubert 1998). The stable association of Vpu with β-TrCP also affects the latter's cellular functions, one of which is to direct the proteosomal degradation of inhibitor of kappa B (IκB) (Bour 2001). This results in inhibition of NFκB activity and the NFκB-dependent expression of anti-apoptotic genes of the Bcl-2 family (Akari 2001). Also, CD4 degradation can be observed in an *in vitro* translation system where no viral proteins other than Vpu are required (Chen 1993). Unlike Nef, Vpu is unable to target CD4 that

has exited the ER. Nevertheless, the rapid degradation of *de novo* synthesized CD4 in the presence of Vpu ultimately leads to a depletion of cell surface CD4 due to the reduction of the total cellular CD4 pool.

Based on mutational analysis of CD4, it is known that deletion of the cytoplasmic domain of CD4 renders the protein insensitive to Vpu, suggesting that this domain of CD4 contains Vpu-responsive sequences (Chen 1993; Lenburg 1993; Vincent 1993). This is supported by the Vpu dependent degradation of chimeric CD8 molecules containing the CD4 cytoplasmic tail. In fact, transfer of a 18 amino acid membrane proximal fragment of the CD4 cytoplasmic domain (aa 403 to 420) into the CD8 cytoplasmic tail was sufficient to confer Vpu sensitivity. While it has been suggested that cytoplasmic sequences of CD4 are sufficient to confer Vpu sensitivity to heterologous membrane proteins, other studies suggest that sequences located in the TM domain of CD4 may also be required (Raja 1994; Buonocore 1994). The importance of the CD4 cytoplasmic domain for this process is undisputed while the importance of the CD4 TM domain remains unclear. The cytoplasmic domain of CD4, which is predicted to form an α-helical structure (Yao 1995), is involved in the physical interaction with the cytoplasmic domain of Vpu (Bour 1995; Schubert 1996a; Margottin 1996). Such interaction between Vpu and CD4 appears to be a prerequisite for CD4 degradation but is in itself not sufficient to trigger CD4 degradation. Mutational analysis of Vpu demonstrated that phosphorylation of Vpu at two conserved seryl residues, Ser52 and Ser56, is essential for its ability to induce CD4 degradation (Schubert 1994; Friborg 1995) but not for its binding with CD4 (Bour 1995). The Vpu TM domain is not required for the interaction with CD4 (Margottin 1996) and alterations in the TM domain have no apparent effect on CD4 degradation, provided the protein retains its ability to properly associate with membranes (Schubert 1996b; Friborg 1994). However, the fact that CD4-Vpu interaction is necessary but not sufficient to induce CD4 degradation indicates that Vpu performs a catalytic function beyond the binding step.

It has also been observed that in cells producing Vpu-defective virus, NFκB activity was significantly increased even in the absence of cytokine stimulation. However, in the

presence of Vpu, this HIV mediated NFκB activation was markedly reduced (Bour 2001). Binding of Vpu with β-TrCP also inhibits degradation of other β-TrCP substrates including β-catenin and ATF4 resulting in upregulation of these substrates. Importantly, β-catenin also accumulates in cells expressing full-length HIV-1 proviral DNA containing Vpu but not in cells expressing an HIV-1 proviral DNA deleted of *vpu* (Besnard-Guerin 2004). In the same study it has also been found that β-TRCP is redistributed from the nucleus to the cytoplasm in cells expressing Vpu. The recognition of all these cellular ligands by β-TRCP requires phosphorylation of one or two serine residues present in a conserved motif, DSG*XX*S for Vpu, β-catenin, and IκB and DSG*XXX*S for ATF4.

Chapter 2

Materials and Methods

2. Materials and Methods

2.1 Materials

Table-1: Reagents and their sources

Bacterial strains *(Escherichia coli)*	
DH5α	Invitrogen, Carlsbad, CA USA
BL21(DE3)	Novagen, Germany
Yeast strains	
AHI09	Clontech, Palo Alto, CA, USA
Cloning Vectors / Clones	
pGEM-T Easy	Promega Life Science, Madison, WI, USA
pGEX 4T-1	Amersham Pharmacia Biotech, Uppsala, Sweden
pGADT7 and pACT2	Clontech, Palo Alto, CA, USA
pGBKT7 and pAS2	
pIRES2-EGFP	
pEGFP-N1, pECFP-N1	
pEYFP-N1, pDs-Red2-N1	
pMALC2	Amersham Pharmacia Biotech, Uppsala, Sweden
pNL4-3	NIH AIDS Research and Reagent Program, NIAID, Rockville, MD, USA
pNL4-3 del Vpu	

Markers	
1kb ladder, 100 bp ladder	MBI Fermentas, Germany
λ/EcoR1-HindIII	Sigma Chemicals, St. Louis, USA
Protein markers	Amersham Pharmacia Biotech, Uppsala, Sweden Bio-Rad Labs, Hercules, CA, USA MBI Fermentas, Germany
Antibodies	
Anti-NL Vpu	NIH AIDS Research and Reagent Program, NIAID, Rockville, MD, USA
Anti-R5 Vpu	ICGEB, New Delhi, India
Anti-MHCII (L243)	Dr. S. Rath, NII, New Delhi, India
Anti-CD74	Serotech, Oxford, UK
Anti-MBP Anti-Erk Anti-pERK	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Anti-Akt Anti-pAKT Anti-mouse IgG-HRP	Cell Signaling Technology, Beverly, MA, USA
Anti-rabbit IgG- HRP Anti-mouse (IgG)-PE Anti-CD74-PE	Santa Cruz Biotechnology, Santa Cruz, CA, USA

Membranes and filter papers	
Nitrocellulose membrane	Amersham Biosciences, UK
Nitrocellulose membrane circles	MDI, Advanced Microdevices Pvt. Ltd., Ambala, India
DE 81 paper discs and paper sheet	Whatman Co., USA
Restriction endonucleases and DNA modifying enzymes	
Restriction enzymes	New England Biolabs Inc., Beverly, MA, USA, Promega Life Science, Madison, WI, USA
Calf intestinal alkaline phosphatase (CIAP), Klenow, T4 DNA ligase	Promega Life Science, Madison, WI, USA GIBCO-Invitrogen Corporation, NY, USA New England Biolabs Inc., Beverly, MA,USA
Yeast medium components	
Yeast nitrogen base	Difco, Maryland, USA
SD agar	Clontech, Palo Alto, CA, USA
Adenine hemisulphate, Amino acids	Sigma Chemical Company, St. Louis, MO, USA
Chlorophenol red-β-D-galactopyranoside (CPRG)	Roche Diagnostics Corp., Indianapolis, IN, USA
Bacto peptone, Bacto yeast extract	Becton Dickinson Company, Maryland, USA Clontech, Palo Alto, CA, USA
Tissue Culture reagents	
DMEM, RPMI-1640,Penn-strep, Sodiumpyruvate, Opti-MEM, Lipofectin.	Invitrogen, Carlsbad, CA, USA
PMA	Sigma Chemical Company, St. Louis, MO, USA

Resins	
Amylose	New England Biolabs Inc., Beverly, MA, USA
Sephacryl S200HR	Amersham Biosciences, UK
Glutathione Sepharose	
Rink amide MBHA resin	Sigma Chemical Company, St. Louis, MO, USA
Others	
General chemicals	Sigma Chemical Company, St. Louis, MO, USA Roche Diagnostics Corp., Indianapolis, IN, USA Promega Life Sciences, Madison, WI, USA Calbiochem, San Diego, CA, USA.
Plasmid mini-kit	Qiagen, Hilden, Germany
Western blot detection system	Pierce Biotechnology, Rockford, IL, USA Santa Cruz Biotechnology, Santa Cruz, CA, USA
Electroporation cuvette	Bio-Rad, Hercules, CA, USA
Macrophage migration inhibitory factor (MIF)	R & D systems, Minneapolis, MN, USA
DIPC (1,3-diisopropyl carbodiimide)	Sigma Chemical Company, St. Louis, MO, USA
HOBt.H_2O (Hydroxy benzotriazole monohydrate)	

Table-2: Composition of common Media and Solutions

Reagents/ Media	Composition
1X Protease cocktail	16 mg/ml benzamidine, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 10 mg/ml pepstatin A, 1 mM PMSF and 10 mg/ml phenanthroline
2X SDS-PAGE buffer	100 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 4% β-mercaptoethanol, 0.01% bromophenol blue
GST binding buffer	20 mM Tris-HCl (pH 7.9), 180 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.1% NP40
MBP binding buffer	20 mM Tris-HCl (pH 7.4), 0.2 M NaCl, 10 mM β-mercaptoethanol, 1 mM EDTA
MBP elution buffer	MBP Binding buffer with 50 mM maltose
LB (Luria Bertani) medium	For 1 liter, 10 g Bacto-tryptone, 5 g Bacto-yeast extract, 10 g NaCl, pH 7.5
YPD (Yeast Peptone Dextrose) medium	For 1 liter, 20 g Bacto-peptone, 10 g Bacto-yeast extract, 20 g dextrose, pH 7.5
PAGE band elution buffer	50 mM NH_4HCO_3 and 0.1 % SDS
Sonication buffer	PBS with 0.02% Triton X-100, 1 mM PMSF
RIPA buffer	50 mM Tris, 100 mM NaCl, 1% (v/v) NP-40, 0.25% (w/v) Sodium deoxycholate, 1 mM EDTA, 1 mM EGTA, 0.1% SDS
1X PBS (Phosphate-Buffer Saline)	150 mM NaCl, 0.27 mM KCl, 10 mM di-sodium hydrogen phosphate (Na_2HPO_4) and 0.2 mM sodium di-hydrogen phosphate (NaH_2PO_4), pH 7.2-7.4
Wash buffer for western blotting (TBST)	0.1% (v/v) Tween-20 in 1X Tris buffer saline (TBS)
Blocking buffer	5% (w/v) Non-fat dry milk (BLOTTO) in 1X TBST

Transfer buffer	25 mM Tris base, 250 mM glycine, 0.01% SDS, 20% methanol
Lysis buffer	20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% triton X-100, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4. Protease inhibitor tablet (Roche, Germany) was added prior to use.
1 X LiAc (Lithium acetate) buffer	10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM LiAc
Buffer I	100 mM HEPES (pH 7.3), 150 mM NaCl, 4 mM L-aspartate Mg salt, 1% BSA and 0.005% Tween-20
Buffer II	27.1 mg of CPRG in 20 ml of buffer I
Buffer Z	0.1 M Na_2PO_4 buffer (pH 7.0), 10 mM KCl, 1 mM $MgSO_4$
PEG/LiAc solution	10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM LiAc, 40% PEG 3350
TE	10 mM Tris and 1 mM EDTA, pH 8.0
TBE	88 mM Tris-borate, 89 mM boric acid, 2 mM EDTA
Stripping buffer	100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl (pH 6.8)
Solution I	50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 µg/ml RNase A
Solution II	200 mM NaOH, 1% SDS
Solution III	3M potassium acetate, pH 5.5
TBS	10 mM Tris , 150 mM NaCl, pH 7.6

2.2 Methods

2.2.1 Competent Cell Preparation and Transformation

A single colony of *E. coli* DH5α or BL21(DE3) cells, as required was inoculated into 5 ml LB medium and was grown for 16 hrs at 37°C with shaking at 200 rpm. Only 1 ml of culture was inoculated in 200 ml of fresh LB medium and allowed to grow upto A_{600} 0.5 to 0.6, of the culture. The cell culture was kept at 4°C for 30 min and pelleted down at 4,000 rpm at 4°C. The cell pellet was resuspended in 25 ml of 100 mM $CaCl_2$ and kept on ice for an additional 30 min. The cells were then pelleted down and resuspended in 1.5 ml of 100 mM $CaCl_2$. For higher efficiency, cells were kept on ice in the cold room for another 24 hrs. The cells were made 20% in glycerol and aliquots were made in sterile microfuge tubes and were stored at –70°C for future use. The competent cells were used for transformation and propagation of various DNA constructs. For transformation about 100-500 ng of DNA or ligation mixture in a volume of 10 µl was added into 150 µl of competent cells in 1.5 ml microfuge tubes and incubated on ice for 30 min. The tubes were transferred to a 37°C water bath for 5 min or a 42°C water bath for 1.5 min followed by 2 min incubation on ice. To this, 850 µl of LB broth was added and the cells incubated at 37°C with shaking for one hr. The cells were then pelleted down, resuspended in 100 µl of LB medium and plated on LB agar plates containing the appropriate antibiotic.

2.2.2 DNA Isolation

2.2.2.1 Small scale plasmid isolation

Miniprep DNA was isolated by alkaline lysis method, as described in Sambrook *et al.* or with Qiagen Columns as per manufacturers instruction.

2.2.2.2 Cesium Chloride (CsCl) density gradient DNA Preparation

Five ml LB medium was inoculated with a single colony from a freshly streaked plate

and grown overnight at 37°C with vigorous shaking. Subsequently 400 ml of Super Terrific Broth containing 100 μg/ml amplicillin or 50 μg/ml kanamycin was inoculated with 5 ml of the overnight grown culture and incubated for 14-16 hrs with shaking. The cells were then pelleted by centrifugation at 4°C in 500 ml bottles in GS3 rotor for 15 min at 5,000 rpm and the pellet was resuspended in 40 ml Solution I (Table 2) and kept on ice for 10–20 min. Then, 80 ml of Solution II (Table 2) was added, mixed and kept at room temperature for 5 min. To this, 60 ml of Solution III (Table 2) was added, mixed well and incubated on ice for 20-30 min followed by the centrifugation at 8,000 rpm at 4°C for 15 min in a GS3 rotor. The supernatant was filtered through cheese cloth and transferred into a fresh bottle. The DNA was precipitated with 0.7 volumes of isopropanol. The DNA pellet was dried and resuspended in 32 ml of TE mixed with 30 g of CsCl and dissolved completely. Ethidium bromide (200 μl; 15 μg/μl) was poured into 30 ml Beckman Quick-Seal tubes and the tubes filled with the DNA-CsCl mixture. The samples were centrifuged at 48,000 rpm in VTi 50 rotor in L8-70M Beckman ultracentrifuge for 16 hrs. The plasmid DNA bands were collected using a 10 ml syringe fitted with an 18 gauge needle and transferred into 5 ml Quick-Seal tubes. The tubes were weighed, sealed and centrifuged at 60,000 rpm in a VTi 65 rotor for 5 hrs. The closed circular plasmid DNA bands were collected as earlier. To remove ethidium bromide from DNA, an equal volume of water-saturated butanol was added, mixed well and the upper phase was removed. This step was repeated until the DNA solution became clear. The DNA was then diluted 4-5 times with TE (pH 8.0) followed by 3-4 rounds of ethanol precipitation. The DNA concentration was estimated and kept as 1 mg/ml.

2.2.3 Spectrophotometric Estimation of Nucleic Acid

The quantity and purity of nucleic acids in solution was determined by measuring the absorbance at 260 and 280 nm. The concentration of nucleic acids was calculated by taking 1 A_{260} = 50 μg/ml for DNA, 40 μg/ml for RNA and 33 μg/ml for single stranded oligonucleotides. The purity of nucleic acids was checked by their A_{260}/A_{280} ratio.

2.2.4 Purification of DNA Fragments from Agarose Gel

DNA samples were resolved by electrophoresis on 0.8-1.5% of agarose mixed with low melting agarose (50:50) gel. The desired fragment was identified using standard molecular weight markers (1 kb ladder) and purified using GFX gel extraction column. To the cut pieces of agarose gel containing the desired DNA fragment, 350 µl of capture buffer, as supplied with GFX PCR DNA and Gel band purification kit (Amersham Pharmacia Biotech Inc. Piscataway NJ, USA) was added. The gel pieces were dissolved by heating at 55°C for 10 min. The mixture was loaded onto a GFX spin column and spun briefly at 10,000 rpm. The flow through was discarded and the column was washed once with wash buffer at 10,000 rpm for 1 min followed by a dry spin. The purified DNA fragment was eluted with 30 µl sterile water.

2.2.5 Cloning and expression of Vpu

2.2.5.1 Cloning of *vpu* gene

The *vpu* gene was PCR amplified with Pfu polymerase (Stratagene, La Jolla, USA) using as template either the pNL4-3 plasmid DNA (NIH AIDS Research and Reference Reagent Program) or a 3.5 kb fragment encompassing the *vpr* to *env* region previously amplified and cloned from a primary isolate of HIV-1 subtype C. The PCR primers used were as follows (with the restriction sites in italics): For the NL4-3 *vpu* gene, Vpu-NL-F, *GGATCC*ATGCAACCTATAATAGTAGCAATA and Vpu-NL-R, *GAATTC*ACTACAG ATCATCAATATCCCAAG; for the R5 *vpu* gene, Vpu-R5-F, *GGATCC*ATGTTAAA TTTAGATTATAAATTAGGAGTAGG and Vpu-R5-R, *GAATTC*ATTACAAATCAT TAACATCCAAAAGCC. The amplified fragments designated as NL *vpu* and R5 *vpu* respectively, were cloned in plasmid pGEMT-Easy (Promega, Madison, USA) and sequenced in both directions. The gene fragments corresponding to the transmembrane (TM) domains were assembled from the following synthetic oligonucleotides: For the NL4-3 *vpu* TM region, NL-TM-F, CATGGAGATGCAACCTATAATAGTA GCAATAGTAGCATTAGTAGTAGCAATAATAATAGCAATAGCTGTGTGGTCCA

TAGTAATCATAGAATAGG and NL-TM-R, AATTCCTATTCTATGATTACTATGG ACCACACAGCTATTGCTATTATTATTGCTACTACTAATGCTACTATTGCTACA T TATAGGTTGCATCTC; for the R5 vpu TM region, R5-TM-F, CATGGAGATGTTA AATTTAGATTATAAATTAGGAGTAGGAGCATTGATAGTAGCACTAATCATAG CAATAGTCGTGTGGACCATAGTATATATAGAATAGG and R5-TM-R, AATTCC TATTCTATATATACTATGGTCCACACGACTATTGCTATGATTAGTGCTACTAT CAATGCTCCTACTCCTAATTTATAATCTAAATTTAACATCTC.

The cytoplasmic domains were PCR amplified using specific primers for NL *vpu* and R5 *vpu* as follows: for the NL *vpu* cytoplasmic region, NL-Cyto-F, *CCATGG*AGTATAGG AAAATATTAAGA and Vpu-NL-R (shown above); for the R5 *vpu* cytoplasmic region, R5-Cyto-F, *CCATGG*AGTATAGGAAATTGGTACAAC and Vpu-R5-R (shown above).

2.2.5.2 Bacterial expression and purification of MBP-Vpu protein

The *vpu* gene was cloned as a *BamHI-EcoRI* fragment into the pMal-C2 vector (New England Biolabs, Beverly, and USA) and transformed into BL21(DE3) *E. coli* cells to express maltose-binding protein-Vpu fusion (MBP-Vpu). One colony of BL21(DE3) cells containing pMal-C2 vpu construct, was inoculated into 5 ml LB medium containing 100 μg/ml of ampicillin and grown overnight at 37°C with shaking. The culture was diluted 100-fold into fresh 500 ml LB medium containing 100 μg/ml ampicillin and grown at 37°C with shaking upto A_{600} of 0.6 to 0.8. Protein expression was induced by addition of isopropyl-β-D-thiogalactopyranosidase (IPTG) to a final concentration of 1 mM. The cells were then grown for an additional 4 hrs at 37°C. The cells were harvested by centrifugation at 6,000 rpm for 10 min at 4°C in a GS3 rotor (Sorvall). The *E. coli* pellet was washed twice with PBS and either kept frozen or processed further. The cells were resuspended in sonication buffer and lysed by 5 cycles of freeze-thaw followed by sonication 8-10 times with 30 sec pulses, and centrifuged at 12,000 rpm in SA600 rotor (Sorvall) for 15 min. To the clarified lysate, 1.5 ml of amylose resin (New England Biolabs, Beverly, USA), pre washed with PBS was added. The clarified lysate containing MBP-Vpu protein was mixed with amylose resin for 2 hrs at 4°C to allow binding, and

the MBP-Vpu bound resin was then packed in a column. The column was washed with 10 volumes of MBP binding buffer (Table 2) to remove non-specifically bound proteins. The protein was eluted using 5 ml of elution buffer and fractions of 500 µl each were collected and analyzed by SDS-PAGE for presence of the protein. Fractions containing the protein were pooled, dialyzed against PBS. Purity of protein was tested by SDS-PAGE followed by Coomassie Blue staining.

2.2.5.3 Bacterial expression and purification of GST-Vpu protein

The *vpu* gene was PCR amplified, cloned and sequenced from a primary isolate of HIV-1 subtype C. It was subcloned as an EcoRI-BamHI fragment into the expression vector pGEX4T-1 to express GST-Vpu fusion protein. A single colony of each GST-Vpu fusion transformant in *E. coli* was inoculated into 5 ml LB medium containing 100 µg/ml ampicillin and grown overnight at 37°C. Five ml of overnight cultures were diluted at 1:100 into fresh LB medium and grown upto A_{600} of 0.6 to 0.8. Protein expression was induced by addition of isopropyl-β-D-thiogalactopyranosidase (IPTG) to a final concentration of 1 mM. The cells were then grown for an additional 4 hrs at 37°C. The cells were resuspended in sonication buffer and lysed by 5 cycles of freeze-thaw followed by sonication 8-10 times with 30 sec pulses, and centrifuged at 12,000 rpm in SA600 rotor (Sorvall) for 15 min. The protein was mainly found in insoluble form and accumulated in inclusion bodies (IBs). These IBs were purified from clarified cell lysates as pellets in PBS containing 2 M urea. The purified IBs were resuspended in 2X SDS-PAGE loading buffer and the proteins were separated on preparative SDS-10% polyacrylamide gels. A strip of gel was sliced out and stained with Coomassie Blue. The stained sliced piece was aligned with the original gel and the band corresponding to the Vpu-GST protein was sliced out and crushed using a 10 ml syringe. About 10 ml of PAGE band elution buffer (Table 2) was added to the crushed gel pieces. The sample was boiled in elution buffer for 10 min and kept on a Nutator overnight at room temperature. Later, the gel pieces were filtered out using glass wool in a 10 ml syringe. The eluted protein was lyophilized and dissolved in PBS.

2.2.5.4 *In vitro* transcription and translation of Vpu

Proteins were expressed *in vitro* using a coupled transcription-translation system (TNT coupled reticulocyte lysate system; Promega Life Science, Madison, WI, USA). The gene to be expressed was cloned downstream of a T7 promoter in an appropriate expression vector. The plasmid to be transcribed (and translated) was added to the reaction mixture containing T7 polymerase, amino-acid mixture (lacking methionine), appropriate buffer and rabbit reticulocyte lysate. All materials supplied in the kit and were used according to the manufacturer's guidelines. Exogenously added [^{35}S] methionine/cysteine was used (>1,000 Ci/ml) to label the protein as recommended. Reaction was allowed to complete for 90 min at 30°C. Subsequently, the *in vitro* synthesized proteins were analyzed by SDS-PAGE and autoradiography.

2.2.6 Polyacrylamide Gel Electrophoresis of proteins

The polyacrylamide gel electrophoresis (PAGE) of proteins was performed in the presence of 0.1% SDS in the gels. Protein samples were prepared by mixing with equal volume of 2X SDS-PAGE loading buffer (Table 2). Samples were kept in boiling water for 5 min, centrifuged briefly and loaded on the gel. Gels were run at 50 V till the proteins were stacked properly and thereafter gels were run at a constant voltage of 100 V. Following the run, gels were stained with Coomassie Blue or electro-blotted onto the nitrocellulose membrane (Hybond-C, Amersham Biosciences, UK). Electroblotting was performed in transfer buffer (Table 2) at a constant voltage of 70 V for 45 min.

2.2.7 Western blotting

For western blotting, the separated proteins were transferred to a nitrocellulose membrane as described earlier. After blocking with Tris-buffer saline (TBS) containing 5% BLOTTO for 1–2 hrs at room temperature or overnight at 4°C, the membrane was

washed with TBST and incubated with primary antibody appropriately diluted in TBST containing 5% BSA for 2 hrs at room temperature or overnight at 4°C. The blot was then washed four times for 10 min each with TBST and then incubated with horseradish peroxidase-linked anti-rabbit or anti-mouse IgG diluted in TBST containing 5% BLOTTO for 90 min at room temperature. Chemiluminescent detection of proteins was carried out using the Phototope horseradish peroxidase Western blot detection system (Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to the supplier's protocol.

2.2.8 Antibody generation against Vpu

2.2.8.1 Immunization of rabbits

For raising antibodies against Vpu, two New Zealand white rabbits were immunized as follows. Primary immunization was carried out with intramuscular (IM) injection of 400 μg MBP-Vpu in Complete Freund's adjuvant. One month later, the first booster was given IM with 400 μg MBP-Vpu in Incomplete Freund's adjuvant (IFA). Two weeks later, the second booster was given IM with 300 μg GST-Vpu in IFA. Ten days later, the third booster of 100 μg GST-Vpu was given intravenous without any adjuvant. Sera collected from the rabbits ten days after each immunization were assayed for antibody titers in a standard direct ELISA format. Non-specific antibodies were removed from the sera by pre-incubation with lysates of *E. coli* BL21(DE3) and DH5α. Antibodies against MBP and GST were removed by incubating sera with GST and MBP saturated beads respectively. Adsorption was carried out by incubating 5 ml of 1:10 diluted serum for 2 hrs at 4°C with rocking, with either 150 μl of MBP-saturated amylose resin or 50 μl of GST-saturated glutathione sepharose (Amersham Biosciences, UK). Crude and MBP or GST- adsorbed sera were tested to estimate anti-Vpu titers. The unbound material was used as adsorbed serum

2.2.8.2 Analysis of sera by ELISA

Titers of anti-Vpu antibodies in the sera of immunized rabbits were determined by a direct ELISA. A 96-well ELISA plate was coated overnight at 4°C with 200 ng of the recombinant Vpu protein in PBS, per well. GST and MBP coated wells were used as controls. Next day, the plate was blocked with 5% BLOTTO in PBS, 0.1% Tween-20 for 2-4 hrs at room temperature. Following blocking, the plate was washed 4 times with PBS, 0.1% Tween-20 and then incubated with serial log dilutions of the individual rabbit sera for 90 min at 37°C. The wells were washed 5 times with PBS, 0.1% Tween-20 and then incubated with anti-rabbit IgG HRPO at 1:4000 dilution for 1 hr at 37°C. Following the secondary incubation, the wells were again washed five times with PBS, 0.1% Tween-20. The colour was developed with *o*-phenylenediamine (OPD) dihydrochloride (1 mg/ml) in 0.05 M phosphate-citrate buffer. The colour reaction was terminated by addition of 2 N H_2SO_4 and the absorbance was read at 495 nm.

2.2.9 Yeast two-hybrid analysis for Vpu oligomerization

2.2.9.1 Cloning of *vpu* in yeast two-hybrid vectors

The GAL4-based two-hybrid system contained the DNA binding domain vector pGBKT7 and the activation domain vector pGADT7. The NL and R5 *vpu* genes were cloned into the pGBKT7 and pGADT7 vectors as *EcoRI-BamHI* fragments from the pGEMT-Easy clones. The nucleotide sequences corresponding to the transmembrane and cytoplasmic domains of the Vpu proteins were similarly cloned in the two-hybrid vectors as *NcoI-BamHI* fragments, respectively. Expression of the relevant fusion proteins from each of the *vpu* two-hybrid constructs was checked in a T7 polymerase based *in vitro* coupled transcription-translation system (Promega Life Science, Madison, WI, USA) followed by immunoprecipitation with anti-Vpu antibodies.

2.2.9.2 Transformation of Yeast strain AH109

Small-scale transformation of yeast *Saccharomyces cerevisiae* for yeast two-hybrid analysis was performed using the protocol described in the Yeast Protocol Handbook (Clontech). For this, *S. cerevisae* AH109 (*MATa, trp1-190, leu2-3, 112, ura3-52, his3-200, gal4*D, *gal80*D, *LYS2::GAL1UAS-GAL1 TATA-HIS3, GAL2UASGAL2TATA- ADE2*) was used. Five ml of YPD medium was inoculated with a single colony and incubated at 30°C for 18-20 hrs with shaking at 250 rpm to stationary phase ($A_{600} > 1.5$). This primary culture was transferred to 300 ml of YPD medium at 30°C with shaking (250 rpm) until the A_{600} reached 0.4-0.6. The culture was then transferred to 50 ml Falcon tubes and centrifuged at 3000 rpm for 5 min at 4°C. The supernatant was discarded, cell pellets thoroughly resuspended in sterile water, and pooled into one tube to a final volume of 25-50 ml. The cells were collected by centrifugation at 5000 rpm for 5 min. The supernatant was discarded and the cell pellet resuspended in 1.5 ml of freshly prepared, sterile 1X LiAc (Table 2). Three µg each of pGADT7 and pGBKT7 constructs and 10 µg of salmon sperm DNA was added to labeled, fresh Eppendorf tubes and mixed well. To each tube, AH109 yeast competent cells (100 µl) were added and mixed well by vortexing. To this, 300 µl of sterile PEG/LiAc solution (Table 2) was then added and vortexed for 5 sec to mix well. These tubes were incubated at 30°C for 30 min with shaking at 200 rpm. To the tubes, 70 µl of DMSO was added and mixed gently by inversion. The cells were then subjected to heat shock at 42°C for 5 min, kept on ice for 1-2 min, collected by centrifugation at 7,000 rpm for 30 sec at room temperature, and resuspended in 200 µl of sterile 1 X TE buffer. From this, 100 µl was plated on SD leu^- trp^- (synthetic dextrose lacking leucine and tryptophan) plates. The plates were then incubated at 30°C until colonies appear in 2-4 days.

2.2.9.3 Identification of protein-protein interaction

The activation domain (AD; pGADT7) and DNA-binding domain (BD; pGBKT7) vectors with inserts co-transformed into AH109 cells were first selected for co-transformants on appropriate SD leu^- trp^- plates. The colonies that grew on the plate were

replica plated onto SD leu$^-$trp$^-$his$^-$ (synthetic dextrose lacking leucine, tryptophan and histidine) plates to select for colonies showing histidine auxotrophy; this is indicative of an interaction among the AD and BD fusion proteins. Yeast cells alone or cells co-transformed with empty AD and BD vectors, BD fusion constructs and empty AD vector, and AD fusion constructs and empty BD vector were used as negative controls. Yeast cells co-transformed with AD-SNF1 and BD-SNF4 were used as a positive control. Further, colonies were patched on SD leu$^-$trp$^-$his$^-$ with 20 mM 3-amino-1,2,3,-triazole (3-AT) plates which confirms interaction by blocking leaky expression of histidine.

2.2.9.4 Colony filter-lift assay

Using forceps, a clean, autoclaved and dried 90 mm disc of nitrocellulose membrane was placed over the surface of the plate containing colonies to be tested. The filter was gently rubbed with the side of the forceps to help colonies cling to the filter. When the filter was evenly wet, it was carefully lifted off the plate surface using forceps and completely submerged, in a container of liquid nitrogen (colony side facing up) for 10 sec, and then allowed to thaw at room temperature. This freeze-thaw step was repeated 3-5 times, after which, the filter was carefully placed (colony side up) on a filter paper presoaked in 5 ml buffer Z (Table-2), containing 20 µl of 20% X-gal, and 8 µl of β-mercaptoethanol. After this, the membrane was kept at 30°C in the dark. The blue colour developed in 2-3 days.

2.2.9.5 Liquid β-galactosidase assay

The liquid β-galactosidase activity was determined using the substrate chlorophenol red-β-galactopyranosidase (CPRG). The culture was grown in 5 ml SD medium for 16 hrs at 30°C. The cells were vortexed and 1 ml of inoculum was transferred to 4 ml of fresh medium. The cells were incubated at 30°C with shaking at 200 rpm for 3-5 hrs till the cells reached the mid-log phase (A_{600} = 0.8-1.0). Equivalent amounts of culture (1.0-1.5 ml) were taken in microfuge tubes and harvested by centrifugation and washed with 1 ml of buffer I (Table-2). The cells were then suspended in 300 µl of buffer I from which 100 µl suspension was transferred into a fresh microfuge tube. Consequently cells were

permeabilized by 5 cycles of freeze-thaw in liquid nitrogen for 10 sec every time followed by a 37°C water-bath for the same time. To the tube 700 µl of buffer II (Table-2) was added and mixed by vortexing. One ml of buffer II was kept aside to be used as blank. After 1 hr, 0.5 ml of 3 mM $ZnCl_2$ was added to all the tubes to stop the reaction. The tubes were centrifuged at 14,000 rpm and the supernatant was transferred to freshly labeled tubes. The absorbance at 578 nm was recorded for each sample. Only those readings that lie in the linear range of 0.2-1.8 absorbance units were considered. Relative enzymatic activity was calculated as a mean of three independent transformants. Appropriate positive and negative controls and buffer blanks were used.

2.2.10 Pull down assays

For the MBP pull-down assay, the MBP-Vpu protein was expressed in *E. coli* BL21(DE3), and purified as described earlier. To the clarified lysate, amylose resin (New England Biolabs, Beverly, USA) was added and the MBP-Vpu protein was allowed to bind for 2 hrs at 4°C. The resin was washed with 10 volumes of wash buffer containing 20 mM Tris-HCl, pH 7.4, 0.2 M NaCl, 10 mM β-mercaptoethanol, 1 mM EDTA to remove non-specifically bound proteins. To 50 µl of amylose beads saturated with either MBP or MBP-Vpu, 25 µl of ^{35}S-labeled Vpu (prepared using a coupled *in vitro* transcription-translation system) diluted in 500 µl PBS was added and allowed to bind for 3 hrs at 4°C with mixing. The beads were then centrifuged down and washed five times with 500 µl each of PBS containing 0.1% Triton X-100, resuspended in SDS dye loading buffer and subjected to SDS-PAGE and autoradiography.

2.2.11 Gel permeation chromatography

The oligomeric forms of Vpu were also analyzed by gel permeation chromatography on Sephacryl S200HR (Pharmacia-Amersham). A 36 ml column was packed followed by calibration with lysozyme and BSA for molecular size estimation at a flow rate of 0.4 ml/min. The ^{35}S labeled Vpu was *in vitro* synthesized as described earlier using a T7 construct pGBK-vpu(R5). Following *in vitro* expression and ^{35}S labeling, 150 µl of the

reaction mixture was loaded on the column pre-equilibrated in PBS. The column was run at a flow rate of 0.4 ml/min and fractions of 0.25 ml each were collected. Liquid scintillation counting and SDS-PAGE analysis of the peak radio labeled fractions estimated the elution of Vpu.

2.2.12 Confocal microscopy and FRET assays

For confocal microscopy and FRET, the NL and R5 *vpu* genes were cloned as *EcoRI-BamHI* fragments in the Living Colors™ vectors pEGFP-N3, pEYFP-N1 and pECFP-N1 (Clontech). Prior to this, the genes were first PCR amplified, cloned in the pGEMT-Easy vector and sequenced. The following PCR primers (with restriction sites shown in italics) were used: for NL *vpu*, NL-X-F, *GAATTC*ATGCAACCTATAATAGTAGCAATA and NL-R-GFP, *GGATCC*GCGCAGATCATCAATATCC; for R5 *vpu*, R5-X-F, *GAATTC*ATGTTAAATTTAGATTATAAATTAGGAGTAGG and R5-R-GFP, *GGATCC*TGCCAAATCATTAACATCCAAAA.

For colocalization experiments, COS-1 and U2-OS cells were seeded at about 50% confluency on cover slips in 12-well plates, grown for 18 hrs and then cotransfected with expression Living Colors™ vectors expressing Vpu and any of the subcellular markers. At 24 to 48 hrs post-transfection, the PBS-washed cells were fixed with 2% paraformaldehyde in PBS at room temperature for 10 min. These were then mounted using Antifade (Bio-Rad, Hercules, USA) and sealed. Confocal images were collected sequentially using a 60 planapo NA 1.4 objective on a Radiance 2100 laser scanning system (Bio-Rad, Hercules, USA) equipped with a Nikon Eclipse TE2000-U microscope. For FRET analysis, COS-1 and U2-OS cells were similarly transfected with ECFP-vpu and EYFP-vpu expression plasmids. The ECFP-Vpu (FRET donor) and EYFP-Vpu (FRET acceptor) images were acquired sequentially in live cells using the Blue diode 405 nm and the Argon ion 514 nm laser lines, respectively. Images of the ECFP emission were collected using a 500 DCLPXR dichroic mirror with an HQ 485/30 emission filter. The EYFP emission images were collected using a 560 DCLPXR dichroic mirror with an HQ 545/40 emission filter. FRET was detected using the acceptor photobleaching

approach. Two different regions within a cell expressing both proteins were selected as the region of interest (ROI), one for FRET and the other as a control. The ROI was zoomed in and bleached with the high intensity Argon 514 laser. The mean intensities of ECFP and EYFP were simultaneously recorded in the pre-bleach and post-bleach periods as a live graph. An increase in ECFP intensity following EYFP bleaching is indicative of FRET between the donor and acceptor fluorophores. The percent FRET efficiency was calculated using the formula

$$\% \text{ FRET efficiency} = 1 - \frac{\text{(ECFP intensity before photo bleach) X100}}{\text{(ECFP intensity after photo bleach)}}$$

2.2.13 Yeast two-hybrid library screening and assays

The R5 *vpu* gene was cloned into the pGBKT7 vector as an *EcoRI-BamHI* fragment as described earlier. The nucleotide sequences corresponding to the transmembrane and cytoplasmic domains of the Vpu proteins were similarly cloned in the two-hybrid vectors as *NcoI-BamHI* fragments, respectively. Expression of the relevant fusion proteins from each of the Vpu two-hybrid constructs was tested in a T7 polymerase based *in vitro* coupled transcription-translation system (Promega, Madison, USA). A commercial human leukocyte yeast two-hybrid cDNA library (Matchmaker™, Clontech, Germany) derived from mRNA isolated from the normal peripheral blood leukocytes pooled from 550 healthy Caucasians was used for screening. The library containing $2x10^6$ independent clones was amplified to 3X redundancy and titered as suggested by the supplier. From stock, 1µl of 1: 1,000,000 dilutions was mixed with 50 µl of LB and was plated on LB agar plate with ampicilin. The colony forming units (CFUs) were calculated as follows.

$$\text{CFU per µl} = \frac{\text{Colonies on plates x } 10^6 \text{ (Dilution factor)}}{\text{50 (Amount plated in µl)}}$$

Around 6 x 10^6 clones were taken to get a plasmid DNA pool so that each clone has the probability of getting selected. The amount of library stock to be plated was calculated on the basis of CFU per µl of stock. A total of 32 µl of stock was found to be sufficient enough to give 6 x 10^6 colonies. This amount was diluted into 45 ml of LB media, mixed

well and plated on 300 LB agar plates containing 100 μg/ml ampicillin. Plates were incubated at 30°C for 60 hrs as recommended by the supplier. The colonies were scrapped from the plates in LB medium and a pool was allowed to grow in 5 liter of LB medium containing 100 μg/ml ampicillin for 3 hrs. A pool of plasmid DNA was isolated by CsCl density gradient centrifugation. For the screen, plasmids pGBKT7-vpu (BD-Vpu) and pACT-leukocyte cDNA (AD) were co-transformed into the *Saccharomyces cerevisiae* strain AH109, containing the *HIS3* and *lacZ* reporter genes under the control of GAL4-binding sites. The host strain containing plasmids pAS2-SNF1 and pACT2-SNF4 was used as a positive control. Various negative controls that included single or dual transformants were also run in the same assay. The AH109 yeast cells were transformed using the lithium acetate procedure and plated on synthetic dextrose (SD) in the absence of leucine, tryptophan and histidine (SD $leu^-trp^-his^-$) to grow co-transformants containing Vpu interactors. The clones that grew were replica plated on SD $leu^-trp^-his^-$ plates containing 20 mM 3-amino-1,2,3-triazole (SD/LTH^-3AT) to select strong interactors. The β-galactosidase filter-lift assay and liquid β-galactosidase assay were carried out using the substrate chlorophenol red-β-D-galactopyranosidase as described earlier. From individual positive yeast clones on SD/LTH^-3AT plates, the plasmid DNAs were isolated and used to transform *E. coli* DH5α, selected on LB-ampicillin plates to eliminate cells containing plasmid(s) other than pACT2. The plasmid DNAs so obtained were purified, digested with EcoRI and XhoI and analyzed on agarose gels. The inserts from these positive clones were sequenced and the sequences obtained analyzed by BLAST search.

A direct yeast two-hybrid assay was carried out using the pGBKT7-vpu and pACT-CD74 to confirm the interaction, essentially as described earlier. To identify the CD74-interacting domain of Vpu, the assay was carried out using pACT-CD74 and either pGBKT7-vpu(TM) or pGBKT7-vpu(cyto). The AH109 yeast cells were transformed using the lithium acetate procedure and plated on SD/LTH^- plates to test protein interactions. Various negative controls that included single or dual transformants were run in the same assay as also the host strain containing plasmids pAS2-SNF1 and

pACT2-SNF4 as a positive control. The interaction specificity was tested by replica plating on SD/LTH⁻3AT plates and through filter-lift and liquid β-galactosidase assays as described earlier.

2.2.14 Synthesis of CD74 peptide

The 30-amino acid cytoplasmic domain of CD74 was synthesized *in vitro* on 0.2 millimoles of Rink amide MBHA resin using solid phase fmoc (fluoronyl methyloxycarbonyl) chemistry. To synthesize the peptide at 0.2 millimole scale, 0.6 millimoles of each amino acid were used. Rink amide resin was taken in vessel and washed thrice with DMF (dimethyl formamide) followed by three washes with 20% piperidine in DMF for 5, 10 and 15 min respectively and again three washes with DMF. Before coupling of the first amino acid, 20 µl of resin was taken and washed with methanol followed by the Kaiser test to check the availability of free amino groups. In each cycle 94 µl of DIPC (1,3-diisopropylcarbodiimide), 81 mg of HOBt.H_2O (Hydroxy benzotriazole monohydrate) and 0.6 millimoles of amino acid were added in a final reaction volume of 2 ml in DMF. The reaction was allowed at room temperature with constant shaking for 5 hrs. Synthesis was monitored by the Kaiser test in each cycle. Negative Kaiser test showed confirmation of amino acid coupling. The final synthesized peptide was cleaved from the resin with 94.5% TFA, 1.25% EDTA for 2 hrs at room temperature with gentle shaking. The cleavage mixture with resin was filtered and the peptide precipitated with cold ether. The peptide was dissolved in 5% acetic acid and lyophilized for 16 hrs. The size and purity of the synthesized peptide was confirmed by SDS-15% polyacrylamide gel electrophoresis and by mass spectrometry. The peptide sequence was MHRRRSRSCREDQKPVMDDQRDLISNNEQL.

2.2.15 Binding assay to detect interaction of Vpu and CD74

For the binding assay, 200 ng of the CD74 cytoplasmic peptide, a nonspecific ACTH peptide and the MBP-Vpu protein were coated in the wells of an ELISA plate for 12 hrs at 4°C and then blocked for 2 hrs at room temperature with 5% BLOTTO in TBST. The

^{35}S-labeled full length, cytoplasmic and transmembrane Vpu proteins were synthesized as described earlier. The reticulocyte reaction mixtures for ^{35}S-labeled full length, cytoplasmic and transmembrane Vpu proteins were added and incubated for 12 hrs at 4°C. A blank ^{35}S reaction mixture was used as a negative control. After three washes with TBST, the retained ^{35}S proteins were eluted in 100 µl of Tris-buffered saline containing 1% SDS and quantitated by scintillation counting. The MBP-Vpu coated wells were used as a positive control as Vpu is known to form oligomers.

For another binding assay, CD74 and ACTH peptides similarly coated on an ELISA plate were used to evaluate the binding of purified MBP-Vpu or MBP (as control). The purified proteins were diluted in TBST containing 5% BLOTTO and 400 ng of either MBP-Vpu or MBP incubated in each well for 2 hrs at room temperature. After two washes with TBST, 1:1000 diluted rabbit anti-MBP antibodies in TBST containing 5% BSA were added and incubated for 2 hrs at room temperature. This was followed by three washes with TBST and incubation for 90 min at room temperature with 1:2000 diluted HRPO-linked anti-rabbit IgG in TBST containing 5%BLOTTO. Following three washes with TBST, the color was developed with diaminobenzidine and the A_{490} was measured.

2.2.16 Immunoprecipitation and western blotting

Human monocytic U937 cells were infected with HIV-1 wild type NL4-3 and *vpu* defective NL4-3U35 viruses. Cells were collected at various times post-infection, washed with PBS, and ~$4x10^6$ cells were lysed using 450 µl of radioimmunoprecipitation (RIPA) buffer lacking SDS. After incubation on ice for 45 min, the lysates were clarified at 12,000 rpm for 10 min and the supernatants incubated on ice for 1 hr with 2.5 µg of either mouse anti-human CD74 (Serotech, Oxford, UK) or a mouse anti-human EGFR (Santa Cruz Biotechnology, Santa Cruz, USA), the latter as a negative control. To this 50 µl of a 50% suspension of RIPA-washed Protein A-Sepharose beads were added, and the mixture was incubated with constant shaking at 4°C for 90 min. The beads were washed five times with 0.5 ml each of the lysis buffer, resuspended in 30 µl of SDS-PAGE loading buffer, heated at 100°C for 5 min, centrifuged, and the supernatants were loaded

on a SDS-12% polyacrylamide gel. For Western blotting, proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane. After blocking with Tris-buffered saline (TBS) containing 10% BLOTTO for 12 hr at 4°C, the membrane was washed with TBS containing 0.1% Tween 20 (TBST) and incubated for 2 hrs at room temperature with a rabbit polyclonal anti-Vpu antibody diluted 1:1000 in TBST containing 5% BSA. The blot was then washed thrice for 10 min each with TBST and then incubated for 90 min at room temperature with HRPO-linked anti-rabbit IgG diluted 1:2000 in TBST containing 5% BLOTTO. The blot was again washed as above and chemiluminescent detection of proteins was carried out using a Western blot detection system (Santa Cruz Biotechnology, Santa Cruz, USA) according to the supplier's guidelines.

2.2.17 Cell lines

HeLa, BMC-2, COS and U2OS cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1X penicillin-streptomycin, and were subcultured on reaching 90% confluency. The monocytic cell lines U937 were cultured in RPMI 1640 supplemented with 10% FBS and 1X penicillin-streptomycin and subcultured as required. The U937 cells were used for infection experiments.

2.2.18 DNA Transfection

2.2.18.1 Electroporation

HeLa cells were grown in DMEM and 10% FBS. At 75% confluence, about 4-7 million cells were trypsinized and transfected with 10 μg of either proviral DNA or plasmid constructs in a 300 μl total volume of DMEM containing 10 mM glucose by electroporation. The electroporator (Bio-Rad, Hercules, USA) was set to 276V and 975 mF. The electroporated cells were plated into wells of six-well tissue culture dishes in 3 ml of DMEM-10% FBS-1X Penicillin-Streptomycin. Virus production was measured in culture medium using a p24 ELISA (Beckman-Coulter). The monocytic line U937 was

grown in RPMI and 10% FBS. Electropration was carried out under the same conditions except that cells were plated in RPMI-10% FBS-1X Streptomycin.

2.2.18.2 Lipofection

Human osteosarcoma (U2-OS) and HeLa cells were grown in DMEM containing 10% FBS in a 10% CO_2 incubator. The cells were seeded at about 30-50% confluency in 60 mm dishes one day before transfection. On the following day, cells were transfected with CsCl gradient purified or column purified (Qiagen, Hilden, Germany) DNA using Lipofectin (Invitrogen, Carlsbad, CA USA). To 90 µl Opti-MEM (Invitrogen, Carlsbad, CA USA), 10 µl Lipofectin was added and incubated for 45 min (Solution A). To this was added 5-10 µg DNA in 95 µl of Opti-MEM (Solution B). At the end of incubation, Solution A and Solution B were mixed and incubated further for 15 min at room temperature to allow formation of a DNA-lipid complex. Meanwhile, the cells were washed once with PBS and once with Opti-MEM. Finally, to each plate containing 1.8 ml Opti-MEM, 200 µl of the DNA-lipid mixture was added and incubated at 37°C. After six hrs of incubation, the Opti-MEM was replaced with complete medium containing DMEM and 10% FBS. At about 48 hrs post transfection, the cells were harvested and used for various experiments.

2.2.19 Virus production and infection

HIV-1 viral stocks were generated by electroporation of HeLa cells with the infectious molecular clone pNL4-3 or the Vpu-defective clone pNL4-3U35. After transfection, growth medium was added. Virus stocks were harvested 72 hrs post-transfection and filter sterilized. The viruses were titrated by p24 ELISA. For infection, U937 cells were washed with RPMI and starved for 90 min without serum. For each infection $1X10^6$ cells were infected with 0.1 MOI of viruses. After a 4 hrs adsorption, the cells were washed and kept in complete medium. At different time points post-infection, cells were washed with PBS and harvested for further studies.

2.2.20 Macrophage migration inhibitory factor (MIF) Signaling in Vpu expressing cells

Around 20 million U937 cells were transfected by electroporation with 20 μg of pEGFP-vpu or pEGFP as a control. After 36 hrs, cells were starved for 3 hrs followed by MIF stimulation for 2 hrs. The final concentration of MIF was 100 ng/ml. Equal numbers of unstimulated cells were used as controls. The cells were washed twice with PBS, 20 μl of SDS dye was added and the samples were boiled. MIF-dependent phosphorylation of ERK-1/2 (p44/p42) was measured by Western blotting of lysates with anti-phospho Erk (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:1000 dilution. The blot was then stripped and reprobed for total Erk using mouse anti-Erk (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody at 1:1000 dilution.

2.2.21 Flow cytometry

Infected U937 cells were collected at different times post-infection, washed with PBS and stained for total and surface MHCII. The L243 monoclonal antibody recognizes mature MHCII while anti-CD74 measures levels of immature MHCII as well as free CD74. The U937 cells were washed twice with PBS and then fixed with 100 μl of fixative (Dako-Intra Stain kit) for 15 min at room temperature. To this, 100 μl of L243 culture supernatant or 5 μg of anti-CD74 was added and incubated at room temperature for 15 min. The cells were washed in PBS and stained with 100 μl of 1:200 diluted PE-conjugated secondary antibodies for 15 min at room temperature. For total MHCII intracellular staining the cells were fixed and permeabilized with 100 μl of permeabilization solution (Dako-Intra Stain kit) for 15 min and stained for MHCII. The cells were then washed twice in PBS containing 1% BSA and resuspended in 500 μl of the same for acquisition. The stained cells were acquired using a Cyan-ADP flow cytometer (Dako, Denmark) and data analyzed using the Summit software version 4.3. U937 cells transfected with expression vectors for a Vpu-EGFP fusion protein or EGFP as a control, were similarly stained for surface or total levels of MHCII or CD74.

2.2.22 Antigen-presentation assay

Around 8 million murine macrophages BMC-2 cells were cotransfected with 15 μg each of p-EGFP-vpu and *myc*-p-tagged OVA to ascertain that a majority of OVA-*myc*-p-expressing transfected cells also expressed Vpu. After 12 hrs of transfection, the dead cells were removed by flotation on Ficoll-Hypaque. The live cotransfected BMC-2 cells obtained ($3x10^6$ cells/ml) were then used as APCs to stimulate OT-II TCR transgenic mice splenic CD4+ T cells ($1x10^6$ cells/ml) for 24 hrs. Activation-induced proliferation of CD4+ T cells was measured by [^{3}H]-thymidine incorporation. As controls for cell death during the assay, the transfected BMC-2 cells were stained with propidium iodide at 12 hrs and 24 hrs into the stimulation assay, and apoptotic cells were quantitated by flow cytometry.

Chapter 3

Results and Discussion 1

3. Results and Discussion 1

Summary

The HIV-1 Vpu protein is an oligomeric integral membrane protein essential for particle release, viral load and CD4 degradation. While the exact mechanism of the enhanced virion release is unknown, some studies have associated this property with the transmembrane (TM) domain of Vpu (Schubert 1996a). The TM domain of Vpu was shown to form ion channels selective for some monovalent or divalent cations (Ewart 1996; Schubert 1996a). This ion channel activity was localized to the TM domain and is independent of the cytoplasmic domain in enhancing viral release from cells (Schubert 1996). In contrast to Vpu-induced degradation of CD4, which is fairly well understood in its mechanistic details, relatively little is known about how Vpu enhances virus release. Vpu after cotranslational insertion into the host membrane can assemble into homo-oligomeric complexes that function as ion-conductive membrane pores *in vitro* (Bour 2003). *In silico* models show Vpu to form pentamers with an ion channel activity. In our work, using Vpu proteins from a primary subtype C and the pNL4-3 subtype B isolates of HIV-1, we show oligomerization of the full-length protein as well as its transmembrane (TM) domain by genetic, biochemical and biophysical methods. Our results provide direct evidence of the presence of Vpu pentamers in a stable equilibrium with its monomers *in vitro*. This was also true for the TM domain of Vpu. Confocal microscopy localized Vpu to the endoplasmic reticulum and Golgi regions of the cell, as well as to post-Golgi vesicles. In fluorescence resonance energy transfer (FRET) experiments in live cells we show that Vpu oligomerizes in what appears to be either the Golgi region or intracellular vesicles, but not in the ER. This argues against an existing model of the role of Vpu in virus particle release by collapse of the ER membrane potential and acceleration of membrane fusion and exocytic protein transport.

3.1 Antibody generation Against Vpu

It has been difficult to express Vpu in a soluble form and generate antibodies against it. In other studies, antibodies have been generated either to synthetic peptides corresponding to different regions of Vpu or the cytoplasmic region alone has been used. However, the low immunogenicity of Vpu makes it difficult to raise polyclonal antibodies to the full length protein. The approach we have followed is to express Vpu as a fusion with larger immunogenic partners and to use the purified uncleaved fusion proteins, directly for raising antibodies. The Vpu protein fused to either the maltose-binding protein (MBP) or to glutathione-S-transferase (GST) was used for priming and boosting the animals, respectively. This resulted in the production of anti-Vpu antibodies at good titers. At the same time it also minimized the antibodies against the fusion partner.

The MBP-Vpu protein was expressed in *E. coli* BL21(DE3) cells as a soluble protein and was purified on amylose resin (Figure 3.1A). The GST-Vpu protein expressed in DH5α cells was insoluble and was purified from inclusion bodies (Figure 3.1B) by gel elution as described in Materials and Methods.

For raising antibodies, two New Zealand white rabbits were immunized as described earlier. The antibody titers measured in a standard direct ELISA format showed good titers for Vpu antibody in crude sera as well as in sera passed through MBP and GST saturated beads (Figure 3.2). Following primary immunization with MBP-Vpu and the first booster dose, there was a modest rise in antibody titers, but this was largely against MBP. After the GST-Vpu booster, anti-Vpu titers were found to increase. A large rise in anti-Vpu antibody titers was obtained after intravenous booster with GST-Vpu (Figure 3.2). Anti MBP and anti-GST titers were also significant in crude sera but minimized levels of same antibodies were observed after passing sera through glutathione beads and amylose resin saturated with GST and MBP respectively.

To test antibody titers, the purified GST, Vpu-GST and cell lysate from MBP expressing cells were loaded on 12% SDS gel and western blotting was done with crude and adsorbed sera using different dilutions (1:400, 1:1000 and 1:2000) as shown in Figure 3.3. Western blotting results showed that crude sera contains antibody against Vpu, GST and MBP (Figure 3.3 Upper panel). After passing the sera through GST and MBP saturated resin the antibody against GST and MBP are minimized while antibodies against Vpu are retained (Figure 3.3 lower panel). This strategy results in anti-Vpu antibodies of increasing titers. Antibodies against MBP and GST are also generated, but can be removed from the sera by a simple adsorption protocol. This gives a serum preparation that shows good reactivity to the target protein (Vpu) and is useful for most applications such as western blotting, immunoprecipitation and indirect immunofluorescence.

3.2 Cloning and expression of Vpu

The *vpu* gene was PCR amplified using as template either the pNL4-3 plasmid DNA (AIDS Reagent Program) or a 3.5 kb fragment encompassing the *vpr* to *env* region previously amplified and cloned from a primary isolate of HIV-1 subtype C. The amplified fragments designated as NL *vpu* and R5 *vpu* respectively, were cloned in plasmid pGEMT-Easy and sequenced in both directions. The translated amino acid sequences (Figure 3.4) showed the predicted TM and cytoplasmic helices of the Vpu protein. Multiple clones of R5 Vpu showed it to be 82 amino acids in length with two additional amino acids at the N-terminus and a deletion at residue 67 compared to NL Vpu. The gene fragments corresponding to the TM domains were assembled from synthetic oligonucleotides and those for the cytoplasmic domains were PCR amplified using specific primers for NL *vpu* and R5 *vpu*.

3.3 Homotypic and heterotypic interactions of full-length and truncated Vpu proteins using Yeast two hybrid

To test for the interaction between Vpu monomers, the full-length and truncated *vpu* genes were subcloned into the yeast two-hybrid expression vectors as fusions to the GAL4 DNA-binding domain (BD) and activation domain (AD) (Figure 3.5A). The expression of the full-length or truncated Vpu fusion proteins from these constructs was verified by T7 RNA polymerase mediated coupled *in vitro* transcription-translation. The yeast two-hybrid assays were performed in *S. cerevisiae* AH109 cells as described in Materials and Methods. A representative set of plates is shown in Figure 3.5B. All transformants grew on nonselective yeast extract-peptone-dextrose (YPD) plates (panel 2). Single transformants and all cotransformants containing AD-Vpu grew on SD/L$^-$ plates (panel 3); similarly, those containing BD-Vpu grew on SD/T$^-$ plates (panel 4) and cotransformants grew on SD/LT$^-$ plates (panel 5). Cotransformants that contain interacting protein pairs fused to AD and BD can transactivate the *HIS3* gene resulting in growth on SD/LTH$^-$ plates. The growth of AD-Vpu/BD-Vpu cotransformants on an SD/LTH$^-$ plate (panel 6) showed homodimerization of the Vpu protein. Colonies were transferred to nitrocellulose filter and a β-galactosidase filter assay was carried out. The presence of β-galactosidase activity only in the positive control and AD-Vpu/BD-Vpu cotransformants (panel 7) further confirmed the Vpu-Vpu interaction.

A more extensive screen whose results are summarized in Figure 3.5C was carried out as above. Both NL Vpu and R5 Vpu showed homotypic interactions. Further, the NL Vpu and R5 Vpu proteins also showed interaction with each other. All these interactions were found to depend upon the TM domains, but not on the cytoplasmic domains of Vpu. The transformants were grown in the presence of 3-amino-1,2,3-triazole (3AT) to further confirm the specificity and strength of the interactions. All cotransformants that grew on SD/LTH$^-$ plates also grew on SD/LTH$^-$ 3AT plates. Compared to the positive control (BD/SNF1+AD/SNF4), the semi-quantitative liquid β-galactosidase assay showed

reasonably strong interactions between the full-length Vpu proteins and slightly weaker interactions between their TM domains but almost no interaction between the cytoplasmic domain of Vpu. The values were higher for homologous interactions (NL vs NL and R5 vs R5) as opposed to heterologous interactions (NL vs R5). A variety of negative controls showed no interaction and only background β-galactosidase activity.

3.4 Biochemical and *in vitro* binding assays

3.4.1 Polyacrylamide gel electrophoresis

We also tested the oligomerization of full-length Vpu (NL/R5) as well as its TM and cytoplasmic domains by polyacrylamide gel electrophoresis. The Vpu proteins were synthesized from the pGBK-vpu and pGAD-vpu plasmid templates using an *in vitro*-coupled transcription-translation assay (TNT; Promega, Madison, USA), in the presence of ^{35}S-methionine. The *in vitro* synthesized proteins were subjected to DTT (100 mM, 1 hr), heat (95°C, 5min) or both treatments and then analyzed by SDS-PAGE. Under these conditions, both full-length Vpu (Figure 3.6A; lanes 2-5) and its TM domains (Figure 3.6A; lanes 7-10), but not the cytoplasmic domains (Figure 3.6A; lanes 12-15), showed oligomers even under fully denaturing conditions (DTT plus heat) and more so under partially denaturing conditions (DTT; no heat). The sensitivity of multimeric Vpu species to heating but not to DTT suggested that the interactions were noncovalent and independent of any disulfides.

When the ^{35}S-labeled proteins were analyzed on native polyacrylamide gels (Figure 3.6B), oligomeric species were prominently observed for full-length Vpu (Figure 3.6B; lanes 1, 2) and its TM domain (Figure 3.6B; lanes 3, 4), but not for the cytoplasmic domain (Figure 3.6B; lanes 5, 6).

3.4.2 Immunoprecipitation of Vpu

The *in vitro* synthesized ^{35}S labeled Vpu was immunoprecipitated using anti-Vpu raised

in rabbits. Following immunoprecipitation, the proteins were subjected to DTT (100 mM, 1 hr), heat (95°C, 5min) or both treatments and then analyzed by SDS-PAGE. Immunoprecipitated full-length proteins also showed high molecular weight oligomers on partial denaturation and some multimeric species on complete denaturation (Figure 3.7). The immunoprecipitation analysis could only be carried out with full-length Vpu and its cytoplasmic domain since the polyclonal antibodies raised against full-length Vpu were found to react very poorly with its TM domain

3.4.3 Pull down assay

We also tested the Vpu-Vpu interactions in a pull-down assay. A maltose binding protein (MBP)-Vpu fusion protein was expressed in *E. coli* and bound to amylose resin (New England Biolabs, Beverly, USA) as described earlier. The ^{35}S-labeled Vpu proteins were synthesized in coupled transcription-translation reactions as earlier and mixed with MBP-Vpu and MBP saturated beads only. Both full-length Vpu and its TM domain were retained on amylose beads saturated with MBP-Vpu, but not with the MBP control (Figure 3.8A and 3.8B). The cytoplasmic domain of Vpu did not bind to MBP-Vpu in this assay (Figure 3.8C). These results further support Vpu oligomerization through its TM but not the cytoplasmic domain.

3.5 Vpu forms a pentamer *in vitro*

The gel permeation chromatography of ^{35}S-labeled full-length Vpu protein or its TM domain was done to characterize their oligomeric states. The proteins synthesized by *in vitro* coupled transcription-translation reactions were separated on a pre-calibrated Sephacryl S200HR column followed by scintillation counting for ^{35}S in different eluted fractions. Two prominent peaks of radioactivity were eluted for full-length as well as the TM domain proteins (Figure 3.9). The faster eluting peak corresponding to the oligomer typically contained about 10% of the radioactivity. The peak oligomer and monomer fractions were further analyzed by SDS-PAGE to confirm the presence of Vpu (Figure 3.9 inset). Based on their elution profiles, the calculated molecular masses were as follows: full-length Vpu monomer 15.3 kDa, oligomer 77.6 kDa; TM domain monomer

10 kDa, oligomer 47.8 kDa. Thus, the *in vitro* oligomers most closely represented pentamers for the full-length and TM domain Vpu proteins. These results also showed that Vpu monomers and pentamers existed in a stable equilibrium *in vitro* in the absence of other cellular components.

3.6 Subcellular localization and FRET analysis

It has been observed that Vpu localizes primarily to the cytoplasmic endomembrane structures in infected (Klimkait 1990) as well as transfected cells (Lopez 2002). We tested subcellular localization of the Vpu protein in transfected COS-1 and U2-OS cells. The cells were cotransfected with EGFP- or ECFP-vpu and either DsRed-ER, DsRed-mito or EYFP-Golgi expression vectors. The confocal images were sequentially acquired and merged for colocalization. In both cell types, the Vpu protein colocalized with the ER and Golgi markers, but not with the mitochondrial marker (Figure 3.10). A three-colour analysis of cells transfected with the ECFP-vpu, DsRed-ER and EYFP-Golgi expression plasmids also showed Vpu distribution to the ER and Golgi (Figure 3.10C). While a majority of the Vpu protein was found to be associated with the ER, significant amounts were also found to be associated with the Golgi.

To detect intimate protein-protein interactions *in vivo*, we used fluorescence resonance energy transfer (FRET). This non-radiative energy transfer between donor and acceptor fluorophores is critically dependent upon the distance and dipole orientations of the two partners, and is taken as evidence of an interaction between them (Xia 2001). We cotransfected COS-1 or U2-OS cells with vectors expressing Vpu proteins fused to the cyan (ECFP) and yellow (EYFP) colored variants of the enhanced green fluorescent protein as the donor-acceptor FRET pair (Siegel 2000). To measure FRET in cells, we followed an acceptor photobleach protocol wherein the mean fluorescence intensities from the donor (ECFP) and acceptor (EYFP) fluorophores were recorded before and after EYFP photobleaching. Two patterns of Vpu expression were observed in transfected COS-1 cells. In cells expressing low levels of the protein, a punctate and vesicular distribution was noted (Figure 3.11A; upper panels). However, cells expressing high

levels of Vpu were found to accumulate this protein in intensely staining subcellular structures present on one side of the nucleus (Figure 3.11A; lower panels); these structures were also marked with the transfected DsRed-ER marker (Figure 3.10). Additionally, Vpu was also detected in more distal vesicular structures. In U2-OS cells, Vpu was similarly distributed in the ER, Golgi and punctate vesicular structures (Figure 3.11B).

We carried out FRET measurements in live cells showing various patterns of Vpu subcellular distribution. Two different areas within the same cell, one showing colocalization and another where no colocalization was observed were subjected to FRET analysis. As expected (and seen in the merged image), the Vpu-ECFP and Vpu-EYFP proteins colocalized in transfected cells (Figure 3.11A; upper panels). On simultaneous scanning of the two fluorophores, there was an increase in cyan (donor) fluorescence following bleaching of the yellow (acceptor) fluorophore. Multiple FRET measurements were carried out in more than one region of the same cell with similar results (not shown). In COS-1 cells showing this pattern of Vpu distribution, the mean fluorescence intensities of ECFP-Vpu before and after EYFP-Vpu photobleaching were 111.95$\pm$5.7 and 137.30$\pm$6.2, respectively. This gave an average FRET efficiency of 18.5%. On the other hand, no FRET was observed in the ER region in COS-1 cells expressing high levels of tagged Vpu proteins (Figure 3.11 A; lower panels). In U2-OS cells, the FRET analysis was carried out in two separate regions of the same transfected cells (Figure 3.11B). No FRET was measured in the intensely staining ER region (Figure 3.11B, set A). However, FRET between ECFP-Vpu and EYFP-Vpu was observed reproducibly in regions of the cell that appeared to be either Golgi or unidentified vesicles (Figure 3.11B, set B). The mean fluorescence intensities of ECFP-Vpu before and after EYFP-Vpu photo bleaching were 70.29$\pm$4.26 and 95.04$\pm$4.8, respectively. This gave an average FRET efficiency of 26%. For technical limitations in the imaging, it was not possible to also cotransfect these cells with subcellular markers to positively identify, in the same cell, those subcellular structures that support Vpu-Vpu FRET and those that do not. Overall, our FRET experiments provide strong *in vivo* evidence of Vpu-Vpu interaction

in a live cell. Further, based on these results, Vpu appears to form oligomers in distinct subcellular locations, primarily in the Golgi and vesicular regions but not in the ER.

DISCUSSION-1

Lentiviruses encode a number of unique accessory proteins that are important for viral replication and pathogenesis, but are not encoded by other retroviruses. The versatility of HIV accessory proteins arise from their ability to function as adaptor molecules that connect various viral and cellular proteins to pre-existing cellular pathways, modulate these pathways and control processes important for viral replication. Thus, protein-protein interactions are an important aspect of the functioning of these proteins. The Vpu protein of HIV-1 is known to bind CD4 and β-TRCP, the latter being a component of the E3 ubiquitin ligase complex. This association is instrumental in dislocation of CD4 from the ER, its ubiquitination and subsequent degradation by the proteosome (Margottin 1998). The other function of Vpu is to promote the release of progeny viruses from infected cells. This appears to be dependent upon the ability of Vpu to form oligomeric complexes with an ion channel activity in cellular membranes (Bour 2003).

The nature of the Vpu oligomer is important due to its functional significance in virion release. An earlier study used chemical cross-linking to demonstrate the oligomerization of Vpu (Maldarelli 1993). Here we have used various genetic, biochemical and biophysical approaches to characterize the oligomerization of Vpu *in vitro* and *in vivo*. Molecular dynamic simulations and conductance studies have shown that the Vpu TM domain is sufficient for its ion channel activity (Schubert 1996) and the pattern of channel activity is characteristic of the self-assembly of conductive oligomers in the membrane (Marassi 1999). This suggested that the TM domain of Vpu would also be required for its oligomerization. We provide here direct evidence that the hydrophobic N-terminal TM domain, and not the charged cytoplasmic domain, is critical for Vpu oligomerization. The two-hybrid and MBP pull-down analysis further showed that full-length Vpu proteins as well as their TM domains derived from two different HIV-1 subtypes interacted efficiently with each other. The isolated TM domains also showed

stable interaction with the full-length Vpu protein.

Molecular-dynamic simulations of ion channels formed by the Vpu TM domain predict the most favourable channel assembly to be a pentamer, but higher and lower oligomeric species were also predicted (Lopez 2002; Moore 1998). Recently, Becker et al (2004) have used synthetic proteins containing a carrier template to which four or five peptides corresponding to the Vpu TM domain were attached, to demonstrate ion channel formation by oligomerization of the TM domain. In our analysis, multimeric species, including pentamers were evident on SDS and native gel electrophoresis of the *in vitro* synthesized Vpu proteins and their TM domains. Further, molecular sizing of *in vitro* synthesized Vpu by gel permeation chromatography clearly showed it to assemble into a pentameric species. Thus, while complementing earlier studies (Becker 2004; Lopez 2002; Moore 1998) we provide direct evidence for pentamerization of the full-length Vpu protein as well as its TM domain. *In vitro* synthesized Vpu proteins, in the absence of other cellular components, demonstrated a stable equilibrium between monomers and pentamers. This demonstrates the inherent ability of Vpu to oligomerize and pentamers appear to be the thermodynamically most stable form of these oligomers.

Earlier studies have shown Vpu to be localized to the perinuclear region of the cell that includes the ER and Golgi (Klimkait 1990; Schubert 1996a). Using CD4-Vpu fusion proteins and Endoglycosidase H resistance, an earlier study has provided evidence for Vpu movement beyond the ER (Raja 1996). A similar fusion protein was also used to tease out the apoptotic pathway (Akari 2001). Recently, the imaging of a Vpu-EGFP fusion protein has also localized it to the ER, Golgi and plasma membrane (Pacyniak 2005). So, there is evidence that Vpu has the ability to be transported to post-ER membranes. Several models have been proposed for a role of the Vpu channel in the budding of new virions (Marassi 1999). It has been suggested that oligomerization of Vpu at the ER could form conducting channels leading to a collapse in the membrane potential across the ER cisternae and acceleration of membrane fusion and protein traffic in the exocytic pathway. Alternatively, at the ER/mitochondrial junctions, Vpu is proposed to collapse the mitochondrial membrane potential and promote apoptosis. It is

also possible that Vpu channels in the plasma membrane may attenuate the cell resting potential, promoting the fusion and release of new virions. We have used confocal microscopy and FRET analysis to test these models. Using transfected fluorescent protein-tagged Vpu fusion proteins and subcellular markers we show that Vpu localizes to the ER and Golgi regions but not to the mitochondria. In the absence of its mitochondrial localization, it would be difficult to support a direct effect of Vpu on the mitochondrial pore transition complex or transmembrane potential (Hengartner 2000). One possibility is the effect of Vpu channels on Ca^{2+} release from its intracellular stores in the ER (Rizzuto 2004). The FRET analysis in this study showed no oligomerization of Vpu associated with the ER, arguing against the role of ER directly or indirectly in this process.

Oligomerization of Vpu was observed by FRET in structures that were distal to the ER. These structures could not be identified positively due to the technical limitation of not being able to use chromophore-tagged subcellular markers together with the FRET donor-acceptor pair. However, separate stainings in two different cell types suggested that these structures might either be Golgi or vesicles associated with exocytic protein transport. The Vpu ion channel activity would be detrimental to ER function, inducing ER toxicity, stress and apoptosis of the infected cell. This would go against the plan of survival of productively infected cells followed by retroviruses and so nicely exemplified by HIV (Peterlin 2003). It would therefore make sense for Vpu oligomerization to occur downstream of the ER, enroute to the plasma membrane. Our FRET analysis in live cells supports this model. Since the cytoplasmic and not the transmembrane domain of Vpu is required for CD4 relocation from the ER and its subsequent degradation (Schubert 1996), this scheme would not affect the CD4 downmodulation function of Vpu. However, contrasting results have recently been presented wherein Vpu with a scrambled transmembrane domain was unable to downmodulate CD4 from the surface of transfected cells (Hout 2005). Whether this is due to the inability of mutant Vpu to oligomerize, or due to an altered protein structure or its arrangement in the membrane, remains to be seen.

In summary, we have used genetic, biochemical and biophysical methods to complement earlier studies on Vpu oligomerization and the role of its N-terminal transmembrane domain in this oligomerization. While theoretical modeling studies (Lopez 2002; Moore 1998) and synthetic peptides (Becker 2004) had earlier predicted pentameric Vpu channels, we provide here direct evidence for the existence of a Vpu pentamer in stable equilibrium with its monomer. This was also true for the Vpu transmembrane domain. Finally, subcellular localization and FRET analysis argue against an earlier model of Vpu-mediated virion release based on channel formation in the ER. Besides channel formation and its effect on virion release, oligomerization would also influence the ability of Vpu to interact with host cell proteins towards regulating the intracellular environment for efficient viral replication, assembly and release.

We have therefore targeted this aspect of Vpu biology by screening for novel cellular partners as the next step in our study.

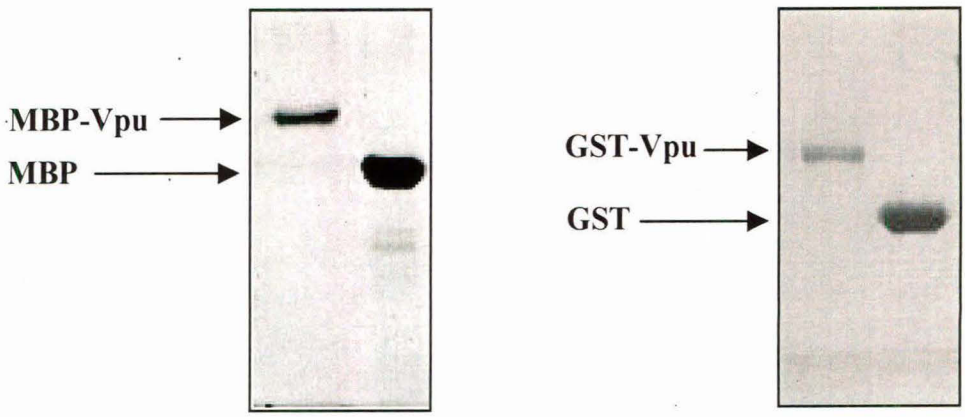


Figure 3.1: Purified proteins run on 12% SDS gel: (A) MBP-Vpu and MBP expressed in E.coli BL21(DE3) and purified on amylose resin, (B) GST-Vpu and GST expressed in DH5β. GST was purified on glutathione sepharose beads while GST-Vpu was purified by gel elution.

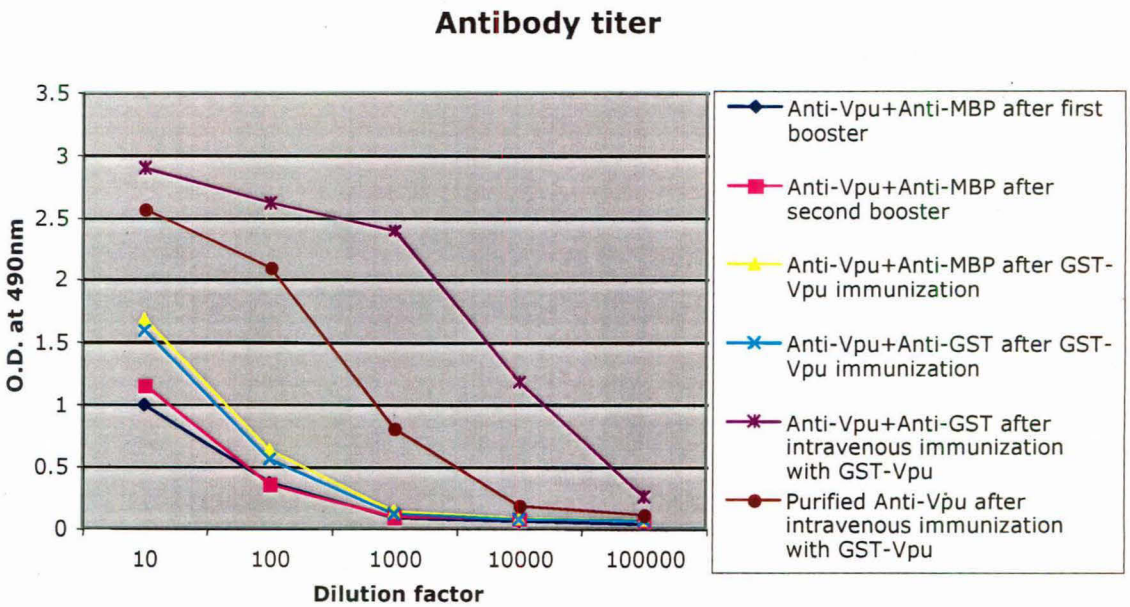


Figure 3.2: Antibody titers after different stages of immunization and antibody purification. Titers of anti-Vpu antibodies in the sera of immunized rabbits were determined by a direct ELISA. After every immunization different dilutions of antibody were used for adding in Vpu-coated wells. Result shows moderate increase in anti-Vpu and anti-GST while a lesser increase in anti-MBP. After passing through MBP and GST saturated beads, the purified anti-Vpu titers were still found good.

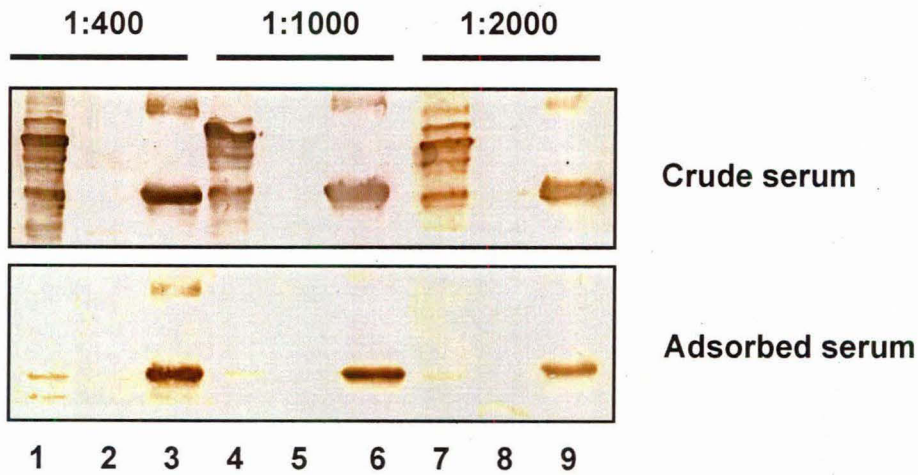


Figure 3.3: Western blot of MBP expressing cell lysate, purified GST and GST-Vpu using different dilutions of post immunized crude sera (upper panel) and purified sera after passing through MBP and GST saturated beads (lower panel). Lanes 1,4,7 contains MBP expressing cell lysate, lanes 2, 5, 8 purified GST and lanes 3,6,9 purified Vpu-GST. Different dilutions of crude and purified sera were used as shown at the top.

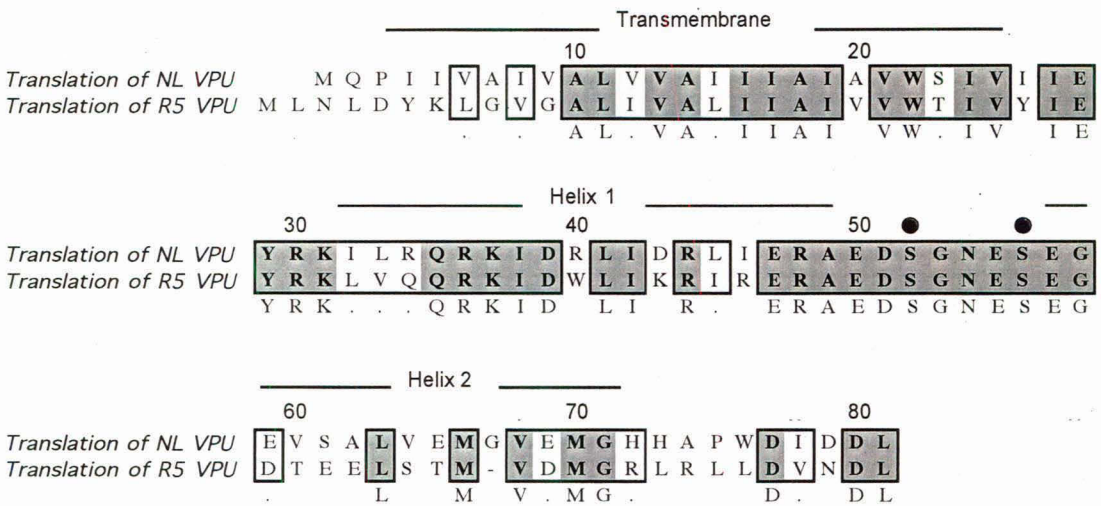
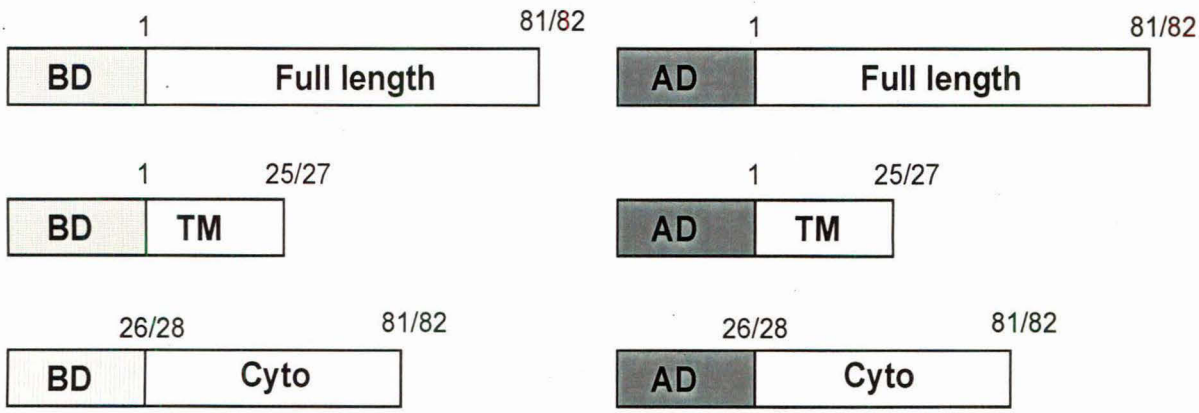


Figure 3.4: An alignment of the NL-Vpu (subtype B) and R5-Vpu (subtype C) protein sequences is shown. Boxes show sequence conservation; dark shading indicates sequence identity and light shading indicates conservative changes. The transmembrane domain, cytoplasmic helices and phosphoserine residues (•) are indicated. Residues are numbered according to the NL-Vpu sequence.

A.



B.

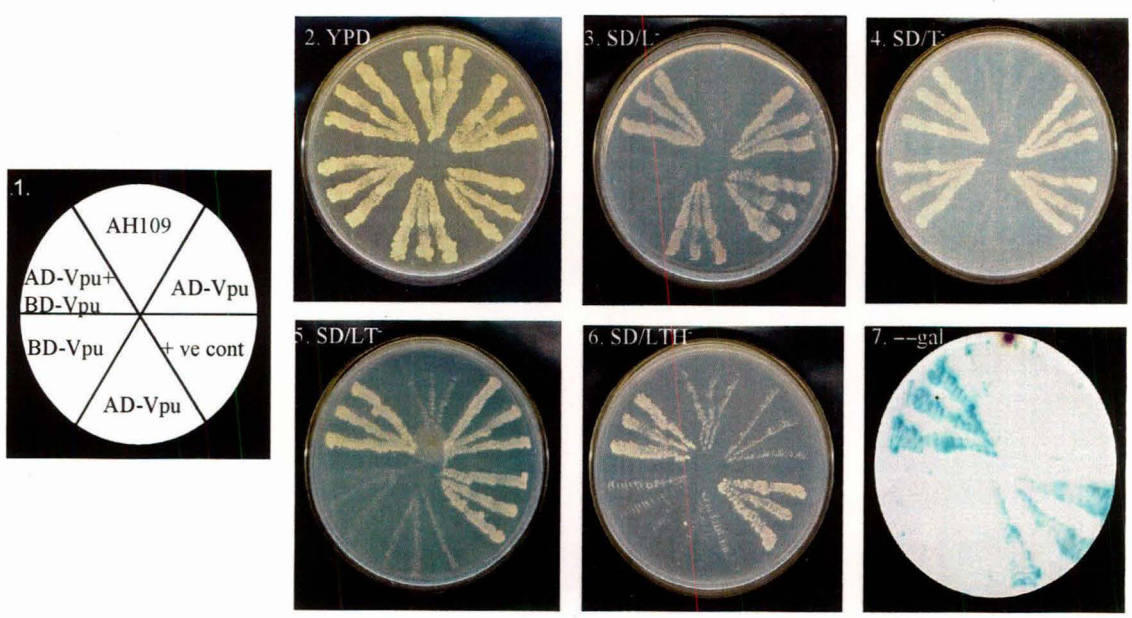


Figure 3.5: (A) Full-length and truncated vpu genes in yeast two-hybrid expression vectors as fusions to the GAL4 DNA-binding domain (BD) and activation domain (AD). (B) Representative plates showing homotypic interactions of the R5-Vpu protein. Panel 1 shows the template for panels 2 to 7 that in turn show transformants (2 to 6) streaked in each section of the following plates: 2, YPD; 3, SD Leu⁻; 4, SD Trp⁻; 5, SD Leu⁻ Trp⁻; 6, SD Leu⁻ Trp⁻His⁻; 7, β-galactosidase filter assay. Growth is seen as light streaks on a dark background (2 to 6). The β-galactosidase signal (7) is seen as dark streaks on a light background.

Plasmids	YPD	SD/ L^-	SD/ T^-	SD/ LT^-	SD/ LTH^-	SD/ LTH^- 3AT	β-gal (OD value)
Full length Vpu							
BD/R5 + AD/R5	+	+	+	+	+	+	+ (0.899)
BD/NL + AD/NL	+	+	+	+	+	+	+ (0.883)
BD/R5 + AD/NL	+	+	+	+	+	+	+ (0.621)
BD/NL + AD/R5	+	+	+	+	+	+	+ (0.629)
TM domain Vpu							
BD/R5 + AD/R5	+	+	+	+	+	+	+ (0.699)
BD/NL + AD/NL	+	+	+	+	+	+	+ (0.576)
BD/R5 + AD/NL	+	+	+	+	+	+	+ (0.401)
BD/NL + AD/R5	+	+	+	+	+	+	+ (0.462)
Cyto domain Vpu							
BD/R5 + AD/R5	+	+	+	+	-	-	- (0.076)
BD/NL + AD/NL	+	+	+	+	-	-	- (0.068)
BD/R5 + AD/NL	+	+	+	+	-	-	- (0.094)
BD/NL + AD/R5	+	+	+	+	-	-	- (0.083)
Controls							
BD/R5 + AD/-	+	+	+	+	-	-	- (0.054)
BD/- + AD/R5	+	+	+	+	-	-	- (0.070)
BD/NL + AD/-	+	+	+	+	-	-	- (0.056)
BD/- + AD/NL	+	+	+	+	-	-	- (0.066)
BD/R5 only	+	-	+	-	-	-	- (0.067)
AD/R5 only	+	+	-	-	-	-	- (0.060)
BD/NL only	+	-	+	-	-	-	- (0.046)
AD/NL only	+	+	-	-	-	-	- (0.072)
BD/SNF1 + AD/SNF4	+	+	+	+	+	+	+ (1.179)
BD vector + AD vector	+	+	+	+	-	-	- (0.065)
AH109 cells only	+	-	-	-	-	-	-

Figure 3.5(C): Complete results for the entire screen using NL-Vpu and R5-Vpu full-length, transmembrane domain and cytoplasmic domain fusions to the Gal4 protein DNA-binding domain (BD) or activation domain (AD). Growth (+) or no growth (-) of transformants on various media is shown. LTH^- 3AT represents growth on SDLeu^- $Trp^-$$His^-$ plates containing 20 mM 3-amino 1,2,3-triazole. The β-galactosidase filter assay results are indicated as + or – and the liquid β-galactosidase assay values are shown in parentheses as an average of two independent measurements. Various negative and positive controls are also shown.

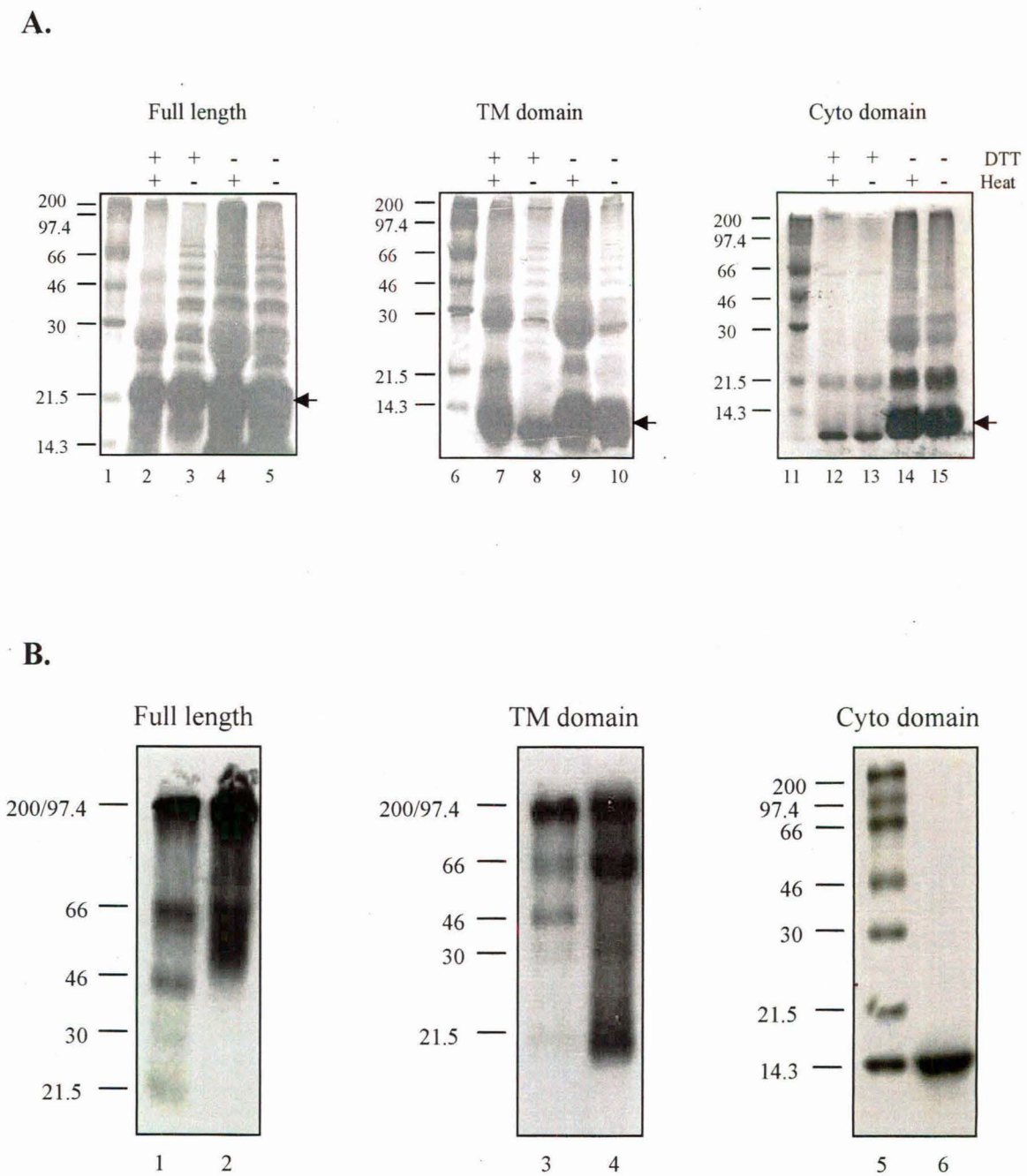


Figure 3.6: The full-length R5-Vpu protein and its transmembrane and cytoplasmic domains were synthesized and labeled with ^{35}S-methionine in a coupled in vitro transcription-translation system. (A) The synthesized proteins were subjected to heating and/or reduction with DTT, as indicated, and analyzed by SDS-PAGE. Arrowheads indicate monomeric forms of the full-length and truncated Vpu proteins. Lanes 1, 6 and 11 show molecular size markers (in kilo Daltons). (B) The proteins were also analyzed on native polyacrylamide gels without heating or DTT treatment. Lanes 1,3 and 5, markers; lanes 2,4 and 6, Vpu full-length, TM domain and cytoplasmic domain, respectively. The molecular sizes (in kilo Daltons) are indicated.

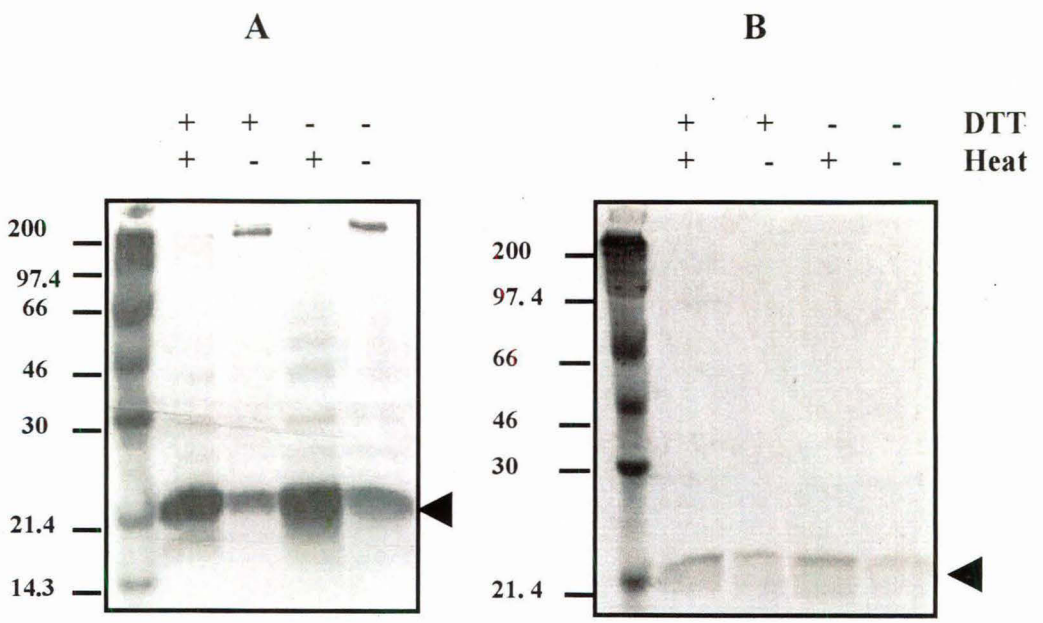


Figure 3.7: Immunoprecipitation of ^{35}S labeled Vpu Full length (A), and cytoplasmic domain (B) under complete and partial denaturating conditions. Immunoprecipitated oligomers were observed more in case of full length Vpu when samples were not boiled but monomeric form of Vpu was observed after heat treatment. The cytoplasmic domain of Vpu after immunoprecipitation showed monomeric form irrespective of heat treatment.

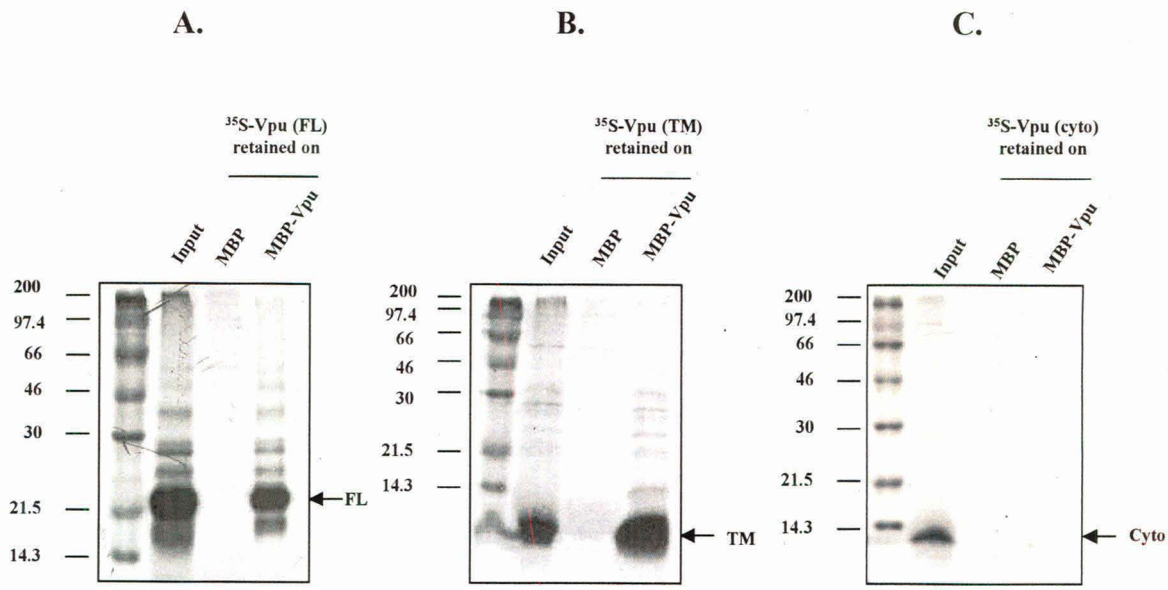


Figure 3.8: For pull-down assays, the ^{35}S-labeled R5-Vpu proteins were synthesized in vitro and bound to amylose beads saturated with either the maltose binding protein (MBP)/R5-Vpu fusion protein or MBP alone as a control. The beads were washed, resuspended in loading dye buffer, boiled and the supernatants subjected to SDS-PAGE. The gels were dried and autoradiographed. Gels A to C show the full-length (FL) R5-Vpu protein, or its transmembrane (TM) or cytoplasmic (cyto) domains, respectively, retained on the beads. Arrows indicate the full-length or truncated Vpu proteins.

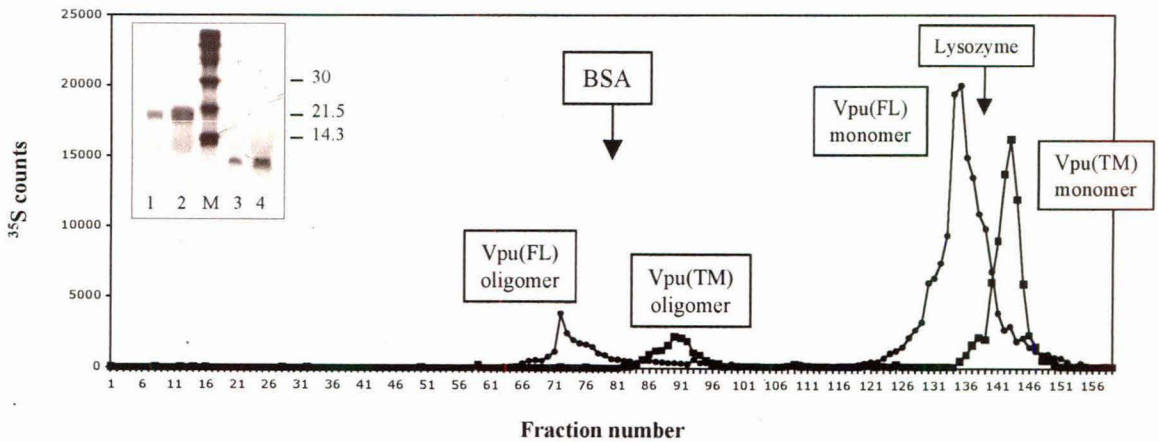
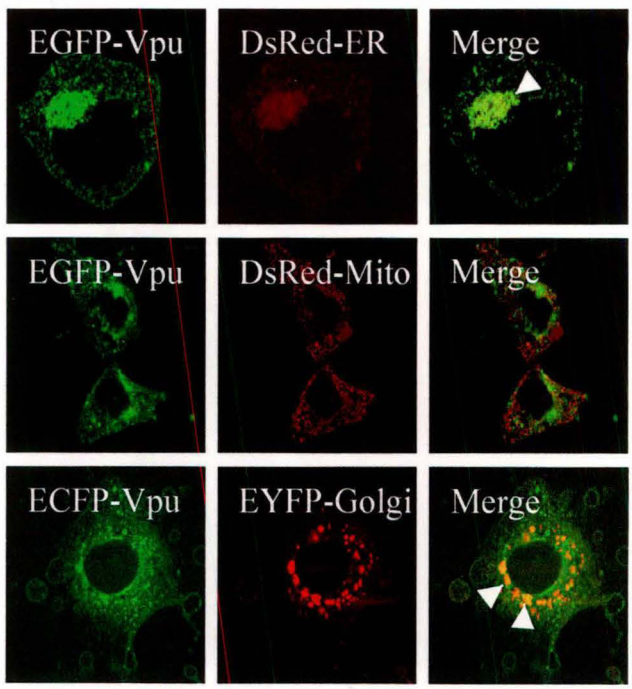


Figure 3.9. Gel permeation analysis of Vpu oligomers. In vitro translated and ^{35}S-labeled Vpu proteins were separated by gel permeation chromatography as described in Materials and Methods. The eluted ^{35}S counts in each fraction are indicated for the full-length (■) or TM domain (■) Vpu proteins. The positions of Vpu monomers and oligomers, as also the marker proteins are indicated. The inset shows SDS-PAGE analysis of the peak fractions. Lanes: 1, Vpu oligomer; 2, Vpu monomer; 3, TM domain oligomer; 4, TM domain monomer; lane M shows molecular size marker as indicated (in kilo Daltons).

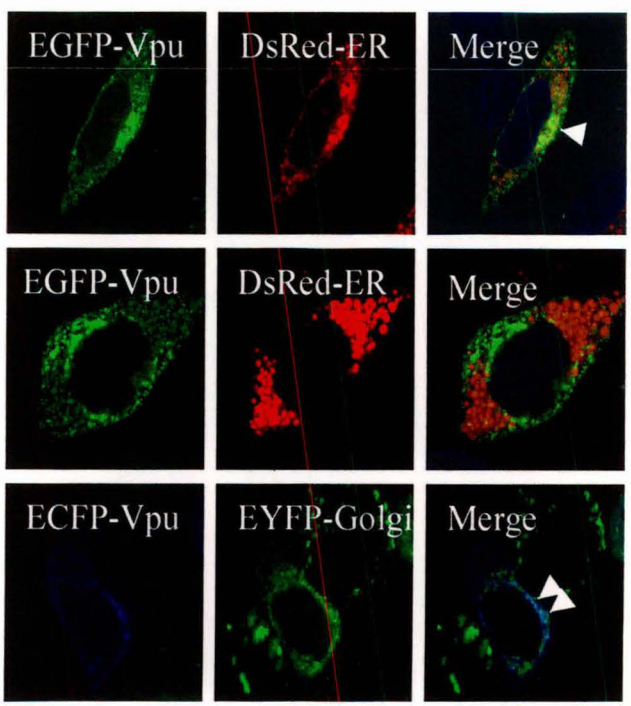
A.

COS-1 cells



B.

U2-OS cells



C.

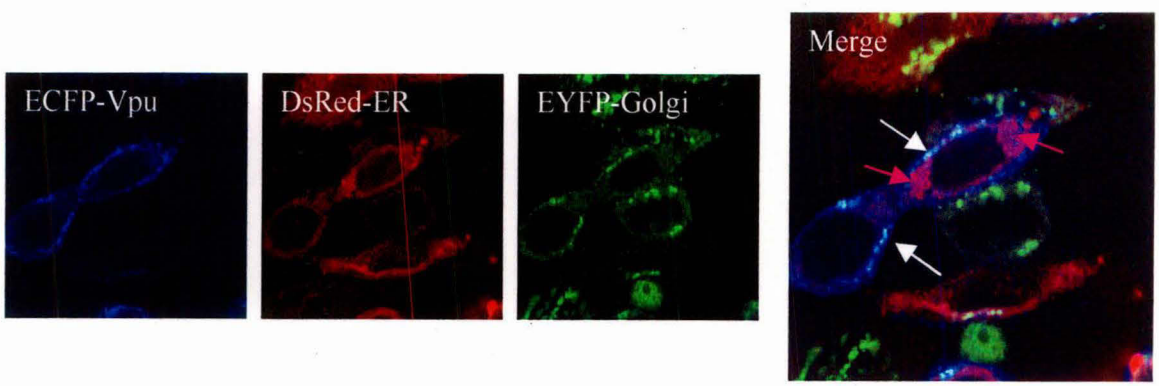


Figure 3.10: Subcellular localization and FRET analysis of Vpu interactions. (A) COS-1 and (B) U2-OS cells were doubly or triply transfected as described in Materials and Methods. The individual and merged images are labeled as shown. The areas of colocalization are indicated with white arrowheads (A and B). (C) In the triple transfection merged image, magenta and white arrows indicate Vpu/ER and Vpu/Golgi colocalizations, respectively.

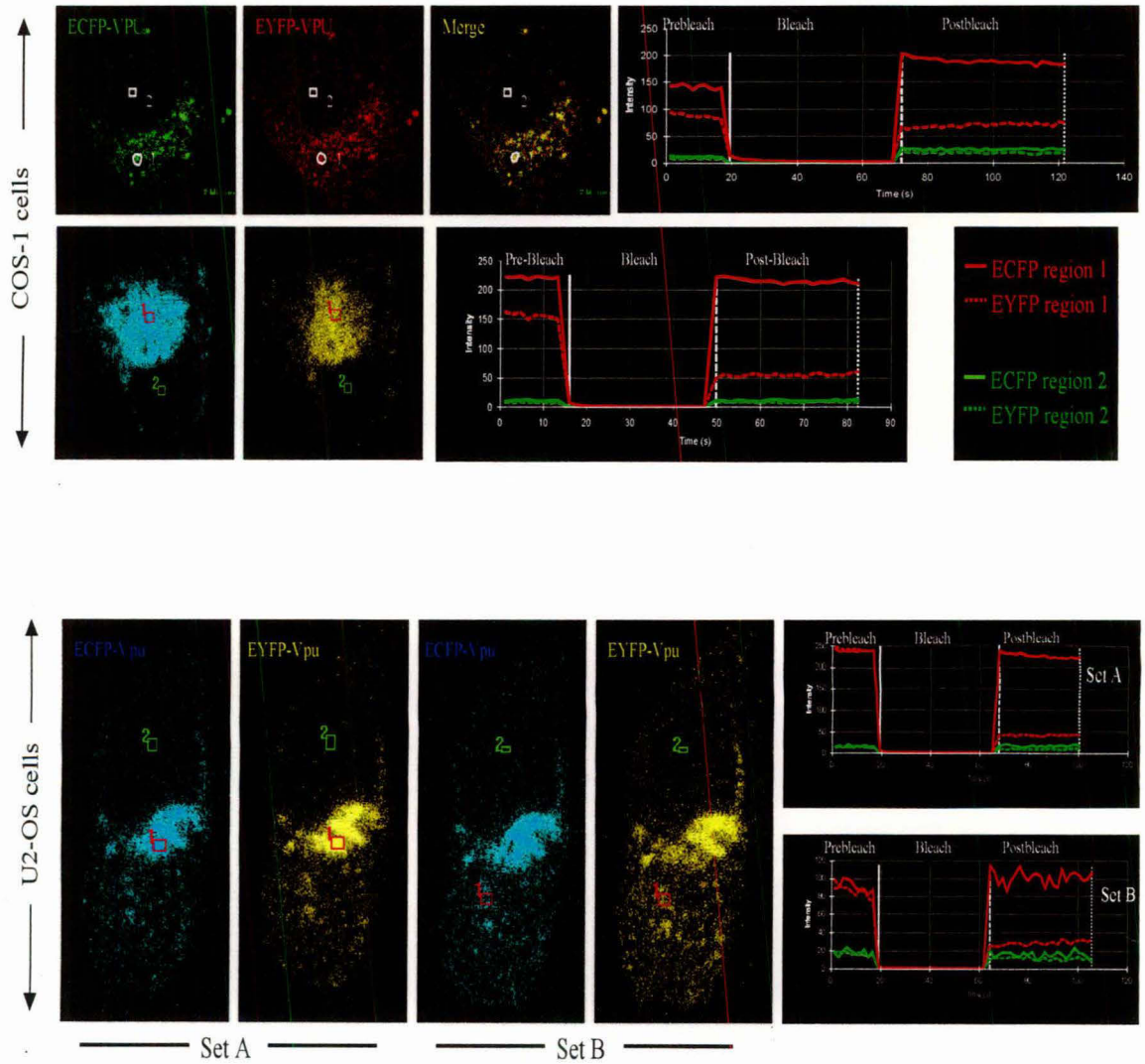


Figure 3.11: FRET assays in live cells. COS-1 and U2-OS cells were cotransfected with Vpu-ECFP and Vpu-EYFP expression vectors and FRET was performed as described in Materials and Methods. Representative images for COS-1 and U2-OS cells are shown together with a kinetic profile of the FRET experiment. For U2-OS cells, sets A and B represent the same cell, with FRET carried out in two different regions of colocalization indicated by the box marked 1; the box marked 2 represents a control region of the cell. A total of 4 independent FRET experiments were carried out in 7-10 different cotransfected cells each time.

Chapter 4

Results and Discussion 2

4. Results and Discussion 2

Summary

During viral infection, the HIV-1 Vpu interacts with various cellular proteins. To search for novel Vpu-interacting proteins, we screened a human leukocyte yeast two-hybrid expression library. Following library screening, sequencing and BLAST search, four novel Vpu-interacting proteins were discovered. The major histocompatibility class II (MHCII) invariant chain, also called Ii or CD74, was found to be one such protein. Here we show direct binding of Vpu and CD74 using the yeast two-hybrid assay and coimmunoprecipitation from cells infected with wild type or *vpu*-defective HIV-1. The cytoplasmic region of Vpu was found to interact with the 30-amino acid cytoplasmic tail of CD74. Human monocytic U937 cells infected with wild type or *vpu*-defective HIV-1 showed that Vpu downregulated the expression of surface as well as total MHCII. This reduction in MHCII correlated with decreased antigen-presentation to T cells in culture. Thus, the Vpu protein may also contribute to viral persistence by attenuating immune responses during HIV infection.

4.1 Library screening for Vpu interacting proteins

In order to identify novel cellular proteins interacting with Vpu, the HIV-1 Vpu protein was used in a yeast two-hybrid screen of a human leukocyte cDNA library. As described in Materials and Methods, the screen was made stringent by selecting only strongly interacting proteins by growing cotransformants on SD/LTH$^-$ plates containing 20 mM 3-amino-1,2-3-triazole and then assaying for the reporter β-galactosidase activity. From an activation domain library of ~$2x10^6$ clones, about 1000 cotransformants with BD-vpu grew on SD/LTH$^-$ plates and this number reduced to 350 cotransformants on SD/LTH$^-$ 3AT plates. Of these, 80 clones showed reporter β-galactosidase activity. Plasmid DNAs isolated from these yeast clones were amplified in *E. coli*, transformed back into *S. cerevisiae* AH109 cells, grown on YPD plates and replica plated on SD/LH$^-$ plates.

Thirty clones did not grow on SD/LH⁻ plates and represented the true interactors. Plasmid DNAs isolated from these were restriction digested with EcoRI and XhoI, and those with unique inserts were sequenced (Figure 4.1). Through this process, the genes for four unique proteins, hitherto not reported to bind Vpu were identified. One of these was CD74, a type II integral membrane protein. It is also known as the invariant chain that binds to MHCII before antigen loading in the endolysosomal compartment. The CD74 protein has an N-terminal cytoplasmic domain of 30 amino acids followed by a 26-aa transmembrane domain and a 160-aa domain that either projects into the lumen or is extracellular depending upon the subcellular localization of CD74 (Figure 4.2). The association of CD74 and MHCII occurs inside the ER, but the complex is also found on the cell surface, possibly enroute to the endolysosomal compartment where CD74 is degraded and MHCII is loaded with exogenously acquired peptides. Since Vpu is also localized to the ER-Golgi region, it is likely that the CD74-Vpu interaction takes place in this compartment.

4.2 Vpu binds to CD74 in HIV-1 infected cells

To detect the interaction of Vpu and CD74 in HIV-1 infected cells, the human monocytic cell line U937 was used. These cells constitutively express endogenous CD74 as well as the MHCII α and β chains. The U937 cells were infected with HIV-1 NL4-3 or the *vpu*-defective mutant NL4-3U35. Cells were collected at 24, 44, 52 and 60 hrs post-infection, the lysates were immunoprecipitated with anti-CD74 antibodies and then western blotted with anti-Vpu antibodies. As shown in Figure 4.3, anti-CD74 antibodies efficiently precipitated Vpu from NL4-3 infected cells while no precipitation was seen from NL4-3U35 infected cell lysates. It is known that Vpu expresses late in viral infection starting at about 40 hrs post-infection. While at early times post-infection (24 hrs) no Vpu could be immunoprecipitated from NL4-3 infected cells, this could be precipitated efficiently later in infection. Direct western blotting of infected cell lysates showed that Vpu was expressed only in NL4-3 infected cells and not in cells infected with *vpu*-defective mutant NL4-3U35. To confirm that Vpu does not bind non-specifically to cellular proteins, we used an isotype-matched anti-EGFR antibody for immunoprecipitation

followed by western blotting for Vpu. No Vpu was immunoprecipitated under conditions that brought down large amounts of EGFR (Figure 4.3). To show that anti-EGFR immunoprecipitation was successful, the blot was stained with anti-EGFR antibody. This also served as a loading control. Overall these results presented in Figure 4.3 confirm a direct interaction between the HIV-1 Vpu protein and host cell CD74 in virus-infected cells.

4.3 Interacting domain mapping

4.3.1 Vpu cytoplasmic domains is required for interaction with CD74

A direct yeast two-hybrid assay was carried out using the *vpu* and *CD74* genes to confirm their interaction. As shown in Figure 4.4, a strong interaction was observed between the two proteins. Besides growing on SD/LTH$^-$ plates containing 20 mM 3-amino-1,2,3-triazole, the AD-CD74/BD-vpu cotransformants showed β-galactosidase reporter activity in a filter assay (Figure 4.4A) as well in a semi-quantitative liquid β-galactosidase chromogenic assay (Figure 4.4B). To identify the CD74-interacting domain of Vpu, regions of the *vpu* gene corresponding to its TM and cytoplasmic domains were cloned in plasmid pGBKT7 as described earliar and, the yeast two-hybrid assay was repeated using pACT-CD74 and either pGBKT7-vpu (TM) or pGBKT7-vpu (cyto). The results summarized in Figure 4.4B show that the Vpu cytoplasmic domain, but not its TM domain, was responsible for the interaction with CD74.

4.3.2 Cytoplasmic domain of CD74 is required for the interaction

Due to the topology of the two proteins, we reasoned that the cytoplasmic domain of CD74 is likely to bind the cytoplasmic domain of Vpu. To test this, a 30-amino acid peptide corresponding to the entire cytoplasmic domain of CD74 (amino acids 1 to 30) was chemically synthesized. During peptide synthesis, the coupling of every amino acid was confirmed manually and mass spectrometry was carried out to confirm the final product. This peptide was then used as the coating antigen on an ELISA plate to test Vpu

binding. A nonspecific peptide corresponding to adrenocorticotropic hormone (ACTH) was used as the coating control. In the first assay, ^{35}S-methionine/cysteine-labeled Vpu proteins were synthesized *in vitro* in a coupled transcription-translation system and used to bind to peptide-coated wells. As shown in Figure 4.5A, significantly higher ^{35}S counts were retained in CD74 peptide coated wells that received either full-length Vpu or its cytoplasmic portion, but not its TM domain. On the other hand, only background counts were retained in wells coated with the nonspecific ACTH peptide.

In another assay, wells were coated with either the CD74 or the control peptide and to this we added either the purified MBP-Vpu or MBP protein. Following washes to remove unbound proteins, the bound proteins were estimated by ELISA with anti-MBP antibody. The MBP-Vpu fusion protein, but not the MBP protein was preferentially retained in CD74 peptide coated wells but not in wells coated with the ACTH peptide (Figure 4.5B). These *in vitro* assays support the yeast two-hybrid results of Vpu-CD74 interaction and show that the cytoplasmic regions of the two proteins are required for their interaction.

4.4 Vpu modulates MHCII levels

Since CD74 is critical for MHC class II presentation, we tested the effects of Vpu on MHCII levels in infected and transfected cells. Human monocytic U937 cells were activated with 10 ng/ml phorbol myristic acid (PMA) for 24 hrs to induce MHCII expression and then infected with either HIV-1 wild type NL4-3 or the *vpu*-defective NL4-3U35 virus. The infected cells were collected 24 and 44 hrs post-infection, stained for surface and total MHCII using either the L243 antibody that recognizes mature MHCII or the MCA2364 antibody that recognizes Ii (CD74) present on immature MHCII, and analyzed by flow cytometry. The anti-CD74 antibody is directed to the lumenal region of the CD74 protein and would also stain the CD74 protein present on the cell surface or in subcellular compartments. The results are shown in Figure 4.6. At 24 hrs post-infection, a decrease in mature MHCII was observed on the surface of cells infected with either the NL4-3 ($p = 0.006$) or NL4-3U35 ($p = 0.01$) viruses compared to uninfected cells, but the differences between these virus infected cells were not

significant. Further, no significant decrease in total mature MHCII staining was observed at this time. At 44 hrs post-infection, a significant decrease in mature MHCII staining was observed on the surface of NL4-3 infected cells compared to uninfected cells ($p = 0.004$). This effect was partially reversed on infection of cells with the *vpu*-defective NL4-3U35 virus ($p = 2.6 \times 10^{-5}$). A similar pattern was observed when mature MHCII levels were estimated in permeabilized U937 cells stained with the L243 antibody. No significant decrease in surface or total levels of immature MHCII (or CD74) were observed in infected cells 44 hrs post-infection. There was a slight increase in immature MHCII (or CD74) levels on the surface of cells infected with NL4-3, but not with the NL4-3U35 viruses ($p = 0.03$). Together, these results suggest that Vpu down regulates the surface as well as total levels of mature MHCII and may increase the levels of immature MHCII (or CD74) in infected cells. This effect was observed late but not early in infection, again suggesting the involvement of Vpu, a late expressing protein during the viral life cycle. The decrease in surface, but not total mature MHCII early in infection was found to be independent of Vpu.

The effects of ectopic expression of Vpu on MHCII were also analyzed. Activated U937 cells were transfected to express either a Vpu-EGFP fusion protein or EGFP as a control. Cells were stained for surface and total levels of either mature or immature MHCII (or CD74) with L243 and MCA2364 antibodies respectively, and quantitated by flow cytometry. In cells gated for EGFP expression, Vpu reduced mature MHCII on the surface of transfected cells ($p = 0.02$), but the apparent differences in immature surface MHCII (or CD74) were not statistically significant ($p = 0.09$) (Figure 4.7). The total levels of mature or immature MHCII (or CD74) were not statistically different between Vpu-expressing and control cells.

4.5 Vpu attenuates T cell activation

Downregulation of mature MHCII is likely to result in reduced antigen presentation to T cells. This was tested using a mouse cell system. The mouse macrophage cell line BMC-2 was cotransfected with plasmids that express a Vpu-EGFP fusion protein and myc-tagged

Ovalbumin (Ova-myc). As controls, BMC-2 cells were also transfected with individual expression plasmids and a cotransfection control comprising of Ova-myc and EGFP expression plasmids. The expression of Ova-myc and Vpu-EGFP proteins in BMC-2 cells are shown (Figure 4.8A, B). The cotransfected BMC-2 cells were then purified to remove dead cells and the live cells were used as stimulator APCs for Ova-specific MHCII-restricted CD4+ T cells from the OT-II transgenic mouse. Stimulator APC-directed T cell activation was measured by [^{3}H]-thymidine incorporation. As shown in Figure 4.8C, BMC-2 cells co-expressing Ova-myc and Vpu-EGFP attenuated activation of the CD4+ T cells compared to BMC-2 cells that co-expressed Ova-myc and EGFP or expressed only the Ova-myc protein. Since Vpu is known to be pro-apoptotic, it was important to also demonstrate that attenuated T cell activation by Vpu-expressing cells was not due to apoptotic death of these antigen-presenting cells. We therefore stained cells co-expressing Ova-myc + EGFP and Ova-myc + Vpu-EGFP with propidium iodide and quantitated the apoptotic cells at different times during the activation assay. Although Vpu-expressing cells showed about twice as much apoptosis as control cells, these were still a very small fraction of the total cells (Figure 4.8D) and would not account for the differences in T cell stimulation observed in Figure 4.8C. Thus, the reduced surface expression of mature MHCII in Vpu-expressing cells is also functionally relevant in downmodulating T cell activation.

4.6 Effect of Vpu on CD74 level and its downstream signaling

4.6.1 CD74 increases in Vpu expressing cells

Our results showed a slight increase in immature MHCII (or CD74) levels on the surface of cells infected with NL4-3, but not with the NL4-3U35 viruses (Figure 4.6). Similar results were also observed for total CD74 in infected cells. This effect was observed 44 hrs post infection but not 24 hrs post infection suggesting the involvement of Vpu. Increased levels of CD74 were also observed in case of vpu-EGFP transfected U937 cells as compared to EGFP tranfected cells when cells were stained for surface and total levels of CD74 with MCA2364 antibodies, and quantitated by flow cytometry (Figure 4.7). To further confirm this, western blotting for total CD74 was done from lysates of vpu-EGFP

transfected cells compared to control EGFP transfected U937 cells. Increased levels of CD74 were observed in Vpu-expressing cells compared to control cells (Figure 4.9).

4.6.2 MIF signaling in Vpu expressing cells

The invariant chain (CD74) is also known to be a high-affinity binding receptor for the cytokine macrophage migration inhibitory factor (MIF). Following MIF binding to the extracellular domain of CD74, there is activation of the extracellularly regulated kinase-1/2 (ERK1/2) MAP kinase cascade. Since CD74 levels were found to be higher in Vpu expressing cells, it was interesting to look for the MIF-induced phosphorylation status of Erk. U937 cells were transfected with vpu-EGFP and also with EGFP as a control. After 36 hrs of transfection, cells were starved followed by MIF stimulation as described in Materials and Methods. Unstimulated cells were also used as controls. The cell lysates were prepared as described earlier and western blotting was done both for pErk and total Erk. Results show increased levels of pErk in Vpu expressing cells stimulated with MIF (Figure 4.10). This increase was not significant in control cells stimulated with MIF. No increase in pErk levels were observed in unstimulated cells whether expressing Vpu or not. Together these results show increased MIF-mediated activation of the Erk pathway in Vpu-expressing cells. This correlates with increased CD74 levels in Vpu-expressing cells.

DISCUSSION-2

Primate lentiviruses encode a number of accessory proteins not commonly found in other retroviruses. While these proteins are not required for viral replication in all cells *in vitro*, they modulate replication, virus spread and survival of the host cell in the background of some cell types. However, *in vivo* studies have clearly demonstrated a role for these proteins in viral pathogenesis, their inactivation leading to dramatic attenuation of disease progression and severity (Greene 2002). These proteins appear to work as multifunctional adaptors that recruit cellular proteins as a means to modulating the host cellular processes. Such modulation translates into optimization of viral replication, through

wide-ranging effects on infectivity, gene expression and production of new virions.

The primary role of Vpu is to increase the synthesis and release of new virions (Binette 2004). This is based on two independent activities of Vpu. The first is its ability to mediate interaction of CD4 with the βTrCP-Skp1-Cul1 complex at the ER, leading to ubiquitination, retrotranslocation and proteosomal degradation of the CD4 protein (Willey 1992). The HIV-1 Vpu protein thus induces rapid degradation of CD4. This prevents formation of a CD4-gp160 complex in the ER allowing the HIV envelope glycoprotein to transit to the plasma membrane. Vpu is also a cation-selective ion channel (Ewart 1996). The Vpu protein of HIV-1 forms cation-selective ion channels and its interaction with the TASK-1 K^+ channel was shown to suppress the latter activity and promote the release of new virions (Hsu 2004). The strongest evidence for a role of Vpu in HIV-1 pathogenesis comes from studies in monkeys infected with SIV/HIV chimeric viruses (SHIV). Deletion of the *vpu* gene in this background led to decreased viral loads in animals infected with the SHIV (Li 1995). Further, an intact *vpu* gene was found to be important for the CD4+ T cell loss during infection with pathogenic SHIV (Stephens 2002). Current models of Vpu action suggest that by increasing viral loads, it contributes to virus spread that in turn results in increased rates of mutation in the *env* and *nef* genes (Singh 2001; Cormick-Davis 2000). Accumulating mutations in *env* would contribute positively to the development of neutralization-escape variants that drive disease progression and virus transmission to a naive host (Narayan 1999).

While searching for novel Vpu-interacting cellular proteins in a yeast two-hybrid screen, we found the MHCII invariant chain Ii or CD74 protein to bind Vpu. It is a type II transmembrane protein that binds the MHCII α and β chains in the ER (Becker-Herman 2005). This complex moves through the Golgi to the MHCII compartment where peptide loading occurs by displacement of the class II associated Ii peptide (CLIP) domain of CD74 before the mature MHCII-peptide complex is presented on the cell surface (Ploegh 1998). The displaced CLIP domain is further cleaved by I-CLIP proteases to release a 42-amino acid cytoplasmic peptide that translocates to the nucleus to modulate transcription of critical survival genes (Leng 2006). The CD74 protein is also expressed as a trimer on

the cell surface and is responsible for mediating signaling initiated by the macrophage migration inhibitory factor (MIF) (Leng 2003). Thus, CD74 or Ii is involved in multiple cellular functions that involve MHCII presentation as well as signal transduction. Here we explored the effects of HIV-1 Vpu on MHCII presentation.

In an earlier report, using the HIV-2 Vpx protein as a bait, Pancio et al (Pancio 2000) similarly pulled out CD74 from a yeast two-hybrid screen. Their results showed that a C-terminal 83-amino acid region of CD74 interacted with a 20-amino acid amphipathic helix in the Vpx protein. We observed that the extreme N-terminal 30 amino acids that comprise the cytoplasmic domain of CD74 interacted with the cytoplasmic domain of Vpu. This region of the Vpu protein is made up of two helices, of which helix 1 is amphipathic and by analogy is the likely interaction site for CD74. Taken together, our results and those of Pancio et al (2000) suggest that binding CD74 is likely to be important for the HIV/SIV group of lentiviruses. While Vpu carries out this function for HIV-1, the Vpx protein fulfils this role for HIV-2 and SIV.

What might be the functional significance of binding CD74? Being the class II invariant chain, this interaction is likely to affect MHCII presentation during HIV infection. In human monocytic U937 cells infected with wild type and *vpu*-defective HIV-1, we observed a slight decrease in surface MHCII presentation early in infection. This effect was Vpu-independent and may be due to Nef, an accessory protein of HIV that is expressed early in infection. Besides promoting the endocytosis of immunologically important cell surface molecules such as MHC class I (Schwartz 1996) and the B7 family proteins (Chaudhry 2005), Nef also downregulates surface MHC class II (Stumptner-Cuvelette 2001), albeit with slow kinetics and a $t_{1/2}$ of about 24 hrs (A. Chaudhry personal communication). At later times following infection, Vpu-dependent downmodulation of surface as well as total MHCII were observed in U937 cells. This also correlated with the timing of Vpu expression and its interaction with CD74 following infection. Ectopic expression of Vpu in transiently transfected U937 cells also showed surface down modulation of mature MHCII in agreement with the infection results. Though there was an apparent increase in surface and total expression of

immature MHCII in Vpu-expressing infected or transfected cells, this showed borderline statistical significance. Due to the antibody specificity, this may also reflect an increase in CD74 levels. Since cell surface CD74 is the MIF receptor and promotes signaling, proliferation and prostaglandin release following ligand binding (Leng 2003), this would have other implications as well. We also observed increased activation of the Erk pathway in a MIF-dependent manner in Vpu-expressing cells.

Viruses have evolved various strategies to evade the host immune response. One critical element in the adaptive immune response is the ability of CD4+ T cells to provide help for differentiation of B cells and CD8+ T cells. This in turn depends upon the ability of CD4+ T cells to recognize exogenously acquired antigens presented efficiently on the surface of APCs in association with MHCII α and β chains. Our results show that HIV-1 Vpu reduces the cell surface levels of mature MHCII molecules in infected APCs and this interferes with the ability of vpu-transfected APCs to efficiently present ovalbumin peptides to Ova-specific MHCII-restricted T cells. However, during HIV infection only a small number of APCs are infected; the remaining uninfected APCs would continue to express normal levels of cell surface MHCII and provide T cell help. While Vpu can down modulate the antiviral immune response and contribute to pathogenesis and viral persistence, this is likely to be a minor pathway. Nevertheless, these results do show the rich diversity and functional redundancy of HIV in its immune evasion strategies.

There are several examples of viral down modulation of the MHC presentation pathway (Ploegh 1998). For example, the adenovirus E3-19K protein binds and retains MHC class I heavy chain in the ER (Burgert 1985), while the herpes simplex virus ICP47 inhibits the ER peptide transporters TAP1/2, thus blocking peptides access to the ER lumen (Fruh 1995; Hill 1995). Cytomegaloviruses (CMVs) encode multiple proteins that interfere with the MHC class I pathway. HIV also uses its Nef and Vpu proteins to interfere with the MHC class I pathway. While Nef, an early protein, promotes endocytosis of MHCI from the surface of APCs (Schwartz 1996), the late expressing Vpu protein interferes with biosynthesis of the MHC class I heavy chain (Kerkau 1997). In studies on viral down regulation of the MHC class II pathway, CMV was shown to interfere with

expression of the MHC class II heavy chain through repression of class II transactivator (CIITA) expression (Heise 1998; LeRoy 1999; Miller 1998). Direct effects of CMV on global protein secretion were also shown to inhibit MHC class II trafficking to the cell surface. Herpes simplex virus infection strongly reduces invariant chain (Ii; CD74) expression (Neumann 2003) and the viral glycoprotein B competes with Ii for binding to HLA-DR (Neumann 2003; Sievers 2002). The Nef protein of HIV also impairs MHC class II presentation by reducing the surface levels of mature MHCII, while increasing the levels of functionally incompetent immature MHCII (Stumptner-Cuvelette 2001). We observe similar effects of the HIV-1 Vpu protein.

We report here a role for the HIV-1 Vpu protein in down modulating MHC class II presentation on APCs. We propose that by binding the class II invariant chain (CD74), Vpu prevents maturation of the MHCII complex, something monitored in our experiments with the L243 monoclonal antibody. This would be a novel strategy for interfering with MHCII presentation, unlike the effects of Nef that are likely to be due to the modification of intracellular MHCII trafficking (Stumptner-Cuvelette 2001). An instance of direct binding of CD74 was demonstrated for the HIV-2 Vpx protein (Pancio 2000), but the functional relevance of that binding for MHCII presentation was not studied. The results presented here add to the growing list of strategies used by HIV to evade the host immune response towards establishing a successful persistent infection.

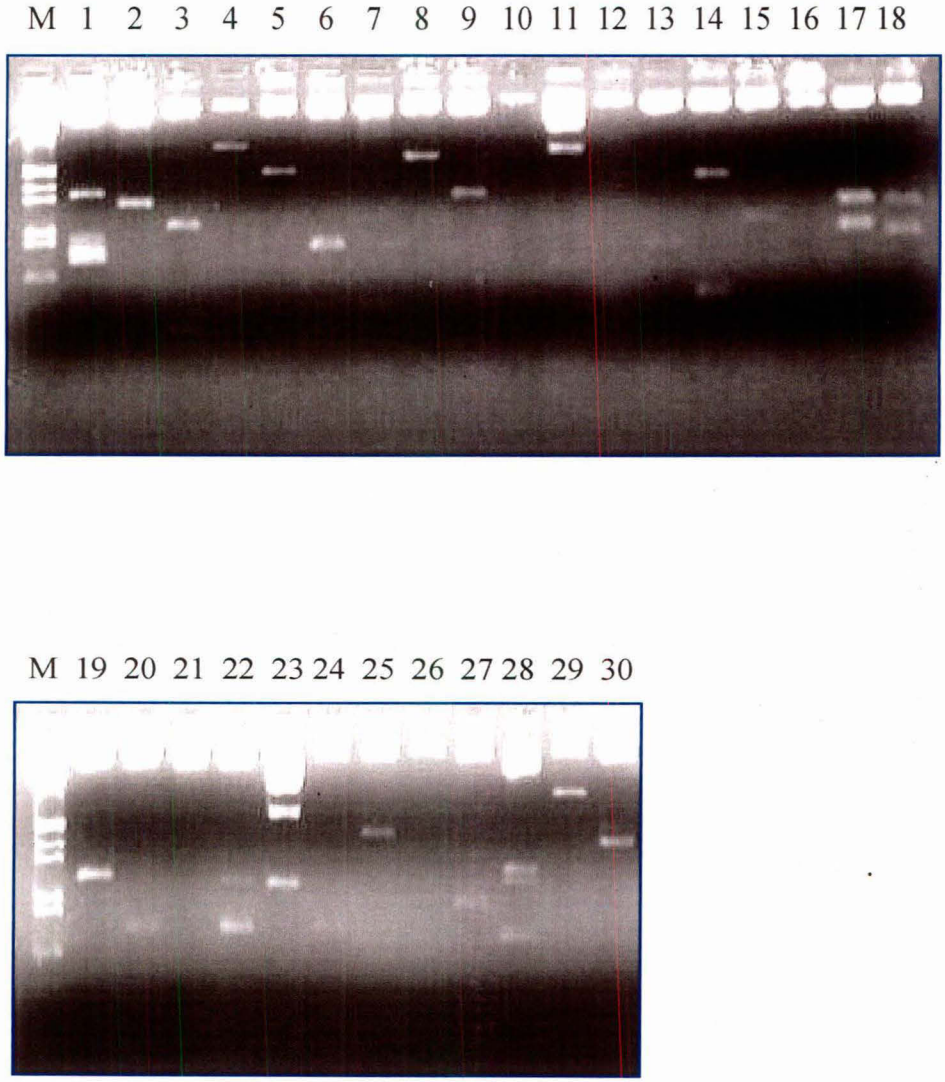


Figure 4.1: Restriction digestion analysis of interacting partners grown on SD LTH⁻ 3AT plates. These were positive for α-galactosidase activity and after amplification in E.coli, and transformation back into S. cerevisiae AH109 cells, grown on YPD plates and did not grow on SD/LH⁻ plates but grew on YPD plates. Numbers at the top show different clones. Sequencing was done for all of these clones except 12, 21 and 26 as those did not give any DNA fragment after restriction digestion.

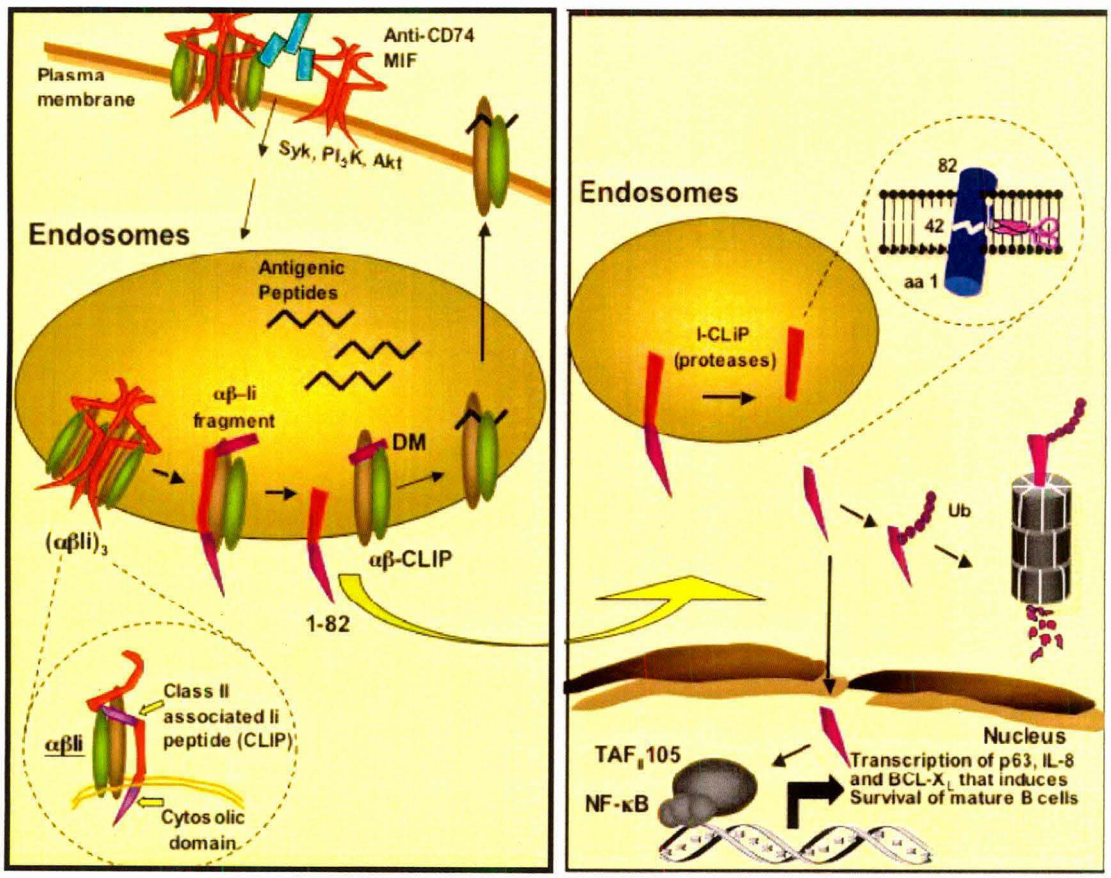


Figure 4.2: CD74, or invariant chain of MHCII. CD74 binds to MHCII before antigen loading in the endolysosomal compartment. The CD74 protein has an N-terminal cytoplasmic domain of 30 amino acids followed by a 26-aa transmembrane domain and a 160-aa domain that either projects into the lumen or is extracellular depending upon the subcellular localization of CD74. Invariant chain binds the MHCII α and β chains in the ER. The complex moves through the Golgi to the endosome where peptide loading occurs by displacement of CLIP domain of CD74 before the mature MHCII-peptide complex is presented on the cell surface. The displaced CLIP domain is further cleaved by I-CLIP proteases to release a 42-amino acid cytoplasmic peptide that translocates to the nucleus to modulate transcription of critical survival genes. The CD74 protein is also expressed as a trimer on the cell surface and modulates signaling initiated by the macrophage migration inhibitory factor (MIF) from the surface. (Source: www.bio.davidson.edu)

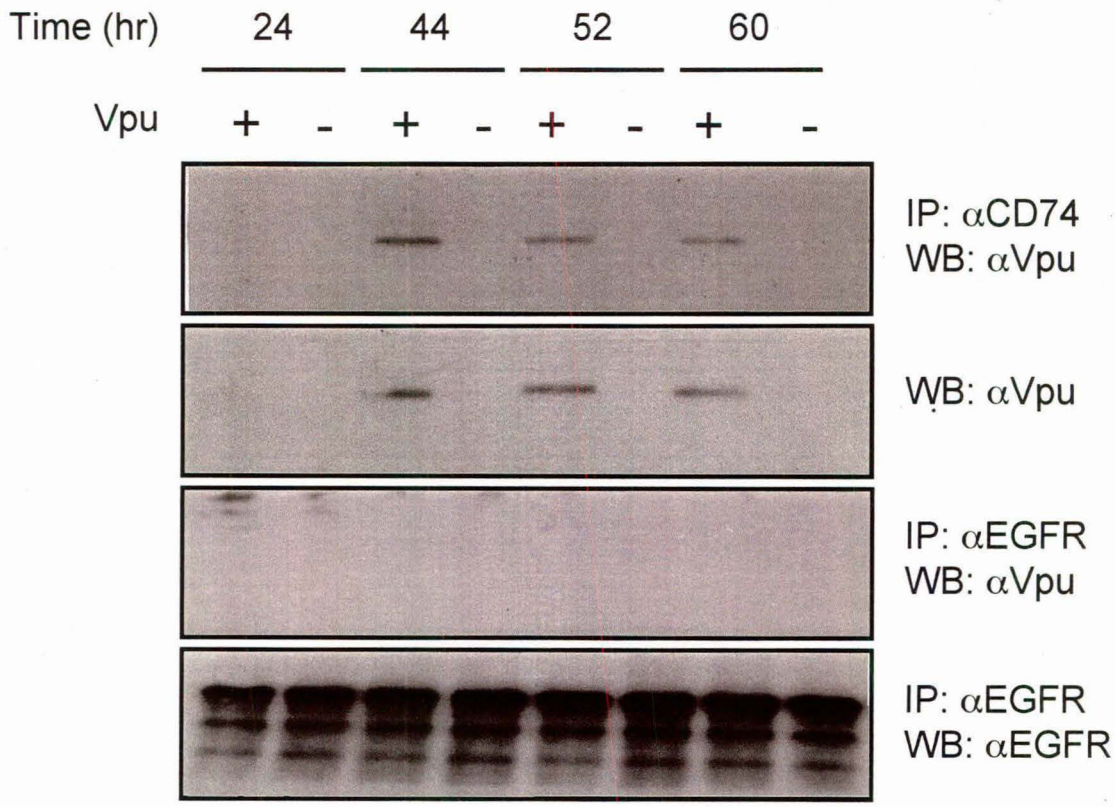


Figure 4.3: Coimmunoprecipitation of Vpu and CD74 in HIV-infected cells. U937 cells were infected with HIV-1 NL4-3 (Vpu+) or NL4-3U35 (Vpu-) viruses and cell lysates prepared at the indicated post-infection times. These lysates were then immunoprecipitated with anti-CD74 followed by western blotting with anti-Vpu. The immunoprecipitated Vpu proteins are shown. Alternatively, infected cell lysates were directly western blotted with anti-Vpu antibodies. As nonspecific controls, the same lysates were also immunoprecipitated with an isotype-matched anti-EGFR antibody followed by western blotting with either anti-Vpu or anti-EGFR antibody.

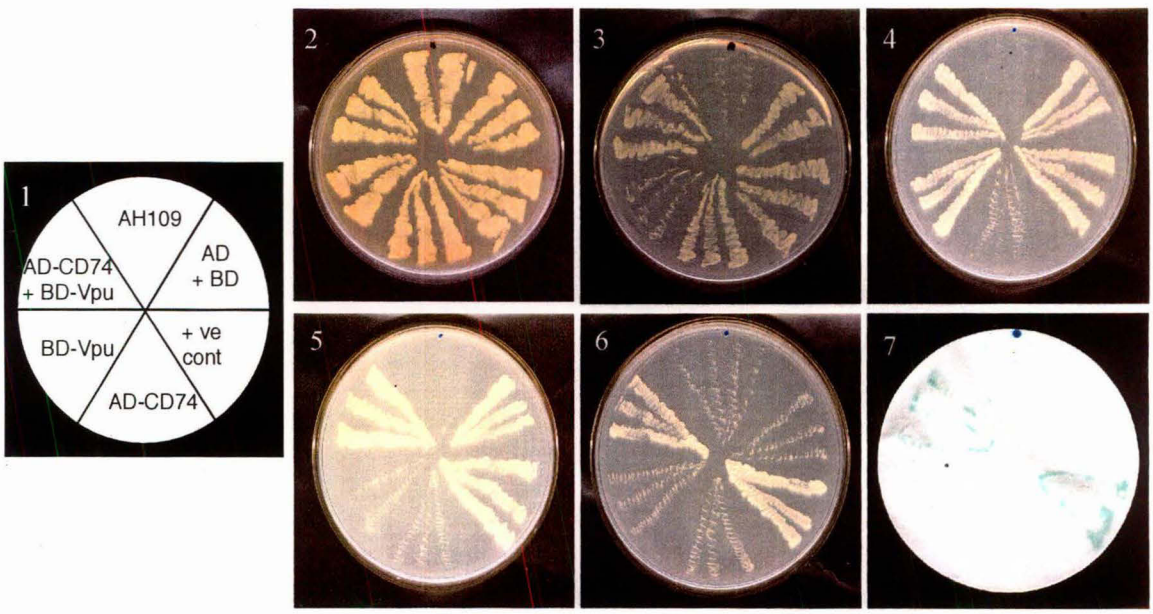


Figure 4.4(A): Yeast two-hybrid analysis of Vpu-CD74 interaction. Representative plates showing interactions of the Vpu and CD74. Panel 1 shows the template for panels 2 to 7 that in turn show transformants streaked in each section of the following plates: 2, YPD; 3, SD Leu⁻; 4, SD Trp⁻; 5, SD Leu⁻Trp⁻; 6, SD Leu⁻Trp⁻His⁻; 7, α-galactosidase filter assay. Growth is seen as light streaks on a dark background (2 to 6). The α-galactosidase signal (7) is seen as blue streaks on a white background.

Co-transforming Plasmids	YPD	SD/L$^-$	SD/T$^-$	SD/ LT$^-$	SD/ LTH$^-$	SD/ LTH$^-$ 3AT	α-gal (A value)
BD-Vpu + AD-CD74	+	+	+	+	+	+	0.793
BD-Vpu(cyto) + AD-CD74	+	+	+	+	+	+	0.546
BD-Vpu(TM) + AD-CD74	+	+	+	+	-	-	0.094
BD-Vpu + AD vector	+	+	+	+	-	-	0.069
BD vector + AD-CD74	+	+	+	+	-	-	0.074
BD-Vpu	+	-	+	-	-	-	0.037
AD-CD74	+	+	-	-	-	-	0.090
BD-SNF1 + AD-SNF4	+	+	+	+	+	+	1.179
BD vector + AD vector	+	+	+	+	-	-	0.065
AH109 cells only	+	-	-	-	-	-	0.068

Figure 4.4(B): Complete results for the entire screen using CD74 and Vpu full-length, transmembrane domain (TM) and cytoplasmic domain (cyto) fusions to the Gal4 protein activation domain (AD) or binding domain (BD). Growth (+) or no growth (-) of transformants on various media is shown. LTH$^-$3AT represents growth on SDLeu$^-$Trp$^-$His$^-$ plates containing 20 mM 3-amino-1,2,3-triazole. The liquid α-galactosidase assay values represent an average of two independent measurements. Various negative and positive controls are also shown

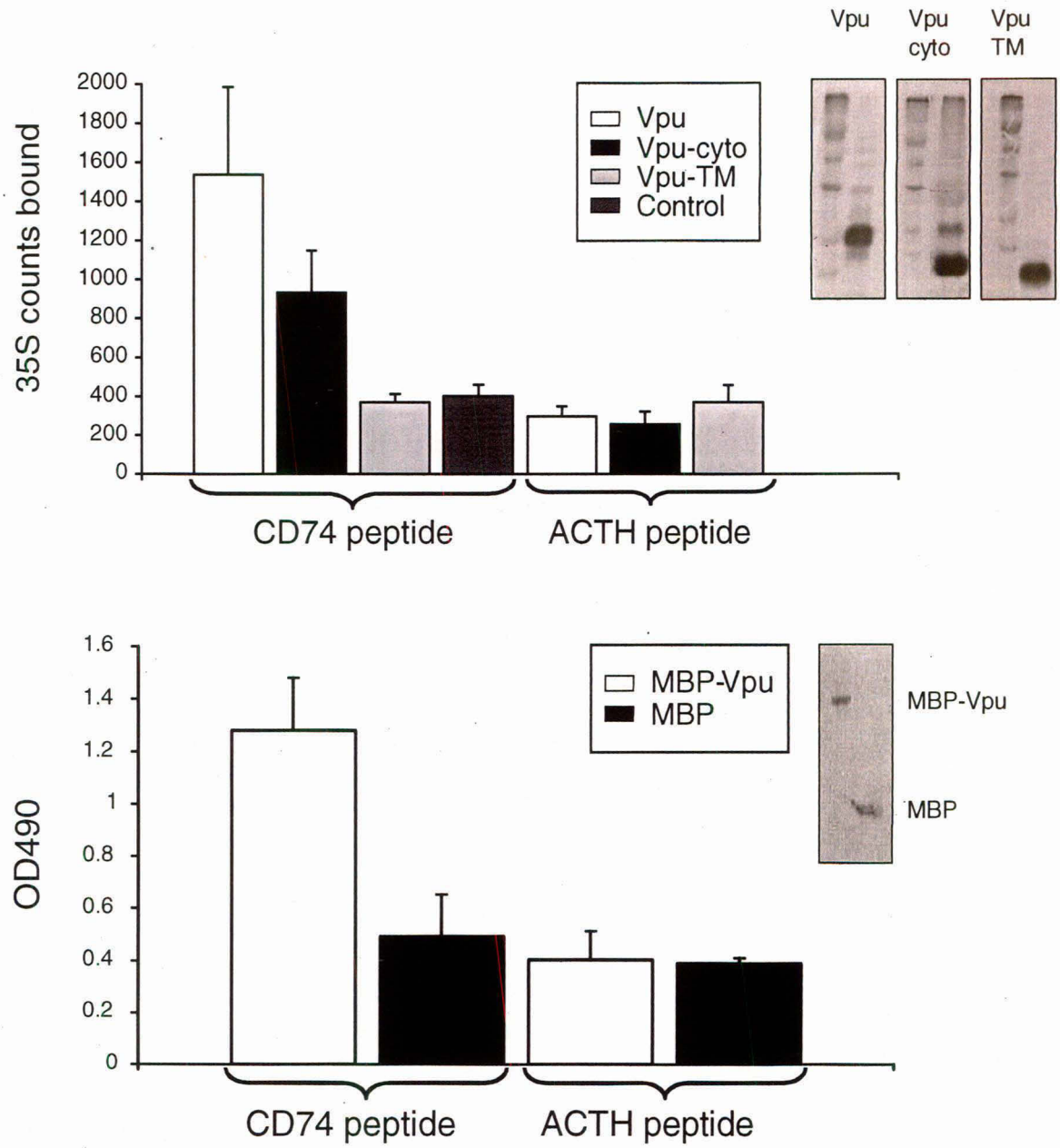


Figure 4.5: Vpu binding to CD74 cytoplasmic domain peptide. (A) In vitro synthesized ^{35}S-labeled Vpu proteins retained in wells coated with either a CD74 cytoplasmic domain peptide or a nonspecific peptide (ACTH). The results shown are an average of triplicate measurements. Autoradiograms on the right show crude in vitro translation mixtures run together with molecular size markers. (B) Purified MBP-Vpu or MBP control proteins retained in wells coated with either a CD74 cytoplasmic domain peptide or the nonspecific ACTH peptide. The results shown are an average of triplicate measurements. Coomassie Blue stained SDS gel on the right show purified proteins.

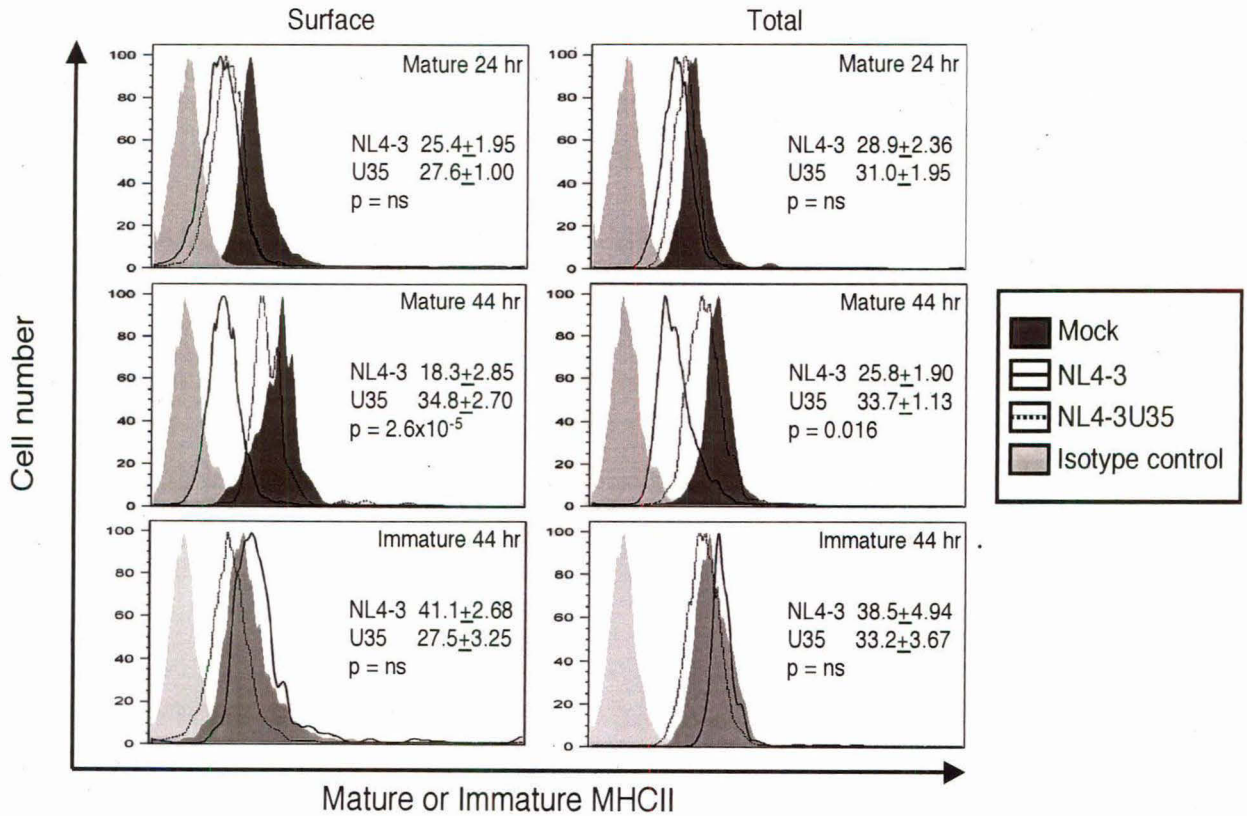


Figure 4.6: Vpu alters MHCII expression in virus-infected APCs. Activated U937 cells were either mock-infected or infected with HIV-1 NL4-3 or NL4-3U35 viruses. At 24 hr or 44 hr post-infection, cells were fixed and labeled with either the L243 antibody (for mature MHCII) or the CD74 antibody (for immature MHCII), either without permeabilization for surface levels or after permeabilization for total levels. Labeled cells were quantitated by flow cytometry. Inset shows mean fluorescence intensities (± SD) calculated over three separate experiments each with duplicate samples. The p values were calculated by the Students T-test and values ≥ 0.05 were considered not significant (ns). The p values shown are for a comparison between NL4-3 and NL4-3U35 infections.

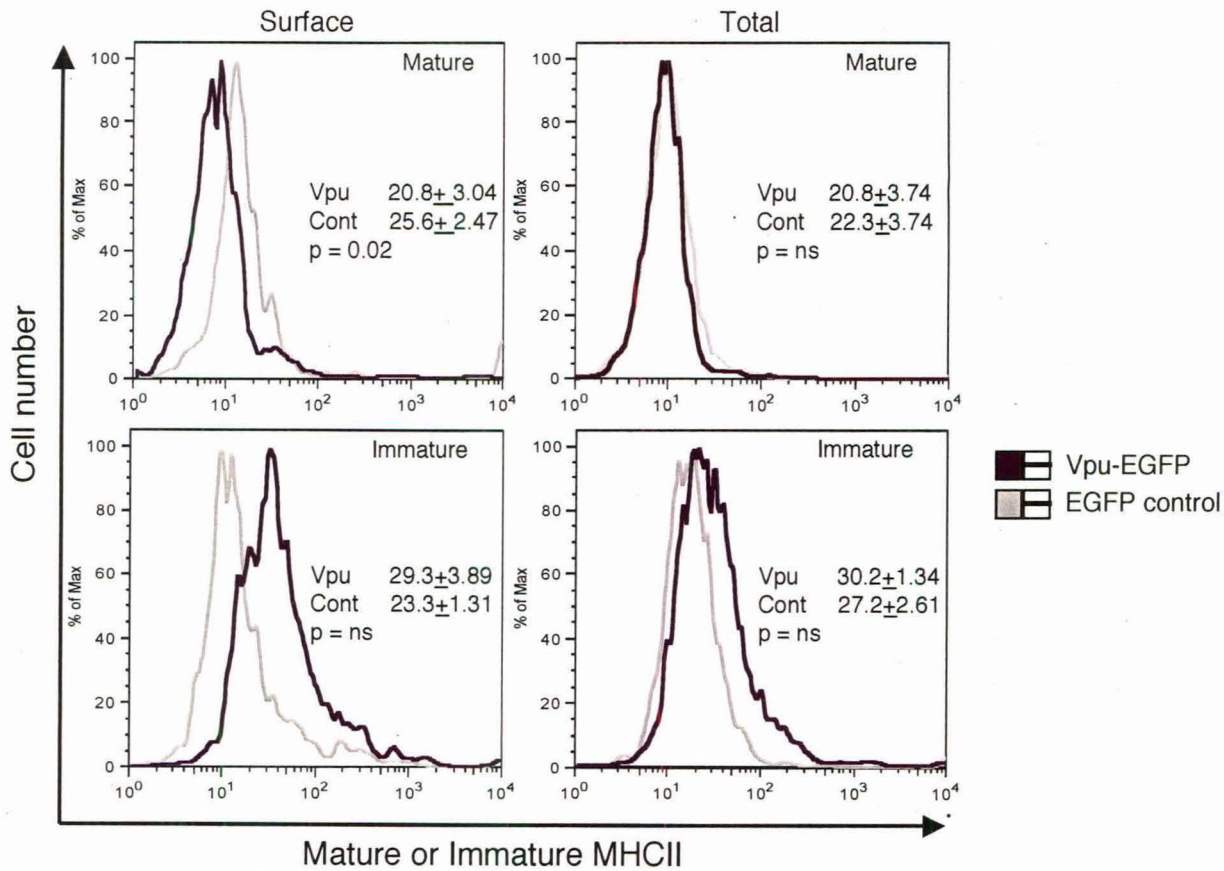


Figure 4.7: Effect of transfected vpu on MHCII expression. Activated U937 cells were transfected to express either Vpu-EGFP or EGFP, and 24 hr post-transfection cells were stained for surface or total levels of mature or immature MHCII as in the previous figure. Single parameter plots are shown for cells gated on EGFP expression. Inset shows mean fluorescence intensities (± SD) calculated over three separate experiments each with duplicate samples. The p values were calculated by the Students T-test and values ≥ 0.05 were considered not significant (ns).

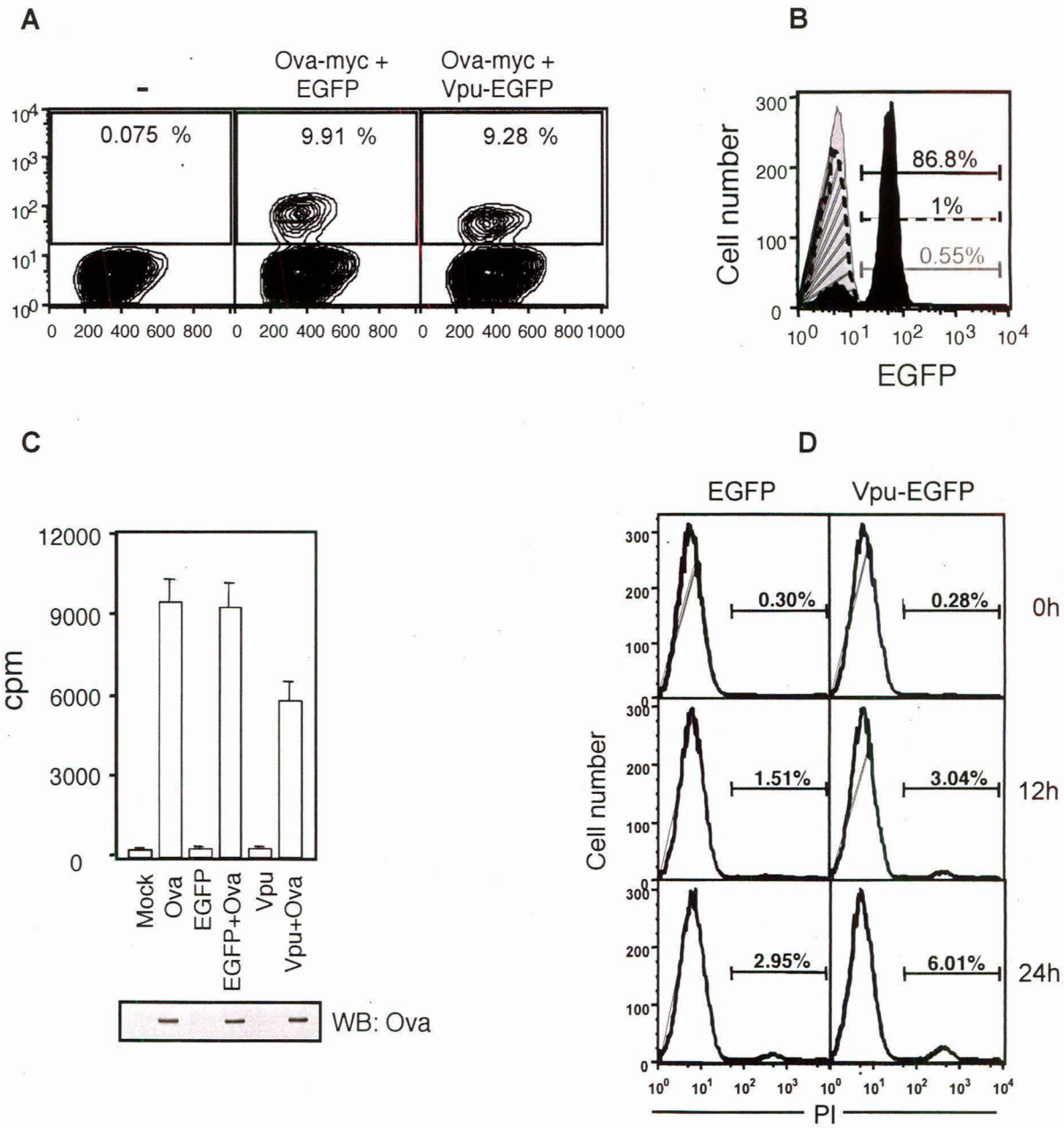


Figure 4.8: Vpu attenuates T cell activation by APCs. (A) Two-parameter plots show frequencies of Ova-myc expression in untransfected BMC-2 cells (-), or BMC-2 cells transfected to express Ova-myc + EGFP or Ova-myc + Vpu-EGFP, as indicated. The gates for OVA-myc-p$^+$ cells are also shown. (B) Histograms show staining levels for Vpu-EGFP in Ova-myc-p+ cells from Ova-myc + EGFP transfected (dotted curve) or ova-myc + vpu-EGFP transfected (black shaded curve) cultures. The grey shaded curve shows the isotype control. (C) Ova-specific MHCII restricted responses of primary T cells from OT-II transgenic mice to BMC-2 cells variously transfected, as indicated. All data are mean ± SE of triplicate cultures. T cell activation is measured as [^{3}H]-thymidine incorporation into cellular DNA. The western blot shows expression of Ova in transfected cells, stained with anti-myc antibodies. (D) Histograms show propidium iodide staining of BMC-2 cells transfected with either ova-myc + EGFP or ova-myc + vpu-EGFP at different times into the stimulation assay. Cells at 0 hr corresponds to those immediately after Ficoll-Hypaque purification or ~12 hr post-transfection.

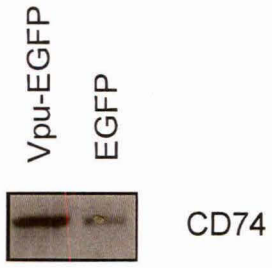


Figure 4.9: Western blotting of total CD74 from cell lysates of vpu-EGFP and EGFP transfected U937cells showing more CD74 in vpu-EGFP transfected cells.

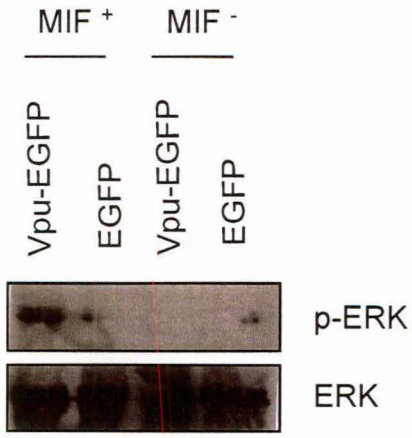


Fig 4.10: MIF induced phosphorylation status of Erk in vpu-EGFP and EGFP transfected cells. Cells not stimulated with MIF were used as control. Result shows more phosphorylated Erk in vpu transfected cells as compared to EGFP transfected cells while in both case MIF stimulation was given (First two lanes, top pannel). In all the cases, total Erk level remain unchanged (Bottom pannel).

5. Conclusion

The first part of the thesis deals with Vpu oligomerisation using different in vitro methods such as pull down assays, electrophoresis, immunoprecipitation and in vivo assays including yeast two hybrid and FRET. Our results provide direct evidence of the nature of Vpu oligomer which appears to be a pentamer. In our analysis, multimeric species, including pentamers were evident on SDS and native gel electrophoresis of the *in vitro* synthesized Vpu proteins and their TM domains. The Vpu pentamers remain in a stable equilibrium with its monomers *in vitro*. This is true for full length Vpu and the TM domain as well. We provided a direct evidence that the hydrophobic N-terminal TM domain, and not the cytoplasmic domain is critical for Vpu oligomerization. The isolated TM domains also showed stable interaction with the full-length Vpu protein. The Vpu-TM domains derived from two different HIV-1 subtypes R5 and NL, interacted efficiently with each other. Confocal microscopy results show localization of Vpu to the endoplasmic reticulum and Golgi regions, as well as to post-Golgi vesicles of the cell. Our FRET results in live cells show Vpu oligomerization to occur downstream of the ER, either in Golgi region or intracellular vesicles.

The second part of the thesis focuses on the cellular proteins which intearact with Vpu and functional impact of these interactions. While searching for novel Vpu-interacting cellular proteins using yeast two hybrid system we found four novel Vpu interacting cellular proteins. One of these was CD74, the invariant chain of MHCII. We showed direct binding of Vpu and CD74 using the yeast two-hybrid assay and coimmunoprecipitation from cells infected with wild type or vpu-defective HIV-1. The cytoplasmic region of Vpu was found to interact with the 30-amino acid cytoplasmic tail of CD74. In infection experiments, cells infected with vpu-defective HIV-1 showed increased surface MHCII levels, suggesting a role for the Vpu protein. This downregulation of MHCII in infected cell was observed late in infection suggesting a role of Vpu as it is known to be a late expressing HIV-1 protein. This effect is likely to be due to binding of Vpu with CD74 in the endoplasmic reticulum (ER), which would prevent the association of CD74 with MHCII in the ER prior to its transport to the surface. Our work also shows a role for the HIV-1 Vpu protein in down modulating MHC class II presentation on APCs and the subsequent help

provided by Vpu-expressing APCs to T-helper cells. This defines a new pathway for immune modulation by HIV. We also observe increased activation of the Erk pathway in a MIF-dependent manner in Vpu-expressing cells. This new effect of HIV-1 Vpu on cellular signaling is being explored further.

Publications

Published work from this thesis:

- **Hussain, A.**, Das, S.R., Tanwar, C. and Jameel, S. "Oligomerization of the human immunodeficiency virus type 1 (HIV-1) Vpu protein – a genetic, biochemical and biophysical analysis." *Virology Journal*, 2007: 4: 81

- **Hussain, A.**, Wesley, C., Khalid, M., Chaudhry, A. and Jameel, S. "The HIV-I Vpu protein interacts with CD74 and modulates major histocompatibility complex II presentation." *J Virol*, 2007: Under Review.

Other published work:

- Chaudhry, A., Das, S.R., **Hussain, A.**, Mayor, S., George, A., Bal, V., Jameel, S. and Rath, S. "The Nef protein of HIV-1 induces loss of cell surface costimulatory molecules CD80 and CD86 in APCs." *J Immunol*, 2005: 1175: 4566-74.

- Padhan, K., Tanwar, C., **Hussain, A.**, Hui, P.Y., Lee, M.Y., Cheung, C.Y., Peiris, J.S.M., and Jameel, S. "The SARS coronavirus Orf3a protein interacts with caveolin." *J Gen Virol*, 2007: In press.

Chapter 6

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6. References

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