ADENOSINE DEAMINASE ACTING ON RNA (ADAR1) IS A NOVEL MULTITARGETED ANTI HIV-1 CELLULAR PROTEIN

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ADAR1 is an RNA editing enzyme which acts on completely or partially double-stranded RNA. Since HIV-1 RNA has such secondary structures, we have examined whether ADAR1 exhibits antiviral activity against HIV-1. Our results indicated that ADAR1 inhibited viral replication and infectious HIV-1 production in various cell lines including 293T, HeLa and Jurkat T cells, and was active against a number of X4- and R5-tropic HIV-1 of different clades. Analysis of the level of intracellular HIV-1 RNA showed no change in levels of intracellular *gag, pol,* and *env* RNA in the presence of ADAR1 despite a significant inhibition of intracellular and virion associated HIV-1 protein production. Furthermore mutational analysis showed that ADAR1 introduced most of the A-to-G mutations in the first exon of *rev* at positions 5998, 6011, 6017, and 6036 and in the Rev Response Element (RRE) binding region (positions 8413 and 8438) of *rev* and *env* RNA. In elucidating the mechanism of ADAR1 inhibition of HIV-1, we observed that A-G mutations in *rev* have a significant negative effect on the expression of Rev. However, all mutations could be complemented by wild type Rev. Furthermore, these A-G mutations in the RRE binding region of *rev* inhibited the binding of Rev to the RRE region in *env* and inhibited transport of primary transcripts like *gag, pol* and *env* from the nucleus to the cytoplasm. Introduction of these specific mutations in *rev* of an infectious molecular clone of HIV-1 by site directed mutagenesis abolished the replication capacity of HIV-1 by inhibiting viral protein synthesis without any effect on viral RNA synthesis, a phenotype exhibited by HIV-1-infected

cells exposed to ADAR1. ADAR1 induced mutations in *env* further attenuated viral infectivity. ADAR1, thus, constitutes a novel class of cellular antiviral proteins with multiple targets in the viral genome thereby providing a new avenue of exploration for therapeutic drugs benefitting public health.

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1.0   INTRODUCTION

1.1   HIV-1/AIDS

Human immunodeficiency virus (HIV), the causative agent of acquired immune deficiency syndrome (AIDS) was first reported in 1981 in four unhealthy homosexual men (47). HIV consists of two types: HIV-1 and HIV-2. HIV-2, the least pathogenic of the two and mostly prevalent in West African populations, has a slower progression rate, leading to the development of AIDS (27). HIV-1 is believed to have originated from simian immunodeficiency virus in chimpanzees (*Pans troglodytes*) and in wild gorillas that got transmitted to humans (22, 43, 52, 140). The evolutionary descendants of HIV-1 formed four phylogenetic groups: M (main), O (outlier), N (non-M, non-O) and P (putative) (52, 110). Group M originated from chimpanzees and group O from gorillas in Cameroon. However, the origins of group P and N are still unknown (146). Group M is the most prevalent globally and has expanded into nine subtypes: A-D, F-H, J and K(145).

1.1.1   HIV-1 epidemiology

HIV-1 is transmitted primarily through heterosexual intercourse, intravenous drug abuse, homosexual intercourse, mother to child transmission, and through blood transfusion or needle sharing. In 2010, the CDC (Centre for Disease Control) estimated that more than one million

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people are living with HIV-1 in the United States. More than 50,000 Americans become infected with HIV-1 every year and more than 18,000 people die of AIDS every year. According to the recent UNAIDS report, 33 million people are currently infected with HIV-1 around the world (137). Of these, 30.8 million adults, 15.9 million women and 2.5 million children (less than 15 years old) are infected. 2.6 million people were newly infected with HIV-1 and 1.8 million deaths have occurred due to AIDs in 2009 (137).

GLOBAL REPORT

Adults and children estimated to be living with HIV | 2009

Western & Central Europe 820 000 [720 000 – 910 000]

Eastern Europe & Central Asia 1.4 million [1.3 million – 1.6 million]

North America 1.5 million [1.2 million – 2.0 million]

East Asia 770 000 [560 000 – 1.0 million]

Caribbean 240 000 [220 000 – 270 000]

Middle East & North Africa 460 000 [400 000 – 530 000]

South & South-East Asia 4.1 million [3.7 million – 4.6 million]

Central & South America 1.4 million [1.2 million – 1.6 million]

Sub-Saharan Africa 22.5 million [20.9 million – 24.2 million]

Oceania 57 000 [50 000 – 64 000]

Total: 33.3 million [31.4 million – 35.3 million]

World Health Organization   UNAIDS

Figure 1: UNAIDS global report on adults and children to be living with HIV-1 in 2009.

UNAIDS 2009 Report of the Global AIDS Epidemic. 2009. UNAIDS (Open access journal)

1.1.2 HIV-1 structure

HIV-1 is a lentivirus containing two copies of a single-stranded positive sense RNA genome. It is a retrovirus, having the characteristic enzyme reverse transcriptase which helps in conversion of positive sense viral RNA to double-stranded DNA (dsDNA). The HIV-1 genome is about 9.7kb and encodes nine viral proteins (Figure 2). They include the structural polyprotein Gag Pr55^{gag}, the proteolytic cleavage of which generates matrix (MA), capsid (CA) and nucleocapsid (NC) protein in mature virion particle. The Gag-Pol polyprotein, pr160 gag-pol, is produced by a site specific frame-shift mutation during viral mRNA translation leading to the overlap of the two open reading frames. Pr 160 gag-pol undergoes self cleavage to generate separate gag and Pol proteins. The enzymatic polyprotein Pol is cleaved into reverse transcriptase (RT, required during viral RNA to proviral DNA synthesis) integrase (IN, required for integration into host genome) and protease (PR, required for cleavage of viral proteins) (9). The regulatory proteins include Tat and Rev which regulate viral gene expression. Tat binds to a specific RNA stem loop structure in LTR known as TAR (Trans Activator Region). Tat facilitates the processivity of RNA polymerase II during early elongation by interacting with cellular kinases. Rev binds to the RRE region (Rev Response Element) in the *env* and transports the single and unspliced RNA from nucleus to cytoplasm where they can be translated into proteins.   HIV-1 also possesses accessory proteins, Vpr, Nef, Vpu and Vif, which facilitate its survival *in vivo* by negatively regulating host defense mechanisms.

The viral genome is flanked by long terminal repeats (LTR) on both ends, which drive viral gene expression. They also play a pivotal role in reverse transcription of the viral RNA and integration of the proviral genome (28). HIV-1 has an outer layer composed of the envelope glycoprotein Env, which is synthesized as a gp160 precursor protein. It is then cleaved to form

gp120 (SU) and gp41(TM) noncovalent heterotrimer. The gp120 acts as the primary ligand that binds to the receptors on target cells and gp41 aids in fusion thereby facilitating fusion and entry into target cells(75). The main structural component of a virion particle, the Gag (Group specific antigen) protein is synthesized as a polypeptide from an unspliced transcript. Posttranslational proteolytic cleavage of this large polyprotein precursor known as p55 gives rise to smaller Gag proteins p24 (capsid protein), p19, p17 (matrix protein) and p6. In a mature virion there is a layer of matrix just below the outer shell. The matrix contributes to the structure and assembly of the virus and the cone shaped core is formed from capsid proteins which contains two copies of the viral RNA genome protected by a coating of nucleocapsid (Figure 3) (42, 80). p6 helps in the release of budding virus particles and interacts with the accessory protein Vpr for its incorporation into virus particles.

The viral protein Pol, is also synthesized as a polyprotein precursor and subsequently cleaved to form three important enzymes: RT, IN and PR. The regulatory protein Tat regulates viral gene expression from the LTR at the transcriptional level by increasing the processivity of RNA polymerase II in its presence. Rev on the other hand monitors the nuclear export of unspliced viral RNA. The accessory proteins on the contrary, are not required for active replication, but perform a myriad of functions essential for virus survival *in vivo* (48, 115). Vpu facilitates the transport and processing of envelope glycoprotein gp160 which gets trapped by CD4 by binding to CD4 at the rough endoplasmic reticulum and degrading it via the ubiquitin-proteosomal pathway (48, 115). Vif increases the infectivity of the virus in certain cell types by counteracting the effects of host restriction factors APOBEC3G, APOBEC3F. Nef, the negative regulator derails the host immune system by down regulating important signaling and regulatory molecules such as CD4, MHC class 1 A, B, C; CD28; and β-chain of CD8αβ receptor. It also

interferes with MHC class II processing of HIV-1 antigen molecules, upregulates FasL and TNF-α (40, 41). Vpr is a pleotrophic protein essential for viral survival and is incorporated into the virion particle. Vpr promotes cellular differentiation and arrest cellular proliferation in the G2 phase of the cell cycle causing enhanced viral replication.
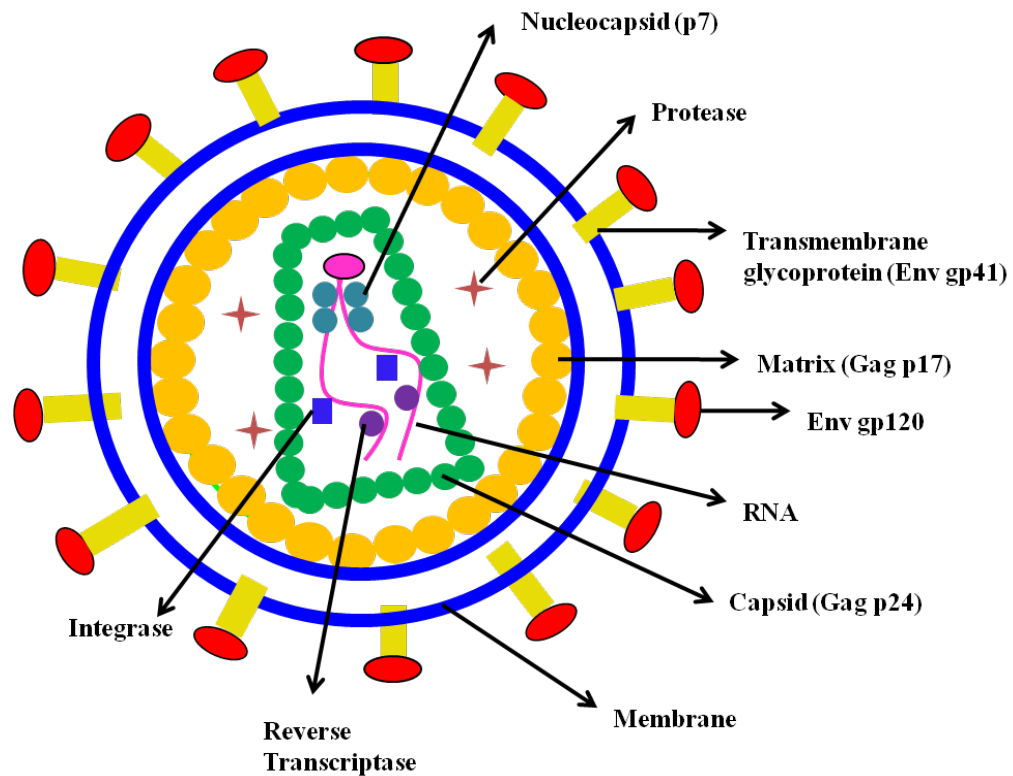
Figure 2: HIV-1 virion structure.
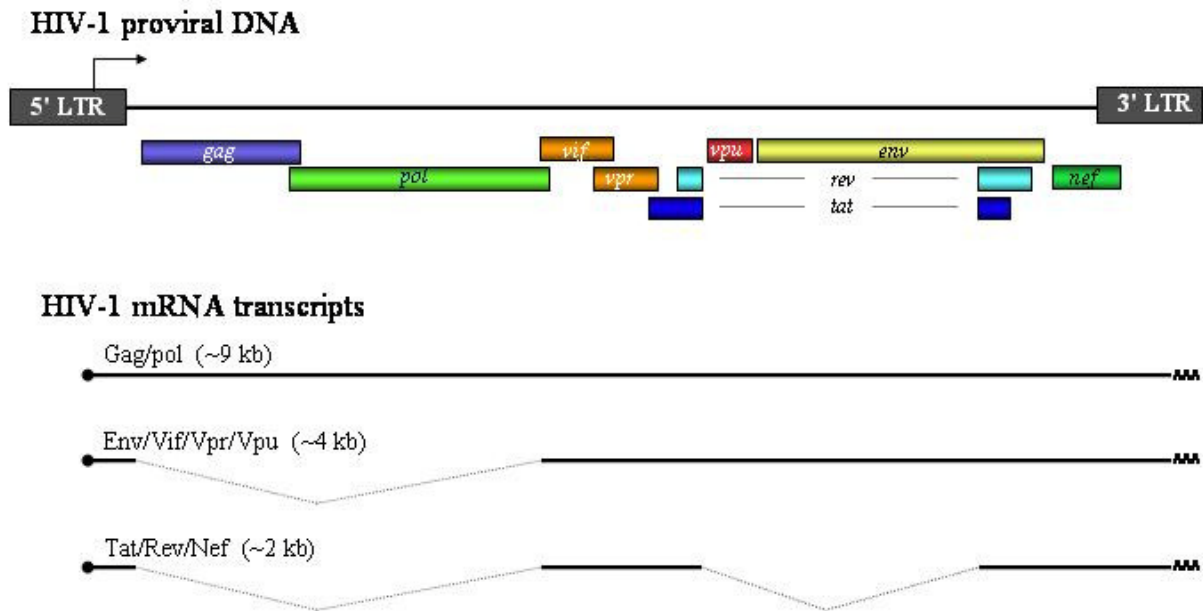
Figure 3: Genomic organization of HIV-1.

1.1.3 HIV-1 life cycle

1.1.3.1 <u>Binding and Fusion</u>

The HIV-1 life cycle starts with binding of gp120 to the host cell receptor CD4 found mainly on T-cells and macrophages, which entails a cascade of structural changes and reorganization of the Env trimer (Figure 4). gp120 also requires binding to a co-receptor, usually CCR5 or CXCR4, which facilitates viral fusion. This results in a conformational change in the transmembrane protein gp41, exposing the hydrophobic ectodomain of this N-terminal fusion peptide. This allows gp41 penetration of the viral cell membrane and fusion with the host cell membrane, causing release of the viral core into host cell cytoplasm.

1.1.3.2 <u>Uncoating, Integration and Reverse Transcription</u>

After virus entry, the capsid core undergoes morphological changes in the cytoplasm and capsid protein subunits dissociate, a process termed as uncoating. This uncoating process has been shown to be imperative for efficient HIV-1 infection and nuclear import and needs host factors like cyclophilin A, which has been shown to increase viral infectivity by binding to capsid proteins during uncoating in some cell lines (35, 101). However host factors like tripartite motif proteins (TRIM) in certain mammalian cell types obstructs viral infection and restricts it at this step by targeting the capsid protein for proteosomal degradation via its ubiquitin ligase activity leading to premature uncoating (2, 39, 135).

The single-stranded plus-sense viral RNA is converted to double-stranded DNA by RT. The genomic RNA serves as the template and using transfer RNA (tRNA) as the primer, viral RNA and tRNA^{lys3} which forms the minimal reverse transcription complex (RTC), a minus strand DNA is first produced. The RNA of the RNA/DNA heteroduplex is then degraded by the

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RNase H activity of RT. A strand transfer event occurs which allows the 3' end of the genomic RNA to be used as a template for continued synthesis of minus strand DNA. A polypurine tract of the vRNA resists RNase H degradation and is used as a primer for plus strand DNA synthesis. Continued plus strand synthesis occurs until the tRNA primer is removed, giving way for the complementation of the plus strand DNA to sequences near the 5' end of minus strand DNA. Another strand transfer event occurs and finally both plus and minus strand DNA synthesis proceeds until a full length double-stranded DNA is formed. After the completion of the double strand DNA synthesis the reverse transcription complex (RTC) becomes competent for integration as is termed the pre-integration complex (PIC) (18, 96).

The 3' terminus of the newly formed double stranded DNA is cleaved by a 32kD protein IN thereby providing the sites for attachment of viral DNA to host DNA. A PIC loaded with viral and cellular proteins along with viral DNA translocates into the host cell nucleus. Viral DNA integrates into host chromosomal DNA by IN, becoming a provirus.

1.1.3.3 Transcription and Translation

Integrated proviral DNA utilizes host RNA polymerase II and the viral transcription transctivator Tat for its transcription, using the viral 5' LTR as a promotor. All newly synthesized viral RNAs undergo post-transcriptional modifications involving 5' capping and 3' polyadenylation. Unspliced transcripts get either packaged into virions as their genetic material or are used as mRNA template for polyprotein precursor synthesis like Gag and Gag-Pol. Multiple spliced transcripts are translated into Tat and Rev protein. Rev shuffles back into the nucleus and bind to the RRE binding region in the *env* and transports single or unspliced transcripts from the nucleus to cytoplasm. These transcripts are then translated into structural and accessory proteins.
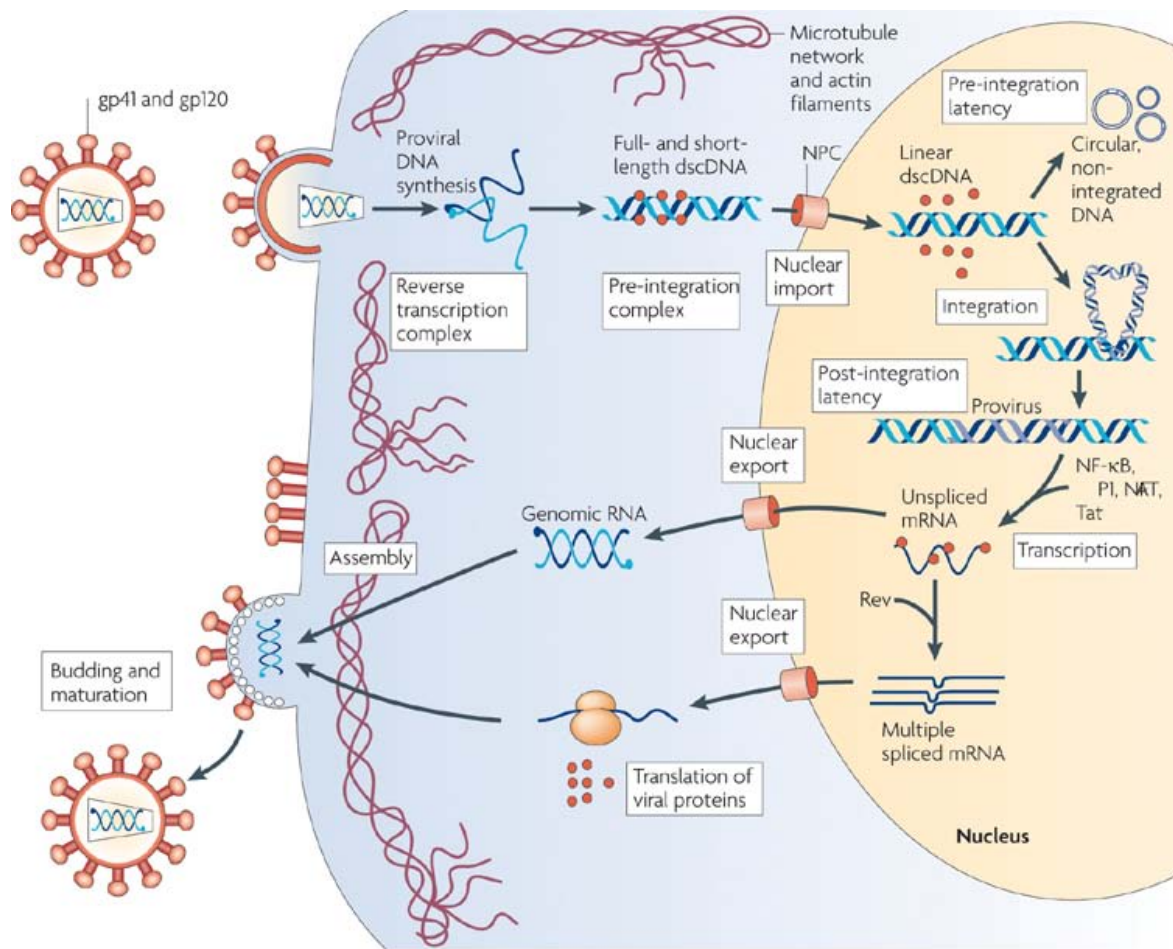
Figure 4: HIV-1 life-cycle.

1.1.3.4 Assembly and Maturation

Synthesized viral proteins, polyprotein precursors and viral RNA are targeted to the plasma membrane for assembly by myristylation of the N-terminal of Gag polyprotein (148). The assembled viral components bud from the plasma membrane. Virion maturation is marked by prompt cleavage of the Gag and Gag-Pol polyproteins by PR after the release of immature

virions (71). Active PR, an essential component for viral infectivity, is a 10kD homodimer composed of two Gag-Pol polyprotein precursors. HIV-1 PR functions as an aspartyl protease by possessing a characteristic active site triad Asp-Thr/Ser-Gly corresponding to amino acids 25-27. The two catalytic Asp25 residues are positioned adjacent to the protein cleft and both subunits of the enzyme contain parallel β sheets called flaps. In their native inactive conformation these flaps overlap to form an intersubunit hydrogen bond that prevent substrate binding, however during catalysis these flaps open up and pin down the substrate in the active site(136).

1.2   HIV-1 PATHOGENESIS

HIV-1 was identified as the causative agent of AIDS in 1983 (6). HIV-1 infection is characterized by an acute immediate symptomatic phase and a chronic asymptomatic phase (Figure 5). Acute infection usually manifests symptomatically within 1 to 4 weeks of infection in the form of brief flu-like symptoms, which include a sore throat, fever, muscle aches, swollen lymph nodes and rash. The acute phase is followed by a chronic asymptomatic phase in which virus replication is maintained without any obvious or apparent illness and is unique for every infected individual, ranging from 3 to greater than 14 years. This chronic phase, however, marks the onset of subtle immunological events such as depletion in CD4 T-cells although in some patients CD4+ T cells count can remain stable for several years. Thus a quasi-steady state precedes a phase of rapid decline in CD4+ T cells. The end of this asymptomatic phase is followed by the onset of AIDS, characterized by depletion of CD4+ T cells, high viral loads, increased genetic diversity, and a variety of immunological disorders that compromise both

cellular and humoral immune responses, leading to increased susceptibility to infections and malignancies.

Upon entry into a new host, HIV-1 targets $CCR5^+ CD4^+$ effector memory T cells majorly present at mucosal sites of the gut lungs and cervicovaginal mucosa. This results in massive depletion of these mucosal resident target immune cells within first few weeks of acute infection (14, 109). Mucosal depletion is soon followed by chronic activation resulting in an increased number of activated and memory T cells, increased production of proinflammatory cytokines, and increased turnover of immune cells. During the acute phase viremia is very high (10^6 to 10^7 copies/ml) in plasma peripheral blood and over 10-100 million infected CD4+ cells die per day
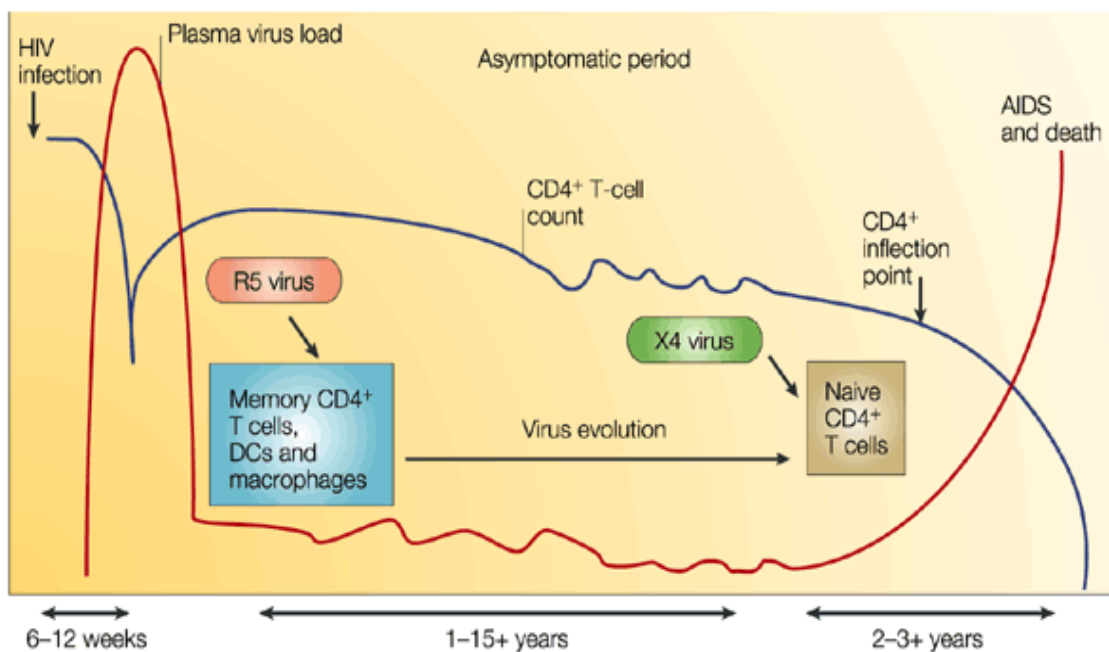
(26).

Figure 5: HIV-1 Pathogenesis.

Figure taken from Sarah *et al.* Nature Reviews Immunology 3, 343-348, April 2003

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During this acute phase, a robust antiviral CD8+ T cell response leads to control of viremia and is a good predictor of the rate of progression to AIDS. This is denoted as the viral set point, low viral set point indicates a slower rate of progression to AIDS. Although an immune response is induced soon after infection to combat the virus, it fails to effectively eliminate the infection. The virus kills the cells of the host immune system and makes way to facilitate its dissemination from the local tissue. Seroconversion normally occurs 1 to 3 weeks after infection and viral set point is reached usually after 3 to 5 months. After 3 to 6 months of primary infection, CD4+ T cell numbers increase, but they never fully recover to pre-infection levels. This marks the beginning of the chronic asymptomatic phase of the viral infection. The decrease in viremia might be a result of immune response mediated by CD8+ T cells killing of virus infected cells. During this chronic phase, which continues for 8-10 years in a typical progressor, there is a persistent level of virus replication in the lymph nodes and peripheral blood, increased viral genetic diversity, and increased viral replicative fitness (14).This viral maintenance is maintained at relatively constant levels by the antiviral CD8+ T cell response. AIDS is marked by an increased viral load, a steady decline in CD4+ T cells to less than 200cells/μl of blood, and a deteriorated humoral and cellular immune responses (14, 109). In the lymph nodes, a massive breakdown of tissue architecture and destruction of follicular dendritic cells occurs (26). All of these events directly precede or coincide with the emergence of more pathogenic, fast replicating viruses and often the use of the CXCR4 coreceptor (in approximately 50% of cases), opportunistic infections and AIDS related malignancies (109).

1.3   RESTRICTION FACTORS

Mammalian evolution has witnessed the development of a plethora of mechanisms to combat viral assault. Host innate and adaptive immune responses provide the first lines of defense. However, due to increased diversity of retroviruses, some inhibitory host proteins can play a formative role in controlling host susceptibility (73,(134). These inhibitors are appropriately termed 'restriction factors', as they block retroviral infection at different stages of their life cycle (25, 134). The discovery of restriction factors was first thought to be limited to mouse cells, but recent studies have shown their widespread prevalence and roles in restricting HIV-1 in human cells (10, 99).

1.3.1   APOBEC and Vif

APOBEC3 (A3) are cytidine deaminases that play a role in the human innate immune response to control retroviruses, retrotransposons, hepadnavirus, and even some DNA viruses like human papillomavirus. APOBEC proteins (*apo*lipoprotein *B* mRNA-*e*diting *c*atalytic polypeptide) include APOBEC1 (located on chromosome 12), APOBEC2 (chromosome 6), and seven APOBEC3 proteins (chromosome 22; APOBEC3A, APOBEC3B, APOBEC3C, APOBEC3DE, APOBEC3F, APOBEC3G, and APOBEC3H) (45, 117). APOBEC3G and APOBEC3F are expressed mainly in T lymphocytes and target HIV-1. APOBEC3 proteins are packaged into viral particles via interaction with viral Gag protein in the absence of Vif. These proteins act during reverse transcription to deaminate deoxycytidine residues to deoxyuridine (dU) in the growing minus-strand viral DNA. These dU-rich transcripts either undergo degradation or form G-to-A hypermutated, non-functional viral DNA (12, 130). HIV-1 Vif binds specifically to

APOBEC3G and recruits the ubiquitination complex consisting of Cul5, RbX1 and Elongin B, C. This results in polyubiquitination and proteasomal degradation of APOBEC3G. Thus Vif reduces the amount of cellular APOBEC3G available for packaging in the budding virus (12, 130). Additional proteasomal degradation independent inhibitions of APOBEC3G by Vif have also been reported. In the absence of Vif, APOBEC3G is active against a broad range of retroviruses and can also block the hepatitis B virus (HBV) (31, 67).

1.3.2 TRIM5α and TRIM-Cyp

It was discovered that TRIM5α protein obtained from Old World Monkeys can successfully block HIV-1 as well as N-tropic Murine Leukemia virus (N-MLV). However human TRIM5α falls short in that it can only restrict N-MLV infection. The most common TRIM5α variants obtained from New World monkeys can effectively restrict SIV mac but not HIV-1 with the exception of owl monkey (60). TRIM5α from Owl monkeys can block HIV-1 but not N-MLV or SIVmac infections. Further investigations into their anomalistic behavior lead to the discovery that instead of TRIM5α they possess a TRIMCyp fusion gene generated by the retrotransposition of a cyclophilin A (CypA) mRNA within the TRIM5 locus (142). CypA is a highly conserved peptidyl prolyl isomerase that catalyzes cis/trans- isomerization of the prolyl peptide bonds. CypA has been shown to bind CA via a direct interaction through its active site and an exposed CypA binding loop. CA proteins of other retroviruses like FIV, SIVcpz, SIVagm also bind to CypA but not MLV or SIVmac. Cyclosporin an immunosuppressive drug can potently disrupt CypA-CA interaction by competitively binding to CypA active site. This finding prompted the cloning of an essential factor the TRIM5-CypA fusion gene (83, 123). TRIM5 alpha also shows similar anti-retroviral activity in most primate species like rhesus macaque, cows and rabbits.

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TRIM5 has a RING finger, a B-box and a coiled-coil domain thereby giving it the status of a tripartite motif gene. The B-box and coiled-coil domain promote multimerization and is required for anti-HIV-1 restriction activity (127, 131). However the mechanism for restricting HIv-1infection is still elusive. There are different models hypothesizing the mode of action of TRIM5α. TRIM5 multimer binds to incoming virions and promotes premature uncoating and blocks reverse transcription. TRIM5α then targets the virion for proteosomal degradation by utilizing the ubiquitin ligase activity of its RING domain. It is also dependent on accessory factors to successfully impede retroviral infection in primate cells in a species specific manner. (124, 147).

1.3.3   Tetherin

Bst-2 or Tetherin is an intereferon induced, 20kDa, single pass, cell surface and lipid raft associated type II glycosylated membrane protein that binds the lipid rafts via its COOH-terminal glycosylphosphatidylinisotol (GPI) anchor (46). BST-1 does not exclusively localize to plasma membranes but is also found in internal membranes like trans Golgi network and in recycling endosomes. However unlike other GPI-anchored proteins BST-2 gets endocytosed form the cell surface in a clathrin-dependent manner (74). HIV-1 virions after assembly and maturation leave the infected cell by budding off from the cell surface in a cell-membrane derived envelope. Tetherin which is localized on the outside of the cell surface serves as an anchor being juxtaposed in between the mature virion after it has completely pinched off and the infected cell membrane (7). Tetherin thus is attached to the cell membrane at both ends. Virion and cell-associated Tetherin thereby interact preventing the release of mature virion from the surface. If Tetherin connects with the infected cell's endocytic machinery via one of its

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membrane anchors, it consequently results in the reuptake of the mature virions by the infected cell and its subsequent degradation by the cellular digestive system. Vpu counteracts Tetherin in that it reduces expression of BST-1 at the cell surface and removes it from its site for tethering action by utilizing a cellular ubiquitin ligase (102).

1.3.4   Non-coding microRNAs and RNA-silencing

Non-coding RNAs (ncRNAs) that cover >70% of the human genome have been shown recently to play an important role in restricting retroviral replication (142). Different forms of non-coding RNA (ncRNA), such as microRNA (miRNA), small interfering RNA (siRNA), piwi-interacting RNA (piRNA), are regulated differently. Amongst this varied group, the most intensely studied is miRNA and currently there are more than 800 miRNAs listed in the human miRNA database (16, 70). RNA silencing or RNA interference activity is mediated by the short 21-23 nucleotide miRNA forming a RNA-induced silencing complex (RISC) with an Argonaute protein and additional RNA-binding cofactors like TAR RNA-binding protein (TRBP) (50, 93). This active miRNA-RISC complex makes limited and imperfect base pairing with the 3' UTR of target mRNA and effectively silences it. However miRNAs show redundancy in which a single miRNA through imperfect base pairing can recognize and silence upto 100 different target mRNAs (44). These siRNAs, piRNAS and Dicer processed miRNAs can effectively suppress mammalian endogenous retroviruses in somatic, germ and embryonic stem cells. Bioinformatics analyses have revealed a myriad of human miRNAs that can target many different types of viruses. Additionally recent studies have demonstrated the capacity of human cells to process HIV-1 RNAs into ncRNAs that in conjunction with other cellular RNAi pathways in human T-lymphocytes and macrophages can effectively obstruct HIV-1 replication (17, 55).

1.3.5 ADAR1 (Adenosine deaminase acting on RNA)

ADAR1 catalyzes the conversion of adenosine (A) to inosine (I) in double-stranded RNA by hydrolytic deamination of purine C-6 (Figure 6) which is recognized as guanosine (G) in the DNA. This protein was first discovered in *Xenopus levis* eggs and early embryos as a double-stranded RNA unwinding enzyme (8, 21, 114). During the unwinding reaction, the adenosine residues in RNA are converted into inosine and the I-U mismatch destabilizes the RNA, causing the unwinding of the RNA strands (141).
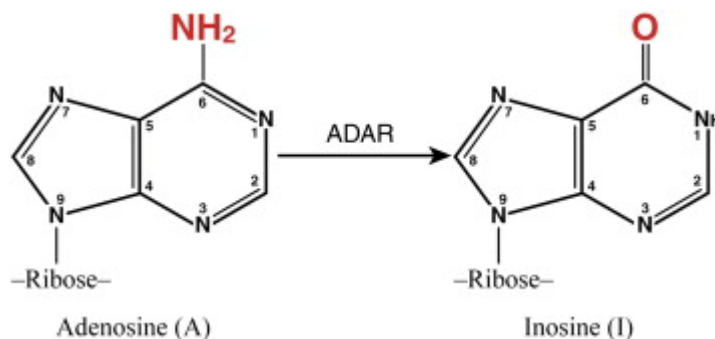
Figure 6: A-to-I editing by ADARs.

Adenosine Deaminases Acting on RNA (ADARs) catalyze the hydrolytic C-6 deamination of adenosine (A) to yield inosine (I) in RNA with double-stranded character.

Figure taken from George *et al*, Journal of Interferon and cytokine research, Volume 31, Number 1, 2011

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Among the wide implications of its hyper-editing capacity the most generic is a direct consequence on the process of mRNA translation. The purine ionosine behaves as a guanosine and base pairs with uridine instead of adenosine. Hence, I in a mRNA codon is read as G by ribosomes during translation, leading to genetic recoding (138)(Figure 7). This event occurs in a highly site-specific manner and leads to amino acid substitution and, ultimately, varied protein

products with potentially altered activity. This A-to-I editing also has a consequence on the RNA structure due to the formation of a less stable I:U wobble base pair from a more stable Watson and Crick A:U base pair (133). This A to I editing generates mutation in RNA viruses that undergo RNA-dependent RNA replication. If ADAR causes another adenosine deamination editing event in the template RNA, a complementary change occurs in the product strand after RNA replication and its secondary structure may be altered (139). Finally A-to-I editing also makes these RNA susceptible to degradation. A ribonuclease was identified that specifically targeted hyper-edited duplex RNAs and efficiently cleaved regions of multiple I:U base pairs.
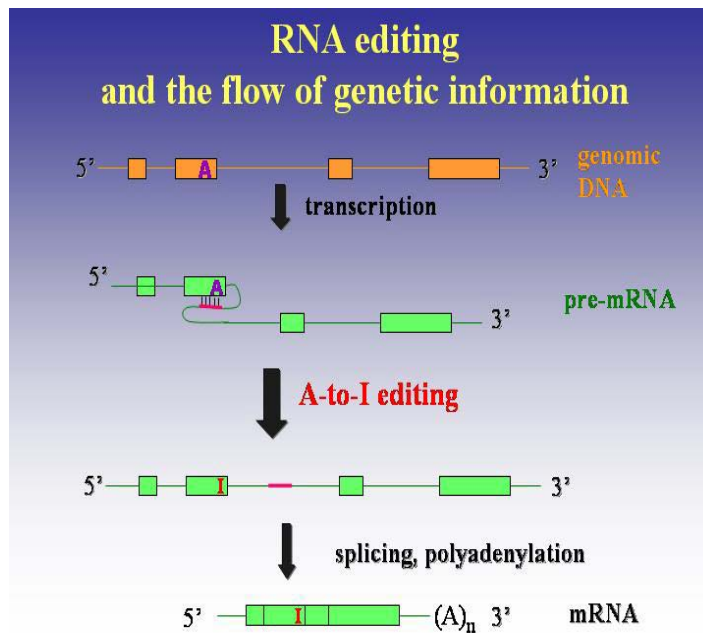
Figure 7: A-to-I editing by ADAR1 and the flow of genetic information.

Figure from Maas Research Group, Department of Biological Sciences, Lehigh University
http://www.lehigh.edu/~swm3/research.html

The first site-specific editing activity of ADAR1 in pre-mRNA was observed in the vertebrate glutamate-receptor subunit, in which the glutamine codon was converted into an arginine codon (76, 107, 125). There are three ADAR enzymes in mammals; ADAR1, ADAR2 and ADAR3. ADAR1 contains three double-stranded RNA binding motifs, followed by a highly conserved C-terminal catalytic domain and a zinc finger binding domain (106). Two major variants of human ADAR1 exist due to different promoter usage and are found in distinct intracellular locations. The full length protein, p150, is expressed from an interferon (IFN) inducible promoter and localizes in the cytoplasm, whereas the N-terminal truncation variant is p110, localized in the nucleus (106).

The catalytic domain of ADAR1 is located at the C-terminal region. Three copies of the double-stranded RNA (dsRNA) binding motif are present in the center of ADAR, designated dsRBMI, dsRBMII, and dsRBMIII, which are highly conserved amongst each other as well as to the dsRBM originally identified in the IFN-inducible PKR (57, 69). In addition to binding single-stranded RNA (ssRNA), ADAR1 also binds Z-DNA (left handed double helical DNA) via its Z-DNA binding motif within its N-terminal region (77, 82, 84). ADAR1 deaminase activity or dsRNA-binding activity is not dependent on its Z-DNA binding activity and conversely the Z-DNA binding activity of ADAR1 is also not dependent on either of them (81, 106).mRNAs can be edited by adenosine deamination to produce an A-to-I substitution in the mRNA template and produce a different transcript. This process has been termed hyper-editing. I is recognized as G and not A by human RNA polymerase and ribosomes and, hence, this type of RNA modification can alter the protein-coding capacity of the edited transcript as well as the sequence of RNAs (8, 141). ADAR1 is implicated in two different kinds of RNA editing depending on the double-stranded regions within substrate RNA (68). The first is the A to I modifications present at

multiple sites in viral RNAs. Viral RNA genomes undergo biased hypermutations during the lytic part of virus life cycles and persistent infections. The, hyper-editing activity is seen in different RNA viruses like measles virus, human parainfluenza virus, respiratory syncytial virus, influenza virus, lymphocytic choriomeningitis virus, Rift Valley fever virus, mumps virus and hepatitis C virus and mouse polyoma virus (73). The second type of ADAR1 editing is the site specific C-6 adenosine deamination catalyzed by ADAR1 as observed in hepatitis delta virus (HDV) RNA and the GluR receptor channel and serotonin pre-mRNAs (125).

1.4   ROLE OF ADAR1 IN MODULATING VIRUSES

Viruses that contain dsRNA or secondary structures in their RNA may be affected by nonspecific adenosine deamination. In 1991, Sharmeen, *et. al.* demonstrated that ADAR1 destabilized the secondary stem loop structure by causing an A-to-I mutation at the +27 position of the TAR region in HIV-1 LTR (126). However, the effect of the mutation on HIV-1 replication is still unknown (11, 126). Editing of A-to-I in pre-mRNA of HDV changes the open reading frame in an individual codon that eventually affects splicing of untranslated regions. Conversion from A-to-I changes a stop codon (UAG) to a tryptophan codon (UIG), resulting in the production of a longer protein, which helps in viral genome packaging(122). ADAR1 is also known to edit dsRNA found in the measles virus. During measles virus replication, RNA editing occurs in the viral matrix protein mRNA, where approximately 50% of the adenosine was converted into guanosine in the viral negative strand RNA (19, 20, 63). The editing inhibited virus assembly and release from the cell, leading to a persistent infection and eventually development of fatal neuropathetic measles infection. Recently, Taylor, *et. al.* showed that upon interferon treatment,

ADAR1 expression was induced, allowing RNA editing and subsequent hepatitis C virus inhibition in replicon cells A-to-G hypermutation was also found in the in parainfluenza virus type 3(100), vesicular stomatis virus (105), Borna disease virus (38), avian leukosis virus (53), and polyomavirus (73) in cell culture systems.

1.5   ROLE OF INTERFERON IN INNATE IMMUNITY

The innate immune system recognizes and responds to pathogens, eventually modulating the immune response. Viral pathogens are recognized mainly by three different pattern recognition receptors: toll like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like helicases (RLHs), and nucleotide-oligomerization domain (NOD)-like receptors (NLRs) (92, 97). Following recognition of foreign molecules through pattern recognition receptors, activation of downstream signaling molecules eventually leads to type I interferon production and an antiviral response. Type I IFNs belong to a family of cytokines that are critical activators of the cellular immune response against viral infection. However, interferons also modulate the immune response through the amplification of antigen presentation to T cells (37). Virus-infected cells secrete IFN-α and IFN-β, while T cells, natural killer (NK) cells and macrophages secrete IFN-γ (4).

IFNs promote expression of antiviral and immune modulatory genes through interactions with cellular receptors. Studies have shown that HIV-1 replication can be restricted by treating infected T cells and monocytes with IFN-α , -β and –γ (58). IFNs can also restrict both early and late stages of the HIV-1 lifecycle through the inhibition of HIV-1 replication in acute and chronically infected cells (1, 3, 24, 54). There are several interferon-inducible proteins that act as

antiviral products, such as dsRNA-activated protein kinase (PKR′), 2 -5′ oligoadenylate synthetase, dsRNA-dependent p68(human)/p65(murine) protein kinase, RNase L and Mx protein (65, 121). These proteins play important antiviral roles at different stages in virus life cycles. These antiviral proteins have been shown to inhibit viral mRNA transcription and translation and degrade viral mRNA. IFN-induced PKR inhibits the translation of mRNA of encephalomyocarditis virus (EMC) (94) and vaccinia virus (78). 2′-5′ oligoadenylate synthetase activates RNase L which degrades RNA of viruses of the Picornaviridae family (21). Recently, studies have shown that a cellular gene, called TRIM22, is up-regulated upon IFN treatment and inhibits budding of the HIV-1 virus particle (5). Other members of the TRIM family, such as TRIM19 and TRIM32, are thought to negatively affect HIV-1 infection by inhibiting trafficking of viral proteins (103).

2.0   SPECIFIC AIMS

The effect of cellular enzymes on the restriction of HIV-1 replication has been well studied. Previously, it was shown that the cellular enzyme APOBEC3G restricts HIV-1 replication in the absence of *vif* by triggering deamination of dC to dU in the first (minus) strand cDNA. ADAR1 is an RNA editing enzyme which acts on dsRNA. The editing of HIV-1 viral RNA has several consequences like introduction of early stop codons, changes in the important regulatory secondary structures of RNA, and changes in the stability and translation of mRNA. Since HIV-1 RNA has secondary structures in its genome, HIV-1 might be a potential target for ADAR1. Our objective was to determine the anti-viral activity of ADAR1 against HIV-1. **My hypothesis for this project is that ADAR1 restricts HIV replication and infectivity by editing viral RNA.** This hypothesis was addressed by the specific aims listed below.

1. To determine the role of ADAR1 in restricting HIV replication and virion production. The goal of this aim was to examine the ability of ADAR1 to restrict replication of different HIV clades and production of infectious virion.

2. To elucidate the mechanism by which ADAR1 restricts HIV-1 infection. The goal of this aim was to investigate the ADAR1 induced mutation in the HIV-1 RNA and the consequences of the A-to-G mutations in HIV-1 replication and infectivity.

3. To evaluate the domains of ADAR1 critical for the inhibitory effect during HIV-1 replication. The goal of this aim was to determine the domains responsible for inhibition of HIV-1 replication and infectivity.

3.0 ROLE OF ADAR1 IN HIV REPLICATION AND IN INFECTIOUS VIRION PRODUCTION

This chapter is adapted from a manuscript submitted to *Virology* (Nabanita Biswas [1], Tianyi Wang[1], Ming Ding[1], Ashwin Tumne[1], Yue Chen[1], Qingde Wang[2], Phalguni Gupta[1]).

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This fulfills Aim 1 and part of Aim 2 of this dissertation.

All these experiments in this chapter were performed by Nabanita Biswas except the Real-time PCR. Real-time PCR was done by Ming Ding.

3.1   ABSTRACT

ADAR1, an RNA unwinding enzyme, catalyzes hydrolytic deamination of adenosine to inosine in complete or partially ds RNA, which are common intermediate products found during viral infections. Results from a few published reports suggest that ADAR1 potentially regulates hepatitis C virus (HCV) and influenza A virus infections. Since ADAR1 is an RNA editing enzyme and HIV-1 RNA has secondary structures in its genome, HIV-1 might be a potential target for ADAR1. Our results indicated that ADAR1 inhibited viral replication and infectious HIV-1 production in various cell lines, including 293T, HeLa and Jurkat T cells, and was active against a number of X4- and R5-tropic strains of different clades. Analysis of the level of intracellular HIV-1 RNA showed no change in levels of intracellular *gag, pol,* and *env* RNA in the presence of ADAR1 despite a significant inhibition of production of intracellular and virion-associated HIV-1 proteins. These results show that ADAR1 inhibits HIV-1 at the stage of post-transcriptional modification of viral protein synthesis. Therefore, ADAR1 could be a novel antiviral factor. Evaluating the RNA editing effects of ADAR1 during HIV infection and understanding the mechanism(s) by which ADAR1 inhibits viral production may provide further insight into the host cell-pathogen interaction and reveal an important host antiviral factor.

3.2   INTRODUCTION

Viruses have developed a number of strategies to circumvent the host's immune response to infections. ADAR1, an RNA editing enzyme, catalyzes hydrolytic deamination of adenosine to inosine in completely or partially dsRNA (64, 68, 104, 106). Editing of adenosine to inosine in pre-mRNA of HDV changed the open reading frame in an individual codon, which eventually affected splicing of untranslated regions, and viral replication (106, 112, 122). ADAR1 is also known to edit double-stranded RNA found in the measles virus, which inhibited virus assembly and release from cells, leading to a persistent infection and development of fatal neuropathetic measles infection (63). Taylor, *et al.* (2005) showed that ADAR1-induced viral RNA editing inhibited Hepatitis C viral replication (132).

Since HIV-1 genome has several putative double stranded secondary RNA structures throughout its genome, HIV-1 RNA was considered a potential target for ADAR1. ADAR1 destabilizes the secondary stem loop structure of the TAR region in HIV-1 LTR, although the effect of the mutation on HIV-1 replication was unknown (11, 126). Therefore, we investigated the antiviral effect of ADAR1 on HIV-1 replication. We found that ADAR1 inhibited HIV-1 replication and infectivity in a variety of cells and against HIV-1 of different tropisms and different clades. We further demonstrated that such antiviral activity was at the posttranscriptional stage of HIV-1 replication and that ADAR1-induced mutation at the *rev* and *env* RNA may be responsible for such posttranscriptional inhibition of viral replication. Our data indicate ADAR1 is a novel cellular factor with anti-HIV activity.

3.3 MATERIALS AND METHODS

3.3.1 Cell Lines and DNA constructs

293T (ATCC CRL-11268), TZM-bl (NIH AIDS research and reagent program Catalog number 8129) and HeLa (NIH AIDS research and reagent program Catalog number 153) cell lines were cultured in DMEM (Invitrogen Life Technologies) supplemented with 10% FBS (HyClone), 1% penicillin, and 10 µg/ml streptomycin. The Jurkat (ATCC®TIB-152TM), ACH-2(NIH AIDS Research and Reagent Program, Catalog number 349), J1.1 (NIH AIDS Research and Reagent Program, Catalog number 1340) cell line was grown in RPMI-1640 medium containing 10% FBS, 2 mM l-glutamine, 1% penicillin, and 10 µg/ml streptomycin.

Initial experiments were done using ADAR1 and ADAR1 mutant constructs as described (77). Subsequently a new ADAR1 construct made in pcDNA 3.1 vector was used. Renilla Luciferase (RLuc) plasmid (Promega) was used as a transfection control (90). Infectious molecular clones pNL4-3 plasmid, pNLAd8 and p89.6 were obtained from the NIH AIDS Research and Reagent Program. pIndie C plasmid was obtained from Dr. Masashi Tatsumi (Tokey, Japan)(98).

3.3.2 Tranfection and p24 ELISA

For transfection in 293T cells, media containing plasmids (pNL43/ pNLAd8/ p89.6, ADAR1 or control RLuc DNAs) and Lipofectamine 2000 (2x of the amount of plasmid) were incubated together at room temperature for 15-20 minutes, and added slowly to the wells containing 293T cells. After 4 hours the medium was replaced with fresh medium containing 5% penicillin and

streptomycin (pen strep), 1% non essential amino acids (NEAA), and 10% fetal bovine serum (FBS). HeLa cells were nucleofected with the Nucleofector Kit R (Amaxa), program I-13 when cells reached 70-80% confluency ($0.5\text{-}1\text{x}10^6$ cells). Jurkat cells were also nucleofected with the same kit, using program A-017.

To measure the production of intracellular and extracellular virus particles, HEK293T cells were co-transfected with cocktails comprising pNL4-3 plasmid, different concentrations of ADAR1, pcDNA 3.1 empty vector and 0.1 ug of RLuc plasmid. Virus-containing supernatants were collected at approximately 48 h post-transfection, and virus production was quantified by analyzing p24 production using ELISA (Perklin Elmer p24 ELISA kit). To evaluate the Renilla luciferase activity, cells were centrifuged at 5000 rpm for 10 min and lysed by incubating with 100 µl 1X lysis buffer (Promega Duel luciferase reporter assay kit, E1910) for 30 min. 30 µl cell lysate was mixed with 100µl Stop and Glo buffer and the luciferase activity was measured using Turner BioSystems Veritas™ Microplate Luminometer. To determine the intracellular virus production, 48 hours post-transfection, the cell pellet was washed twice with phosphate buffered saline (PBS), resuspended in PBS containing 0.5% NP-40, and incubated on ice for 10 min. The mixture was centrifuged and supernatant was used for p24 ELISA.

3.3.3   Infectivity Assay

TZM-bl (JC53-bl) cells are a genetically engineered HeLa cell line that expresses CD4, CXCR4 and CCR5 and contains Tat-inducible luciferase and lacZ reporter genes (NIH AIDS Research and Reference Reagent Program, catalog number 8129). Equal amounts of viruses (as determined by p24) obtained from 293T in the absence and presence of ADAR1, were used to infect TZM-bl cells. Twelve hours post-infection, the cells were washed with PBS and replaced

with DMEM media supplemented with 10% FBS and penn strep. At 48 hours after infection, cells were lysed with lysis buffer (Promega) and the productive infection was measured as the induction of luciferase activity using LARII buffer and the color was measured using the Turner BioSystems Veritas™ Microplate Luminometer (29, 111).

3.3.4 Western Blot and Antibody

Cell lysates were prepared by lysing 293T cells with a lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P40, 50 mM NaF, 1 mM Na_3VO_4, 5 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) supplemented with a protease inhibitor mixture (Sigma) on ice. Lysate was clarified by centrifuging at 14,000 x g for 20 min and subjected to Western blot analysis using ADAR1 monoclonal antibody (1:2000 dilution, Santacruz Biotechnology Inc), anti-actin antibody (1:1000 dilution, Sigma) and rabbit anti-gp120 antibody (1:1000 dilution, Advanced Biotechnologies) or HIV-1 infected patient antisera.

3.3.5 RNA extraction, Cloning and Sequencing

To analyze the editing effect in *rev* and *env* regions, 293T cells were transfected with pNL4.3 construct with the ADAR1 construct or pcDNA 3.1 control DNA. RNA was isolated from transfected cells using RNA Bee (TEL-TEST, INC) according to the manufacturer's instructions. The extracted RNA was treated with RNase free DNase (Roche Applied Science) for 30 min followed by heat inactivation and subjected to RT-PCR. The cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen) and random hexamar (IDT) in the presence of RNase inhibitor (Roche Applied Science). The *rev* and *env* PCR was done using primers listed in

Table 1. The PCR product was cloned in pcDNA3.1 Vector (Invitrogen) between the restriction sites KpnI and XhoI for *rev* and between Mlu1 and Xho1 for *env*. A sample of 12-25 clones were picked from each plate and sequenced using ABI PRISM 3730 automated DNA sequencer. For studies on *rev* the sequences of 24 clones were sequenced and found to fall under 4 groups of similar patterns of mutations, whereas for *env* 24 clones were analyzed and found to fall under 11 groups.

3.3.6 HIV-1 RNA Secondary Structure Analysis

Recently, the architecture and secondary structure of HIV-1 RNA was experimentally determined by Watts *et al.* (143) using selective 2 -hydroxyl acylation analysed by primer extension (SHAPE). The publically available Helix base-pairing data (http://www.nature.com/nature/journal/v460/n7256/extref/nature08237-s2.txt) and SHAPE reactivites (http://www.nature.com/nature/journal/v460/n7256/extref/nature08237-s3.txt) for HIV-1 RNA were used to map the specific nucleotide positions of each ADAR1-induced Adenosine-to-Guanosine (A→G) mutation to the proper HIV-1 RNA secondary structure involved. The open-source software XRNA (http://rna.ucsc.edu/rnacenter/xrna/) was used to compose secondary structure images outlining each specific RNA secondary structure for a given subset of ADAR1-induced mutations generated.

3.3.7 Real-time RT-PCR analysis for Quantification of HIV-1 RNA from Cells

A 20ng sample of RNA was applied for reverse transcription using TaqMan® Reverse Transcription Reagents (Applied Biosystems) according to the manufacturer's protocol. A real-

time RT-PCR assay was performed in a 30 µl reaction mixture consisting of 5µl cDNA with TaqMan® Universal PCR Master Mix (AppliedBiosystems), 900nM each of forward and reverse primers and 250nM FAM/TAMRA or FAM/MGB labeled probe. A real-time RT-PCR assay for the endogenous gene was performed using Eukaryotic 18S rRNA Endogenous Control Reagents (Applied Biosystems). ABI Prism 7000 Sequence Detection System was used to carry out the real-time PCR reactions, using the following cycling conditions: 50 ºC for 2 min, 95 ºC for 10 min, 45 cycles of 95 ºC for 15 sec and 60 ºC for 1 min. The primers and probes used for the real-time RT-PCR were based on HIV-1 pNL4-3 sequence encoding the *pol, gag and env* regions respectively (Table 1). A serial dilution of pNL4-3 plasmid DNA ranging from 10 to 10^6 copies was applied to each real-time PCR assay, serving as an HIV-1 standard. An endogenous gene standard containing serial diluted cellular RNA ranging from 10^0 to10^5 pg was included in each endogenous gene test. Both no RT control and no template control were included in each assay to monitor the genomic DNA contamination and PCR cross contamination, respectively. Each sample was run in triplicate. ABS Prism 7000 SDS Software (Applied Biosystems) was used for PCR data analysis and copy number estimation. To rule out sample input and amplification variations, HIV-1 quantity in each sample was normalized by the endogenous control, and the final result was expressed as HIV-1 RNA copies per microgram of total RNA.

Table 1: HIV-1 *rev* and *env* RT-PCR primers.

Region (Accession number m19921)	Primer Sequence
rev	Forward: ttggtaccatggcaggaagaagcggagaca Reverse: tctcgagctattctttagttcctgactccaatactgtaggag
env (1-652)	Forward: ccacgcgtatgagagtgaaggagaagtatcagcact Reverse: cctcgaggggcacaataatgtatgggaattggc
env (651-1300)	Forward: atacgcgtcccggctggttttgcgattcta Reverse: cctcgagcatacattgcttttcctacttcctgcc
env (1301-1950)	Forward: atacgcgtcccctcccatcagtggacaaat Reverse: cctcgaggctggttttgcgattcttca
env (1951-2566)	Forward: ccacgcgtccagcaagaaaagaatgaacaag Reverse: gctcgagcttatagcaaaatcctttccaagccctgtc

Table 2: HIV-1 real-time PCR primers and probes.

Region		Sequence	HXB2 location
pol	Forward	5'-TGG GTT ATG AAC TCC ATC CTG AT-3'	3241→3260
	Reverse	5'-TGT CAT TGA CAG TCC AGC TGT CT-3'	3296←3315
	Probe	5'-FAM-AAT GGA CAG TAC AGC CTA TAG TGC TGC CAG AA-TAMRA-3'	3265→3293
gag	Forward	5'-CCCATGTTTTCAGCATTATCAGAA-3'	1297→1320
	Reverse	5'-CCACTGTGTTTAGCATGGTGTTTAA-3'	1339←1363
	Probe	5'-FAM-AGCCACCCCACAAGA-MGB-3'	1323→1337
env	Forward	5'-TTCATCAAATATTACAGGGCTGCTA-3'	7559→7583
	Reverse	5'-CAGGTCTGAAGATCTCGGACTCA-3'	7613←7635
	Probe	5'-FAM-AGATGGTGGTAATAGCA-MGB-3'	7592→7608

3.4   RESULTS

3.4.1   ADAR1 inhibits HIV-1 replication in 293T cells

In order to evaluate the effect of ADAR1 expression on HIV-1 production, 293T cells were co-transfected with 0.1µg pNL4.3 HIV-1 DNA and different concentrations of ADAR1 DNA. In each transfection assay, cells were also co-transfected with a luciferase-expressing plasmid DNA to control transfection efficiency. The expression of ADAR1 was also measured by Western blot (Figure 8A). β-actin was used as an internal loading control. Following 48 h of transfection, viral replication was quantified by measuring HIV-1 p24 in culture supernatant and intracellular HIV-1 p24 production in a cell extract. ADAR1 expression inhibited extracellular (Figure 8B) and intracellular (Figure 8C) HIV-1 p24 production in a dose dependent manner. With 0.7 µg of ADAR1-containing plasmid, there was a 8-fold reduction of extracellular HIV-1 p24 production in culture supernatant as compared to control plasmid, pcDNA. The inhibition of viral replication was not due to cellular toxicity by ADAR1 expression as observed by no significant change in cell viability in the presence and absence of ADAR1 (data not shown).
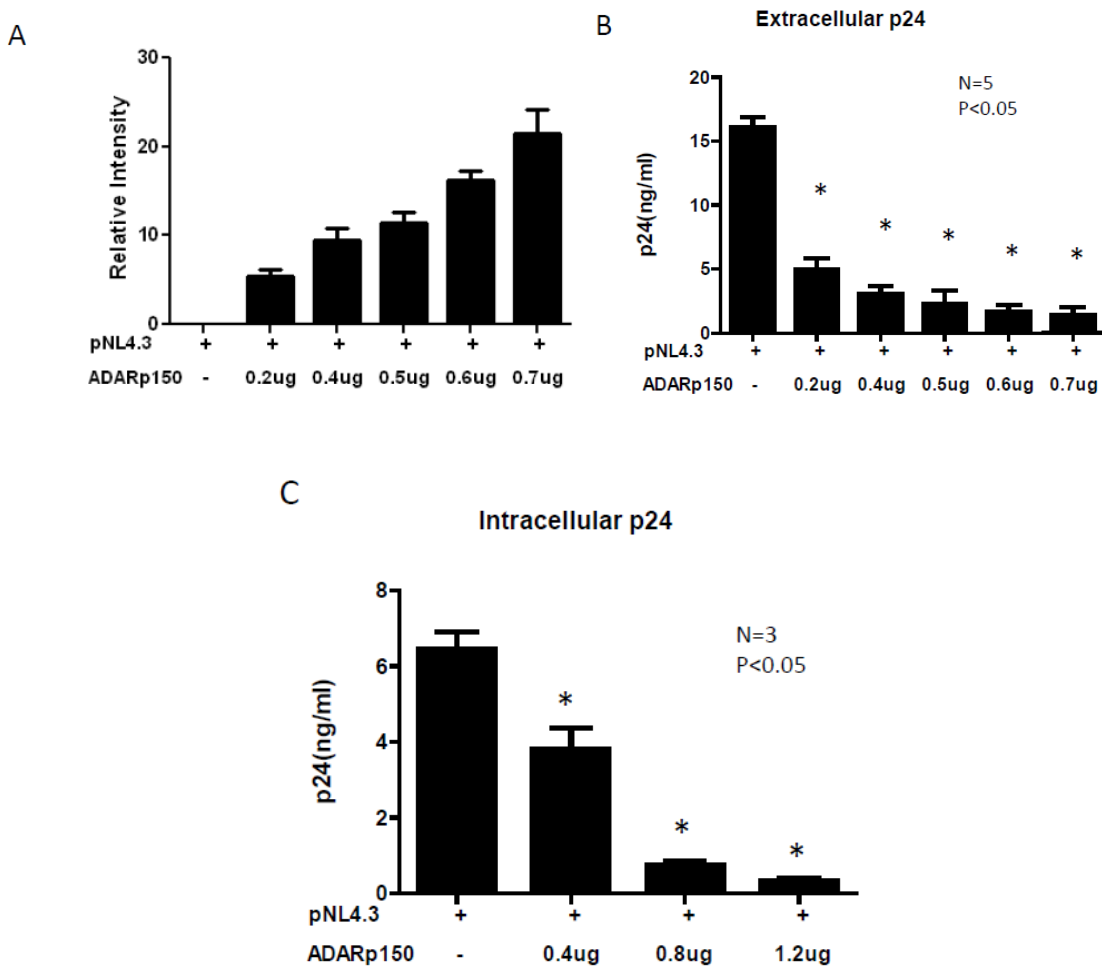
Figure 8: Effect of ADAR1 on HIV-1 replication and infectivity.

293T cells were co-transfected with 0.1ug pNL4.3 plasmid and different amounts of ADAR1 plasmid and the control Renilla Luciferase plasmid. HIV-1 production and infectivity were evaluated after 48 h. The expression of ADAR1 in the transfected cells was determined by Western Blot and normalized against β-actin loading control to show the relative intensity of ADAR1 expression (A). Virus replication was monitored by measuring HIV p24 in culture supernatant (B) and intracellular HIV p24 production (C). Results are given as means ± standard deviation.

3.4.2 ADAR1 inhibits HIV-1 replication in HeLa, Jurkat and CD4 enriched T cells

The antiviral activity of ADAR1 was further evaluated in two other cell lines: HeLa and Jurkat T cells. Inhibition of viral p24 production in culture supernatants was also observed in HeLa (Figure 9A) and Jurkat T cells (Figure 9B). We also examined the effect of ADAR1 expression on HIV-1 replication in primary CD4-enriched T cells, in which viral challenge was done by infection rather than by transfection. For this purpose, PHA-stimulated CD8+ cell-depleted PBMC were nucleofected with 2 µg of plasmid encoding ADAR1. The cells were infected 48 h post-transfection with 0.5 ng/µl, and 1 ng/µl of pNL4.3 virus for 2 h. Virus production in culture supernatant was measured using p24 ELISA 72 h post-infection. As shown in Figure 9C, ADAR1 expression inhibited HIV-1 replication in CD4 enriched T cells at both concentrations of virus.

C

Extracellular

After 72 hrs
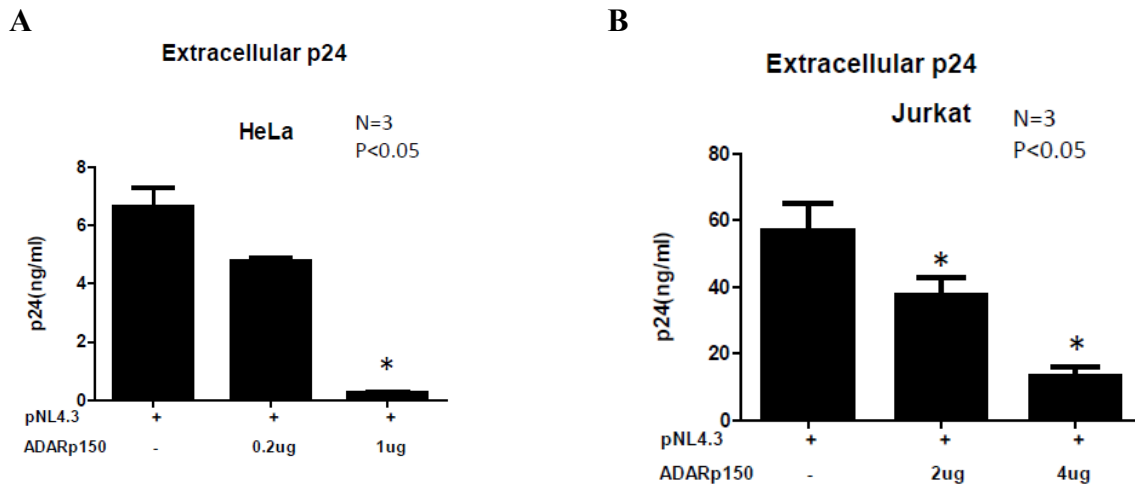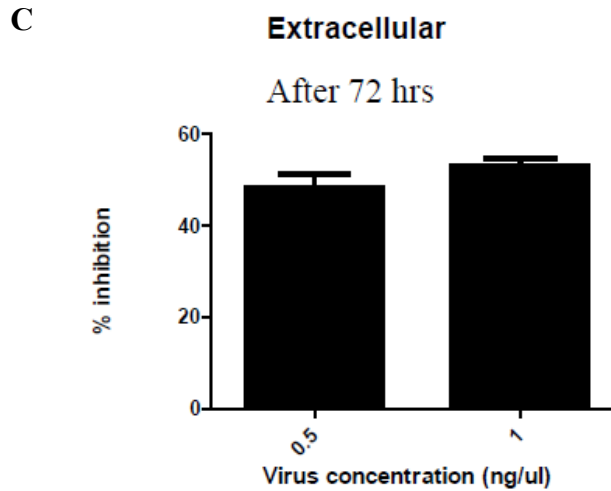
Figure 9: ADAR1 inhibits HIV-1 replication in HeLa, Jurkat and CD4 enriched T cells.

Measurement of extracellular HIV-1 p24 production in transfected HeLa (A) and Jurkat cells (B) in the presence and absence of increasing amounts of ADAR1 in three independent experiments are shown. CD8 depleted PBMC were nucleofected with 2ug of ADAR1. After 48 h cells were infected with 0.5ng/ul and 1ng/ul, of pNL4.3 virus for 2 h. HIV-1 production was evaluated after 72 h using p24 ELISA and calculating the percentage of inhibition of p24 production in the presence of ADAR1 (C).

3.4.3 Knockdown of ADAR1 inhibits ADAR1 mediated HIV-1 replication

To further demonstrate specificity of ADAR1-mediated inhibition of HIV-1 replication, shRNA-mediated silencing of ADAR1 was used to block ADAR1 expression. First, we evaluated the effect of shRNA on the expression of ADAR1. 293T cells were co-transfected with ADAR1 in the presence and absence of two shRNA constructs targeting human ADAR1 p150 (sh1724 and sh228). As shown in Figure 10A, both of the shRNA constructs were able to inhibit expression of ADAR1 p150. As a control, shRNA 526 against an unrelated protein showed no inhibition of human ADAR1 expression. 293T cells were transfected with the pNL4.3 proviral construct and

shRNA against ADAR1 (sh1724 and sh228) or shRNA 526. As shown in Figure 10B, shRNA

against ADAR1 blocked ADAR1-mediated inhibition of HIV-1 replication.

A   B

Figure 10: Knockdown of ADAR1 inhibits ADAR1 mediated HIV-1 replication.

293T cells were transfected with 0.5ug of ADAR1 with 1 µg (1:2 ratio), 2 µg (1:4 ratio), 3 µg (1:6 ratio) of shRNA constructs 1724 and 228 against ADAR1 and 3 µg of shRNA 526 (1:6 ratio), a control shRNA. Western blot was performed using ADAR1 monoclonal antibody to check the expression of ADAR1. β-actin was used as a control (A). 293T cells were transfected with 0.1 µg pNL4.3, 0.5 µg ADAR1 in presence and absence of shRNA 228, shRNA 1724 and 526. Virus replication was monitored by measuring HIV p24 in culture supernatant 48 h post transfection (B).

3.4.4   ADAR1 inhbits replication and infectivity of HIV-1 of different clades and tropism (Clade B and C, R5 and X4 viruses)

Next, the inhibitory effect of ADAR1 expression was examined in 293T cells against HIV-1 that

have different tropisms and of different clades. As shown in Figure 11A, ADAR1 expression

inhibited production of clade C HIV-1 (pIndie C) in culture supernatant, however the antiviral

activity was more pronounced against pNL4.3 (clade B) than pIndie C (clade C). Maximum

inhibition was observed in case of pNL4-3 (28 fold). With the highest concentration of ADAR1 a

3 fold inhibition was observed in case of pIndie C and 8 fold inhibition was observed in case of

AD8. In case of 89.6 construct 5 fold inhibition was observed with highest concentration (0.8ug/ul) of ADAR1. ADAR1 expression also inhibited production of R5-tropic HIV-1$_{AD8}$ and dual-tropic HIV-1$_{89.6}$ (Figure 11A).

The infectivity of the virus from the culture supernatants 293T cells transfected with the ADAR1 plasmid or control plasmid (Figure 8B) were then evaluated for infectivity in TZM-bl cells. A dose-dependent reduction in the viral infectivity in the presence of ADAR1 was observed (Figure 11B), even though equal amounts of virus, as determined by p24 levels, were used.

We then examined the effect of ADAR1 expression on infectivity of HIV-1 produced in culture supernatant from 293T cells transfected with HIV-1 having different tropisms and of different clades (Figures 11A). Equal amounts of viruses, as determined by p24 levels, obtained from 293T cells transfected with the ADAR1 plasmid or the control plasmid were used to infect TZM-bl cells. As shown in Figure 11C, viral infectivity was significantly inhibited in the presence of ADAR1, regardless of viral tropisms and clades. With the highest concentration of ADAR1 a 5 fold inhibition was observed in case of pIndie C and 7 fold inhibition was observed in case of AD8 and pNL4-3. In case of 89.6 construct a 6 fold inhibition was observed with highest concentration (0.8ug/ul) of ADAR1.
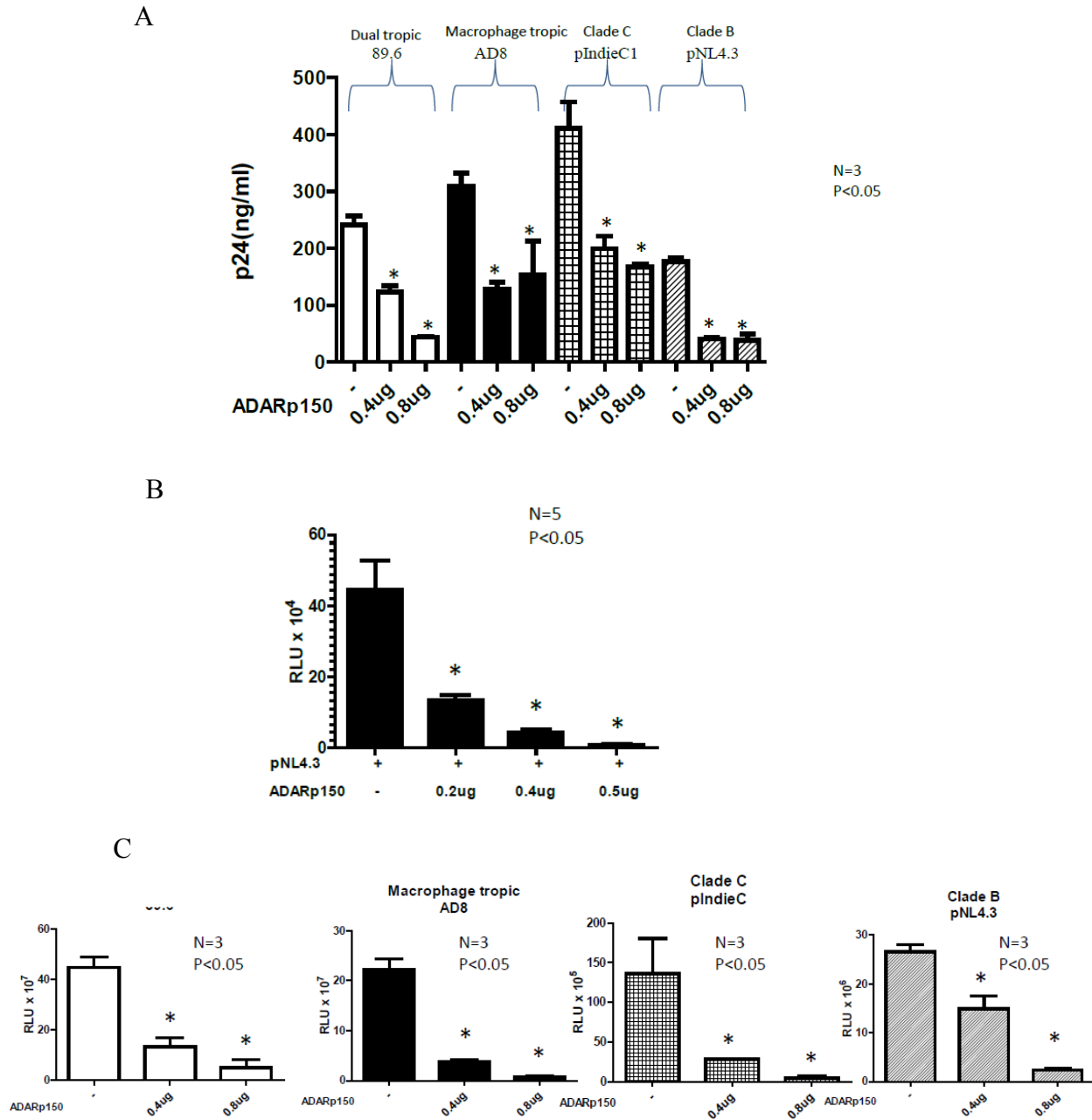
Figure 11: ADAR1 inhbits replication and infectivity of HIV-1 of different clades and tropism (Clade B and C, R5 and X4 viruses).

Production of extracellular HIV-1 p24 in 293T cells co-transfected with HIV-1 89.6, AD8, pIndie C or pNL4.3 plasmids and increasing amount of ADAR1 DNA (A). 3ng HIV-1 p24 equivalent viruses obtained from 293T cells transfected with pNL4.3 alone or together with ADAR1 from Figure 8B were used to infect TZM-bl cell line (B). Productive infection was monitored after 48 h by measurement of luciferase activity. * indicates there is a significant difference (P<0.05) compared to the respective control. Equal amount of viruses (3ng p24 equivalent) (A) were used to infect TZM-bl cell line and productive infection was monitored by measurement of luciferase activity(C).

3.4.5   Expression of ADAR1 in different cell lines

To check the expression of ADAR1 in different cell lines, TZM-bl (modified HeLa cell), ACH-1 (latently HIV infected cell line), Huh7 (human liver cell line) and J1.1 (latently HIV infected cell line) cells were lysed and ADAR1 expression was determined by Western blot analysis. Two major variants of human ADAR1 exist due to different promoter usage and are found in distinct intracellular locations. The full length protein, ADAR1 p150, is expressed from an interferon (IFN) inducible promoter and localizes to the cytoplasm, whereas the N-terminal truncation variant, p110, localizes to the nucleus. It was observed that Huh 7 cells, a liver cell line, express ADAR1 p110 but not p150. TZM-bl cells, a modified HeLa cell line, do not express either ADAR1 p150 or p110. ACH-2 and J1.1 cells, a latently HIV-1-infected cell line, express ADAR1 p110 but not p150 (Figure 12).
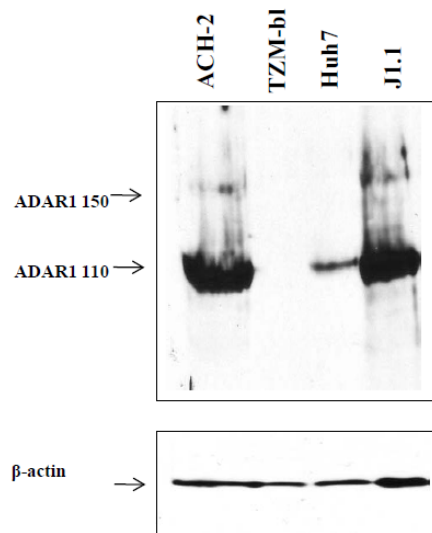
Figure 12: Expression of ADAR1 in different cell lines.

2×10^{6} ACH-2, TZM-Bl, Huh7, J1.1 cells were lysed with RIPA buffer and a Western blot was performed using an ADAR1 antibody to show the expression of endogenous ADAR1. An immunoblot assay was also performed against β-actin to normalize for gel loading.

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3.4.6 Induction of endogenous ADAR1 expression in Jurkat cells following IFNα treatment and its antiviral activity

Because ADAR1 is an IFNα-inducible enzyme, the expression of ADAR1 p150 is expected to increase in the presence of IFNα treatment. Jurkat cells were stimulated with different concentrations of IFNα (100units/ml, 500units/ml and 1000 units/ml) for various lengths of time (12 h, 24 h, 48 h and 72 h). At the indicated time after interferon treatment, cells were lysed and a Western blot was performed using a monoclonal antibody against ADAR1 to evaluate the level of IFNα-induced expression of endogenous ADAR1. Intracellular ADAR1 p150 expression was observed after 24 h of IFNα stimulation with 100, 500 and 1000 units/ml of IFNα and it increased over time. In addition, ADAR1 p150 expression was higher after 72 h with increasing amounts of IFNα (Figure 13A). As ADAR1 p150 is an IFNα inducing enzyme the expression of p150 forms was increased with increasing amount of IFNα. However ADAR1 p110 showed no change in expression level as it is not an IFNα inducible enzyme rather it is expressed constitutively in the cell.

To check whether ADAR1 expression induced by IFNα could inhibit the replication of HIV-1 virus, cells were incubated with 500 units/ml or 1000 units/ml IFNα. After 72 h, cells were infected with pNL4.3 virus. In presence of 500 units/ml and 1000 units/ml of IFNα, approximately 25% and 40% reduction in the replication of virus was observed, respectively, as compared to the untreated cells (Figure 13B). To verify that the inhibition of HIV-1 replication was due to INFα-induced expression of ADAR1, cells were treated with 500 units/ml and 1000 units/ml IFNα for 72 h. After 72 hours, cells were transfected with shRNA against ADAR1 (sh 228) and shRNA against an unrelated RNA (sh 526). As shown in Figure 13C, IFNα-induced

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inhibition of viral replication was at least partially reversed in the presence of shRNA against ADAR1, but not in the presence of shRNA against an unrelated RNA. These results indicate that the inhibition of HIV-1 replication in presence of IFNα was due to expression of endogenous ADAR1 in Jurkat cells (Figure 13C).
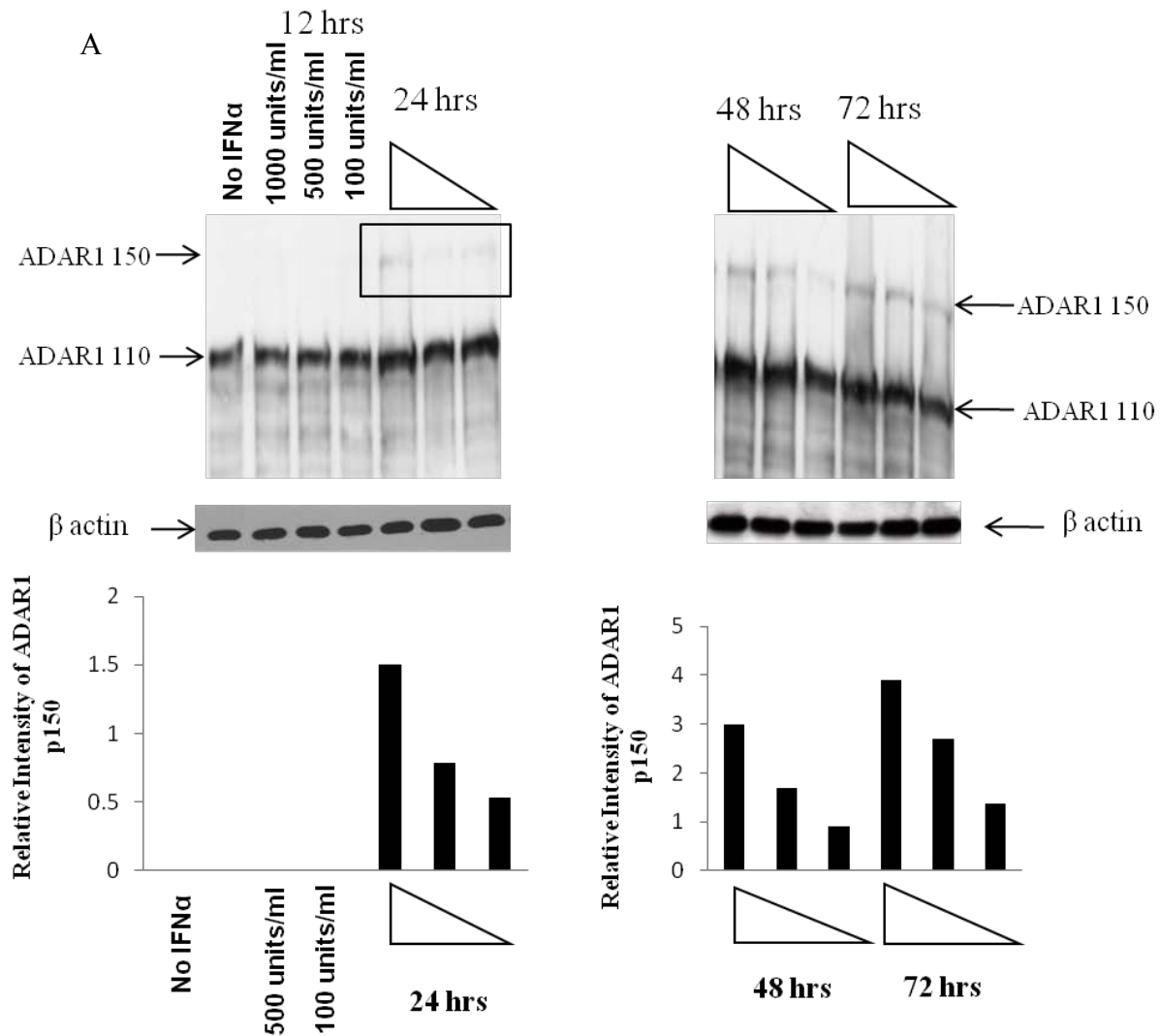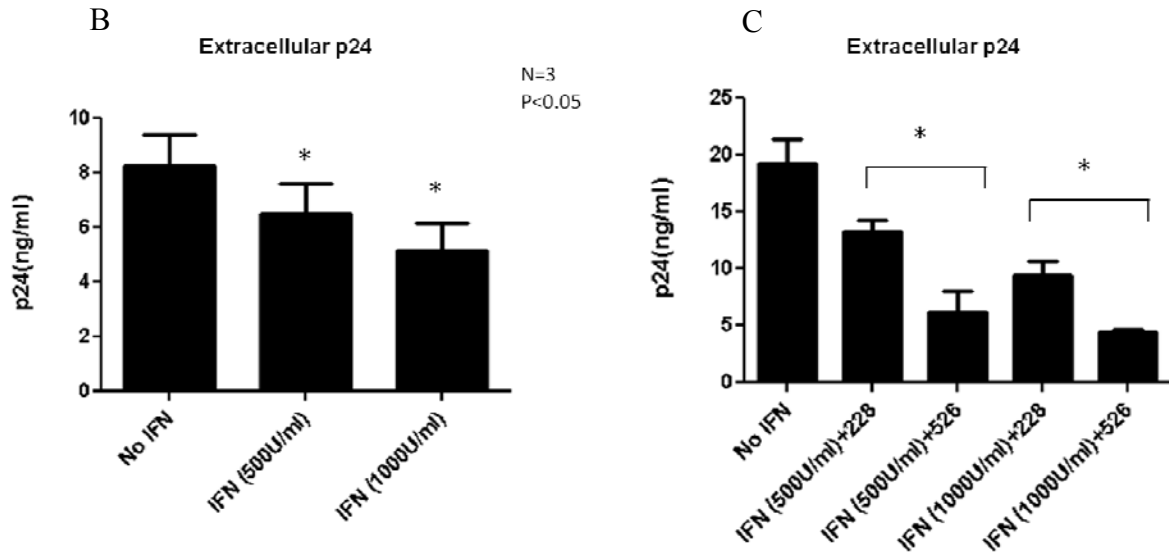
Figure 13: Expression of endogenous ADAR1 in Jurkat T cells and its inhibitory capacity on HIV-1 replication.

Jurkat T cells were stimulated with 100units/ml, 500units/ml and 1000 units/ml IFNα for different lengths of time: 12 h, 24 h, 48 h and 72 h. At each time point, cells were harvested and expression of ADAR1 was determined by Western blot using an ADAR1 antibody and normalized against β-actin, which was used as a loading control (A). Jurkat cells were stimulated with IFNα for 72 h and then infected with 500 ng/ml of pNL4.3 virus. After 48 h, virus replication was monitored by measuring the HIV-1 p24 production in the culture supernatant (B). Jurkat cells were stimulated with IFNα for 72 h. After 72 h, cells were transfected with 2μg of shRNA 228 or shRNA 556 constructs. After 48 h, cells were infected with 500 ng/ml of virus. Virus replication was monitored 48 h after infection by measuring the p24 production in the culture supernatant (C).

3.4.7   Effect of ADAR1 on HIV-1 production and infectivity with time

293T cells were co-transfected with pNL4.3 plasmid (0.1μg/μl), different concentrations of ADAR1p150 (1.2μg/μl), pcDNA 3.1 empty vector, and 0.1 μg of RLuc plasmid. Cell supernatants were collected after 72 h, 96 h and 120 h. Virus production was quantified by measuring p24 production. Equal amounts of viruses (10 ng of p24) were obtained from 293T in the presence and absence of ADAR1, were used to infect TZM-bl cells, and productive infection was measured by luciferase activity. Inhibition of HIV-1 replication in presence of ADAR1 was

observed even after 120 hours of transfection (Figure 14A). Viral production was inhibited 18, 11 and 2.4 fold after 72, 96, 120 h respectively compared to the control. Virus infectivity was inhibited 2.5 fold after 72 h and 120h but 1.5 fold inhibition was observed after 96h (Figure 14B).
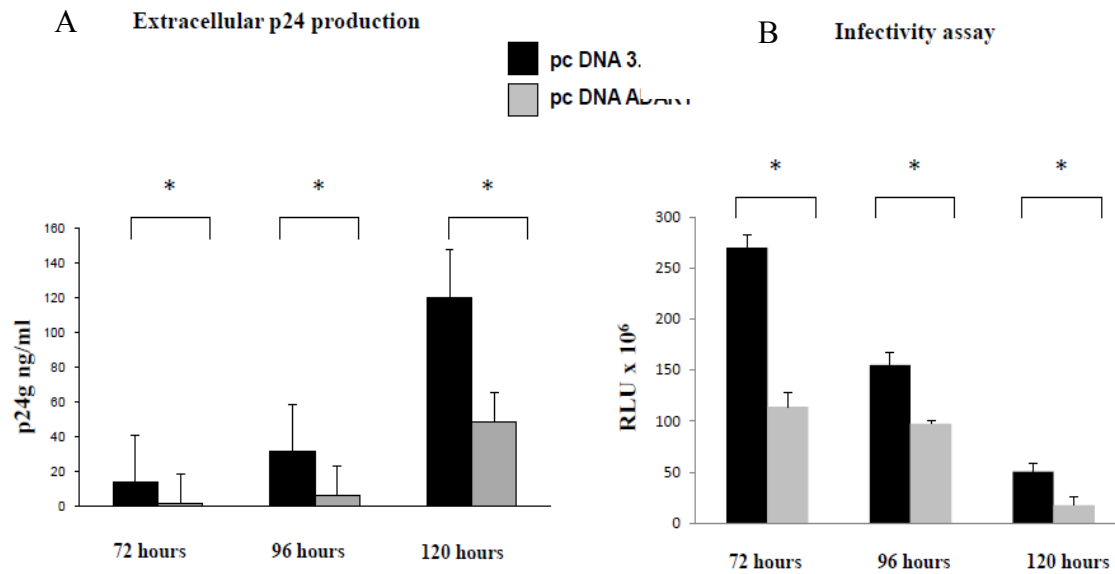
Figure 14: The effect of ADAR1 on HIV-1 production and infectivity at various time points after transfection.

293T cells were co-transfected with cocktails comprising pNL 4.3 plasmid (0.1ug/ul), different concentrations of ADAR1p150 (1.2ug/ul), pcDNA 3.1 empty vector and 0.1 ug of RLuc plasmid. Cell supernatants were collected after 72 h, 96 h and 120 h.Virus production was measured by p24 assay (A). Virus infectivity was measured by TZM-bl assay (B). * indicates there is a significant difference (P<0.05) compared to the respective control.

3.4.8   ADAR1 inhibits HIV-1 viral protein synthesis

Because expression of ADAR1 inhibited extracellular and intracellular p24 production, we investigated the effect of ADAR1 on production of other HIV-1 proteins. Western blots were performed using a monoclonal antibody against gp120 and a polyclonal serum from a single HIV-infected patient to determine the expression of p24, Gag-Pol and gp120 proteins from culture supernatants as well as lysates from cells transfected with or without ADAR1 plasmid.

As shown in Figure 15A, the expression of HIV-1 p24, Env gp41, p65 Pol, and Env gp160 proteins in the presence of ADAR1 expression was significantly reduced in culture supernatants as compared to supernatants of cells not expressing ADAR1. The reduction of Env protein in virions in the presence of ADAR1 expression was further confirmed by Western blot analysis using monoclonal antibody against Env gp120 (Figure 15B). In the presence of ADAR1, there was a 9-fold decrease in the expression of virion associated HIV-1 gp-120 protein in culture supernatant (Figure 15B). In addition, Western blot analysis of cell lysates using a polyclonal serum from an HIV-1-infected patient showed that the levels of p24 and p65 protein were significantly reduced in the presence of ADAR1 (Figure 15C). Similarly, as shown in Figure 15D, a 7-fold decrease in the expression of intracellular gp120 was observed in the presence of ADAR1 compared to the control DNA. However, ADAR1 showed no effect on the synthesis of Nef (Figure 15E). These results indicated that ADAR1 inhibited expression of intracellular and extracellular HIV-1 structural proteins Gag, Pol and Env proteins.
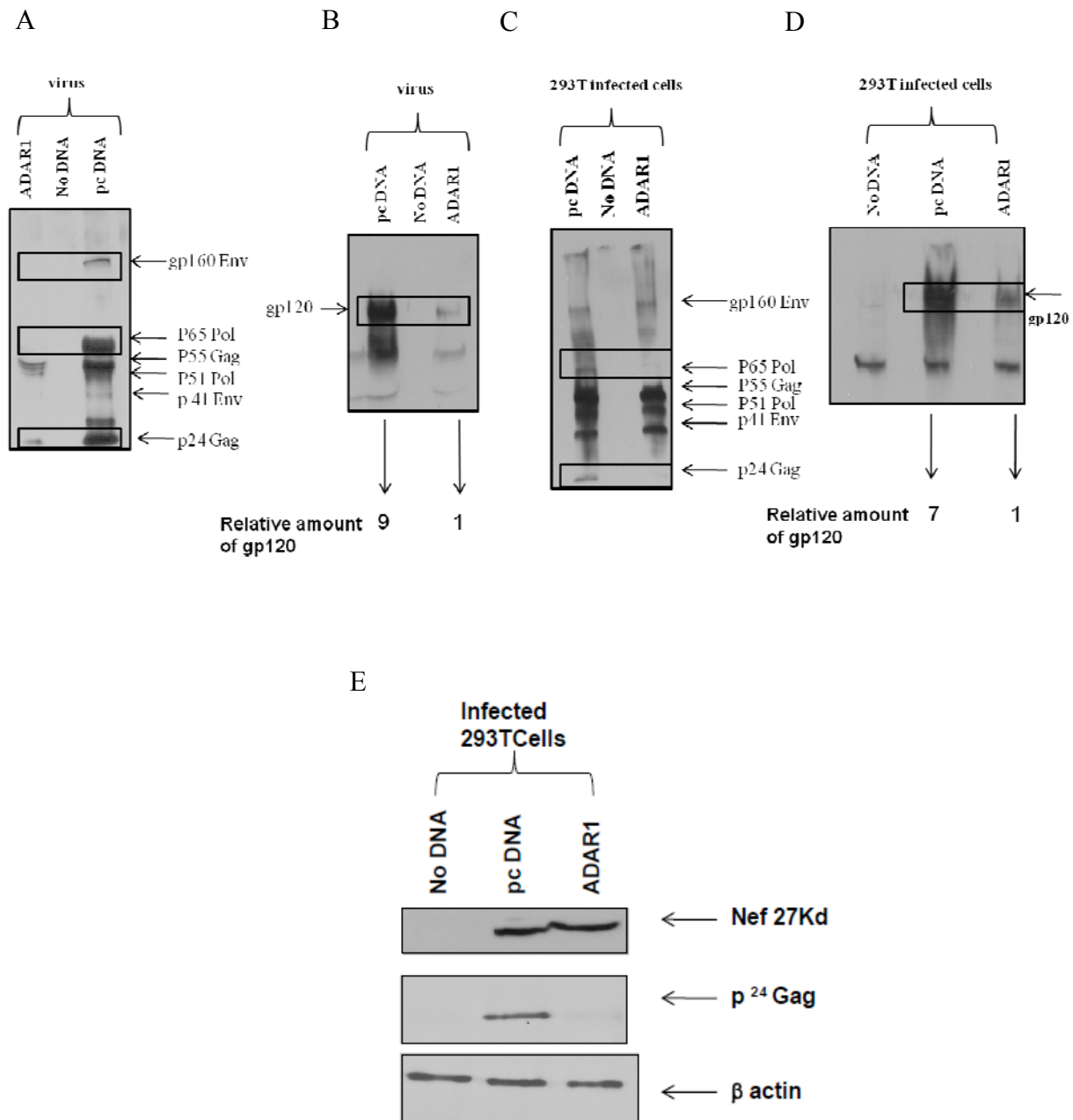
Figure 15: HIV-1 viral protein synthesis in the presence of ADAR1.

293T cells were co-transfected with pNL4.3 plasmid, and ADAR1 or control empty vector (pcDNA 3.1). After 48 h culture supernatant was subjected to Western blot analysis. Western blot analysis of cultural supernatant was done using HIV-1 infected patient's antisera (A) and gp120 polyclonal antibody (Panel B). The numbers below Figure B shows the relative amount of gp120 in the supernatant by the band density. Western Blot was also performed using the cell lysate from the same experiment using HIV-1 infected patient's antisera as an antibody (C) and gp120 polyclonal antibody (D). Number below panel B shows the relative amount of gp120 in the infected cells. Expression of Nef protein in presence and absence of ADAR1 in cell lysate was analyzed by Western Blot using anti-Nef antibody (E).

3.4.9   ADAR1 does not inhibit HIV-1 RNA synthesis

In order to determine whether the inhibition of HIV-1 protein synthesis is due to inhibition of viral RNA synthesis, we quantitated the level of *gag, pol* and *env* RNA by real-time RT-PCR. Cellular RNA was isolated from cells transfected with ADAR1 or control plasmid in the presence of pNL4.3 plasmid and subjected to real-time RT-PCR, as described in the Materials and Methods section. The level of *gag*, *pol* and *env* RNA was not diminished in the presence of ADAR1 expression as compared to cells not expressing ADAR1 (Figure 16A), whereas the production of the HIV-1 p24 protein was significantly reduced in the presence of ADAR1 expression (Figure 16B). These results led us to conclude that the ADAR1-mediated antiviral effect was at a post-transcriptional step in the HIV-1 life cycle.

A
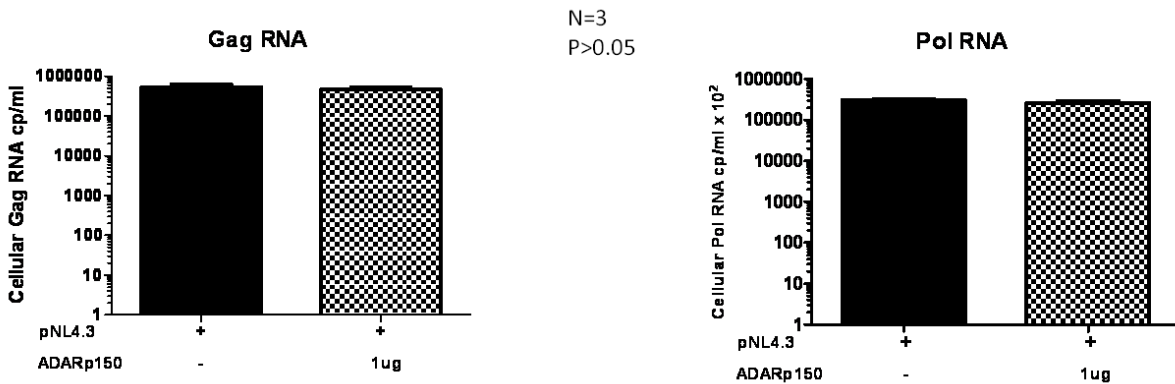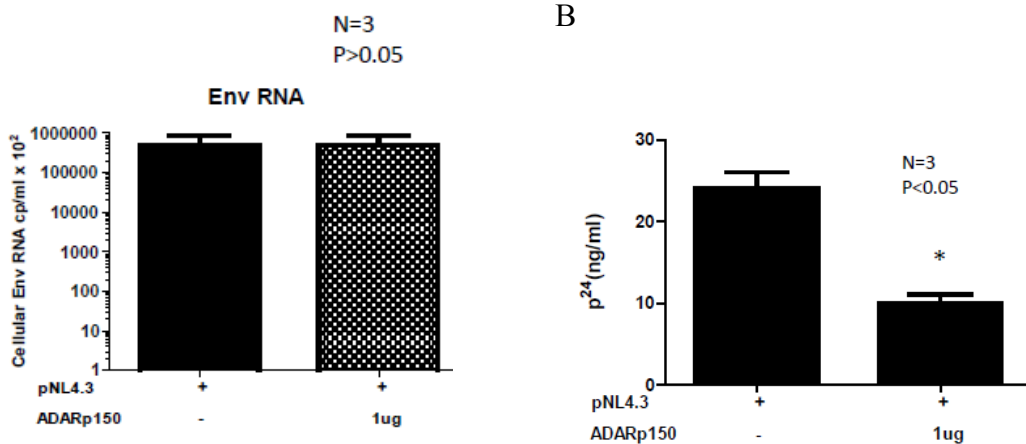
Figure 16: Effect of ADAR1 on HIV-1 RNA synthesis.

Viral *gag*, *pol* and *env* RNA was quantitated in cells transfected with pNL4.3 in the presence and absence of ADAR1 DNA by real-time RT-PCR (A). Production of HIV-1 p24 protein by ELISA in presence and absence of ADAR1 from the same cell culture supernatants used in A (B).

3.4.10 ADAR1 induces mutation in HIV-1 *rev* and *env* RNA

HIV-1 *rev* RNA is involved in post-transcriptional modification of HIV-1 proteins (36, 51, 86, 87, 89, 116). Furthermore, Env and Vif expression are required for infectivity of virus (15, 30, 128, 129). Therefore, we analyzed *rev, env* and *vif* RNA for A-to-G mutations, a hallmark of the editing function of ADAR1. For this purpose, 293T cells were transfected with the pNL4.3 construct in the presence and absence of ADAR1 expression. After 48 h post-transfection, cellular RNA was isolated and was amplified by RT-PCR using gene specific primer pairs. PCR products were cloned in the pcDNA3.1 vector and sequenced. As shown in Figure 17A, specific A-to-G mutations were noted in the *rev* sequences. These mutations were specifically observed in the first exon of *rev* at positions 5998, 6011, 6017, 6036, and the N-terminus of the RRE

binding sites (positions 8413 and 8438 of *rev*). These mutations caused significant amino acid changes, such as arginine to glycine and glutamine to arginine in the RRE binding site (Figure 17B). No A-to-G mutations were observed in the LTR, *vif,* and *tat* regions of the HIV-1 genome (data not shown).

We then investigated whether there was an association between the level of ADAR1-mediated inhibition of replication and the frequency of ADAR1-induced mutations in *rev* RNA. Cells transfected with a lower amount of ADAR1 plasmid (0.2μg/μl) resulted in a lower level of inhibition of viral replication (36%) and a lower frequency of clones with A-to-G mutations in the *rev* gene (30%). In contrast, cells transfected with a higher amount of ADAR1 plasmid (1μg/ul) led to a higher level of inhibition of viral replication (80%) and to a higher frequency of clones with A-to-G mutations in the *rev* gene (56%) (Figure 17C). Since in presence of ADAR1 virus infectivity was diminished and there was inhibition of viral Env synthesis, we also examined the editing activity of ADAR1 on *env* RNA in 293T cells. Since the HIV-1 *env* gene is 2564bp in length, we analyzed the *env* sequences in 4 segments: Segment 1(6221 – 6873), Segment 2 (6872-7521), Segment 3 (7522-8187), and Segment 4 (8188-8803). Specific A-to-G mutations were located at 29 different regions in *env* (Figure 18). No A-G mutations were observed in the absence of ADAR1. It is noteworthy that a number of these mutations were observed in the RRE, a region needed for binding to Rev for its function, and in the variable regions 2 and 4 (V2 and V4).
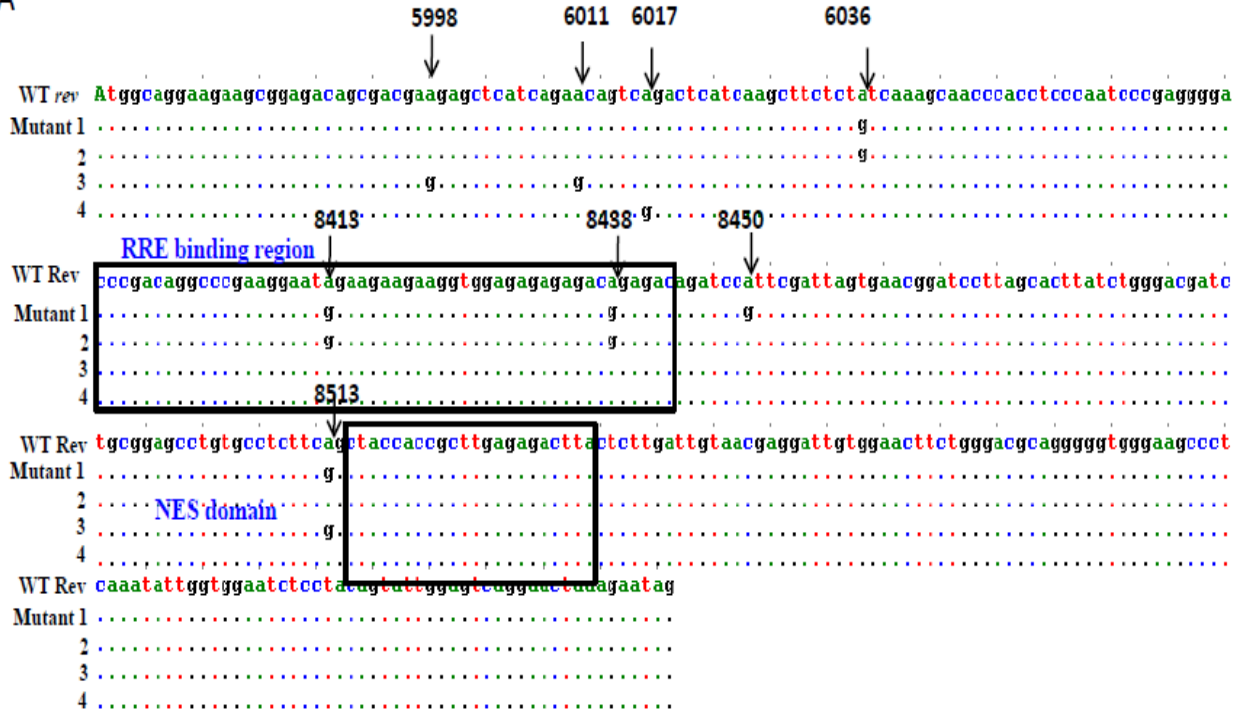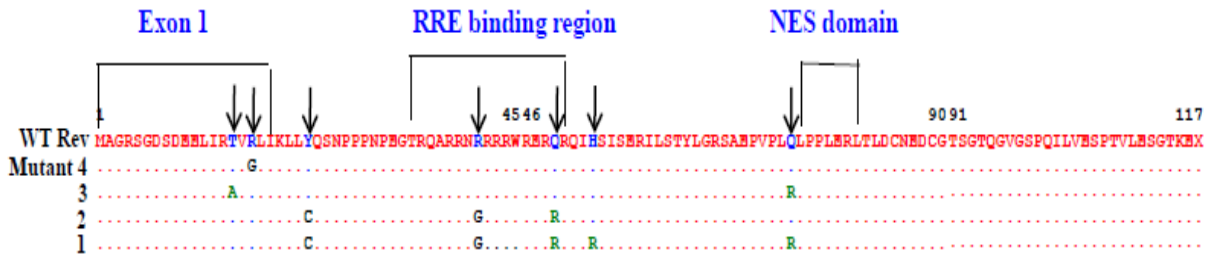
C

% inhibition of HIV-1 production	% clone with A-G mutation in rev
36 (0.5ug ADAR1)	30
80 (1 ug ADAR1)	56

Figure 17: ADAR1 induced mutations in HIV-1 *rev* RNA.

293T cells were transfected with pNL4.3 and the ADAR1 plasmid DNAs. The RNA isolated from transfected cells were subjected to RT-PCR amplification. Amplicons were cloned in pcDNA3.1 and 12-25 clones were picked (in presence and absence of ADAR1) and sequenced. Mutational analysis of the nucleotide sequence of *rev* region of HIV-1 genome (A). Comparison of amino acid sequences of HIV-1 *rev* region in presence and absence of ADAR1 (B). Representation of an association between the levels of ADAR1 mediated inhibition of replication and frequency of ADAR1 induced mutation in *rev* RNA (C).
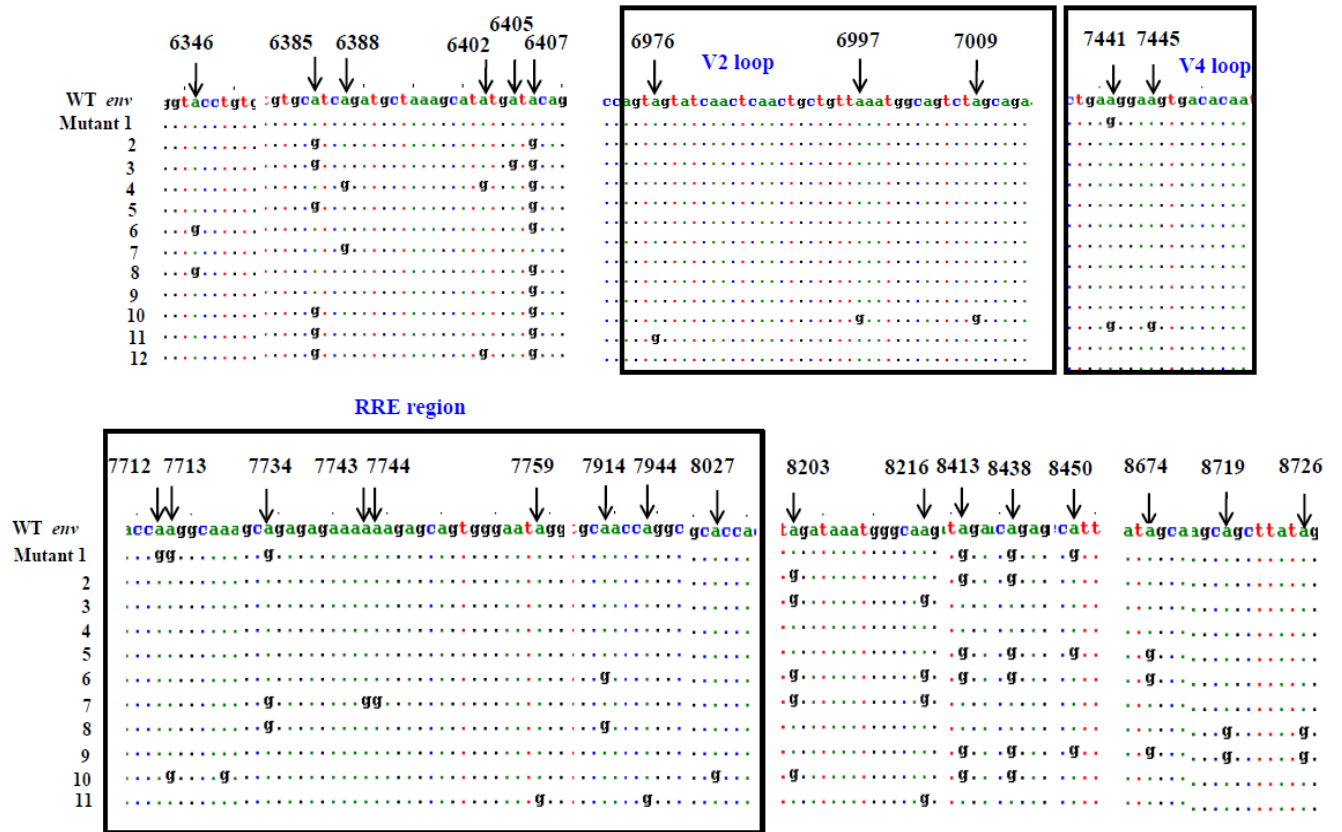
Figure 18: ADAR induced mutations in HIV-1 Envelope.

Mutational analysis of the *env* region of HIV-1 genome in the presence and absence of ADAR1. 293T cells were transfected with pc DNA or ADAR1 constructs and RNA was isolated from infected cells. RT-PCR was done and c DNA of *env* was cloned in pc DNA vector. Each segment of the *env* gene was cloned individually (Segment 1(6221 – 6873), Segment 2 (6872-7521), Segment 3 (7522-8187), and Segment 4(8188-8803). 24 clones were randomly picked from each segment in presence and absence of ADAR1 and sequenced. In case of *env* 24 sequences were computed into 11 groups.

3.4.11 HIV-1 RNA Secondary Structure Analysis

We were interested in determining whether the A-to-G transitions induced by ADAR1 in *rev* and *env* RNA occurred in a specific manner. We hypothesized that RNA secondary structure might play some role in ADAR1 selectivity of certain adenosine residues over others. In analyzing the recently elucidated HIV-1 RNA secondary structure (143), the ADAR1-induced A-to-G transitions observed in *rev* and *env* mapped to eight specific mRNA secondary structures (Figures 19 and 20). These included specific RNA secondary structures within exons 1 and 2 of *rev* (Figures 19A and 19B, respectively), the RRE (Figure 19A), and additional RNA stem-loop structures located in various stretches of the *env* open reading frame (Figures 19C-D and 20B-D). The specific ribonucleotide positions of these ADAR1-induced A-to-G edits indicate a preference of the enzyme for unpaired adenine residues contained within single stranded loops or unpaired bulges (Figure 19E), corroborating previous findings by Doria, *et al*. (32). Additionally, a substantial amount of ADAR1-induced mutations were located at adenine residues immediately adjacent to ssRNA or mispaired bases. Furthermore, no A-to-G transitions were found more than 4 ribonucleotides from a single-stranded region, suggesting steric accessibility might contribute to the selectivity of ADAR1 action on HIV-1 RNA. However, all ADAR1 A-to-G mutations occurred near dsRNA helices. No A-to-G transitions were observed in the numerous stretches of ssRNA within the *env* ORF (143), suggesting dsRNA motifs play some role in site selection.

55

E.

■ Unpaired Adenosine Residue
■ 1 nucleotides from a mispairing
■ 2 nucleotides from a mispairing
■ 3 nucleotides from a mispairing
■ 4 nucleotides from a mispairing
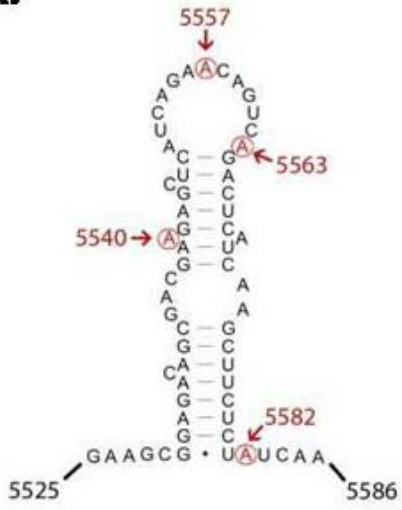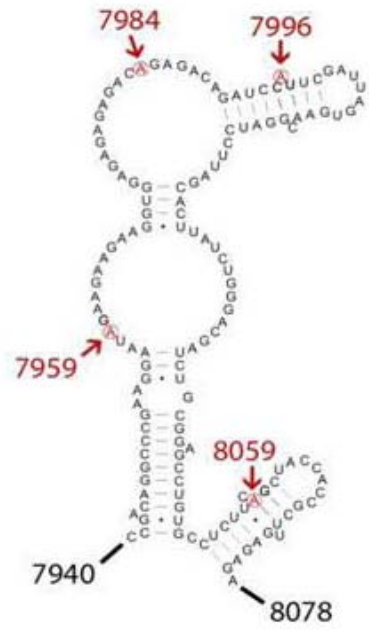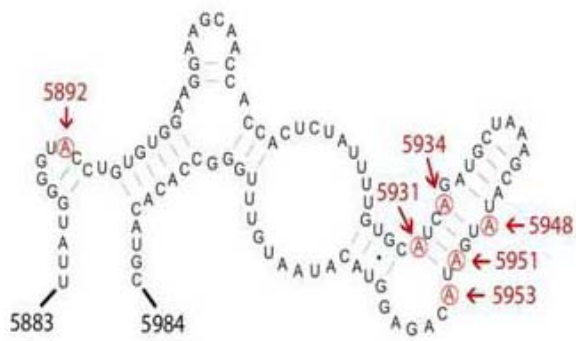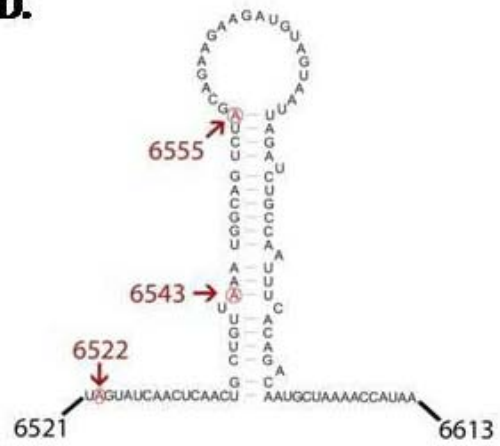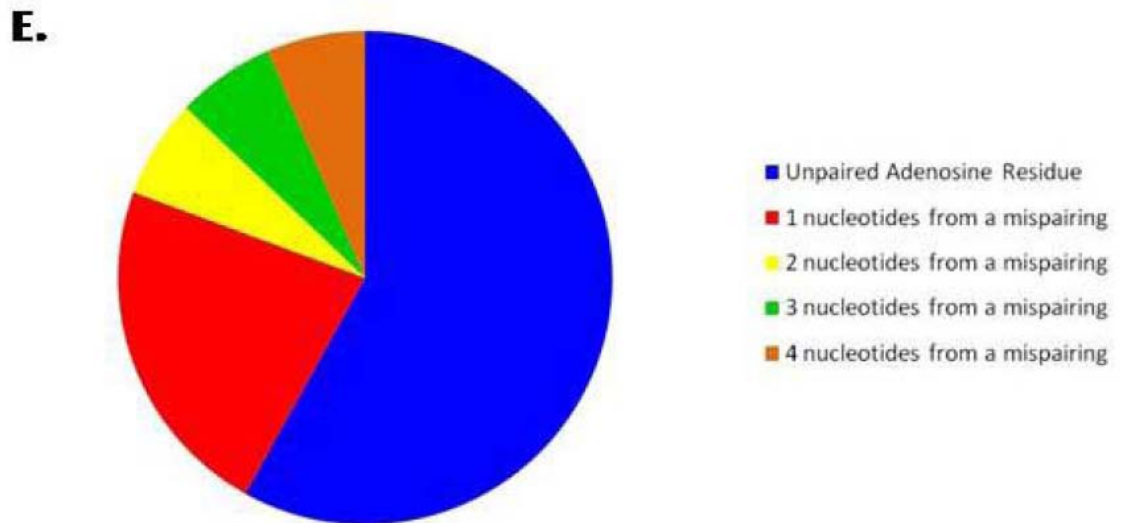
Figure 19: Sites of ADAR1-induced A-to-G transitions within the secondary structure of HIV-1 mRNA for some of the mutations found in *rev/tat* and *env* open reading frames.

Positions of A-to-G transitions are shown for (A) exon 1 and (B) exon 2 of the HIV-1 *rev* gene. Positions of A-to-G transitions for the 5' end of HIV-1 *env* ORF are shown in (C) and (D). The specific base-pairings were derived from the experimentally determined secondary structure for full length HIV-1 mRNA, as outlined in Materials and Methods. Red circles represent the position of Adenosine residues targeted by ADAR1, with arrows indicating position number of the A-TO-G transition sites. Panel E. Proportion of A-to-G transitions occurring at unpaired adenosine residues compared to those appearing within duplex RNA that were 1, 2, 3, or 4 nucleotides from unpaired/mispaired RNA residues (E).
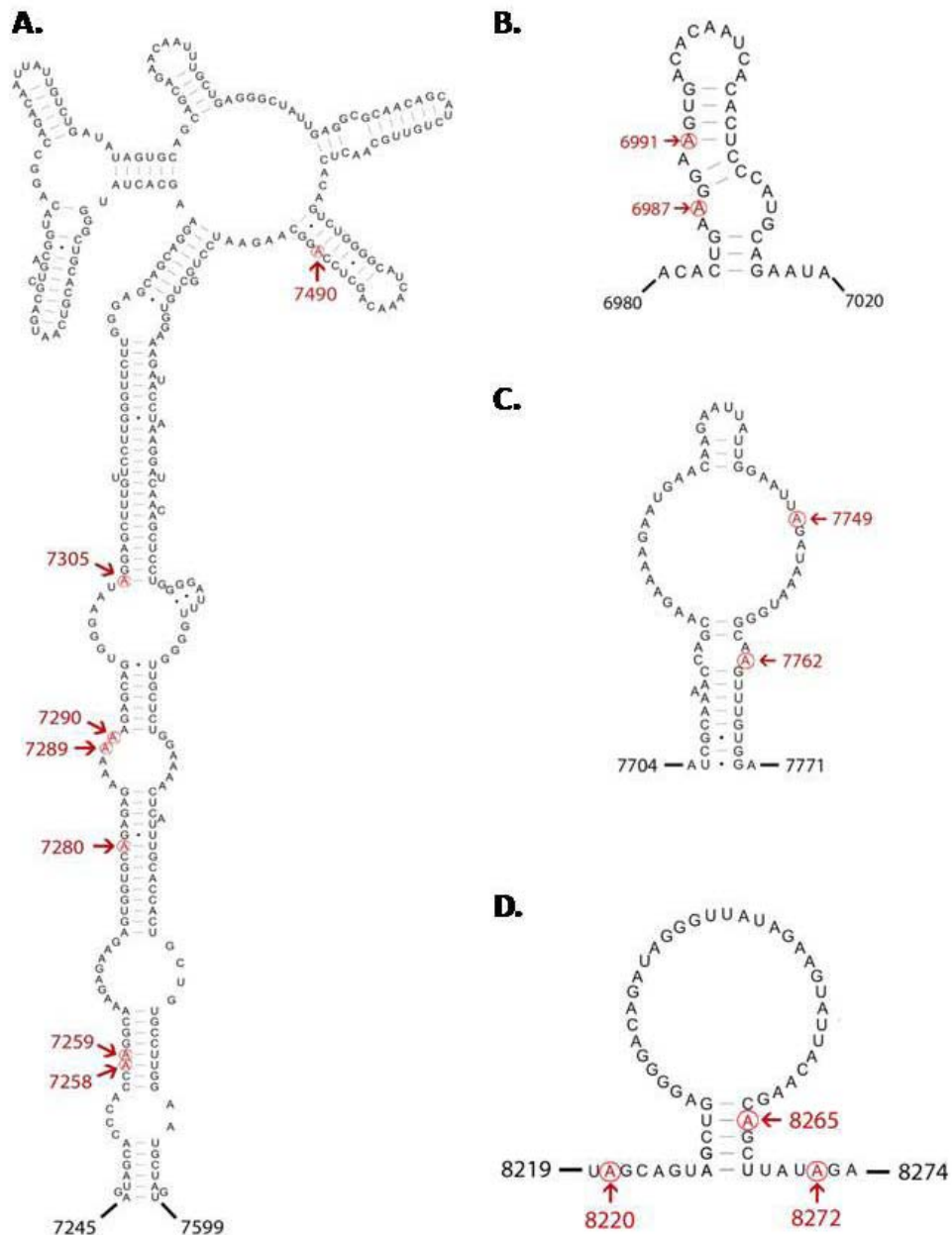
Figure 20: Further sites of ADAR1-induced A→G transitions within the secondary structure of HIV-1 mRNA within the secondary structure of HIV-1 mRNA for mutations found in the remaining segments of the *env* open reading frame.

Positions of A→G transitions are shown for (A) the *rev* response element (RRE) as well as remaining segments of *env* which were sequenced for the set of mutant clones (B-D). Red circles represent position of adenosine residues targeted by ADAR1, with arrows indicating position number of the A→G transition sites. The nucleotide positions shown are in reference to the HIV-1 transcription start site for the unspliced HIV-1 genomic transcript (gene bank accession number m19921). The specific base-pairings were derived from the experimentally verified secondary structure for full length HIV-1 mRNA as outlined in Materials and Methods.

3.5   DISCUSSION

In this study, we provide evidence for the inhibitory effect of ADAR1 on HIV-1 replication and infectivity. Our data show that ADAR1 restricts HIV-1 irrespective of tropism or clade. ADAR1-induced HIV-1 restriction was observed in different cell lines: 293T, HeLa and Jurkat. Such inhibition of viral replication was observed with as little as 0.2 μg of ADAR1 plasmid expression in 293T cells. Our results indicate that ADAR1-mediated restriction of viral replication occurs at a post-transcriptional step of viral replication. Furthermore, we observed that ADAR1 induced A-to-G mutations in HIV-1 *rev* and *env* RNA and such mutations were associated with inhibition of virus replication and production of infectious HIV-1.Since ADAR1 inhibits HIV-1 replication, presumably due to mutations in *rev* RNA, it is worth considering how such mutations could perturb viral replication. It is known that fully spliced HIV-1 mRNA encode the viral regulatory proteins Tat, Rev and accessory protein Nef, whereas partially spliced (4kb) or unspliced (9kb) mRNA encode the viral structural proteins Gag, Pol and Env (49, 113). Rev is involved in regulating synthesis of the structural proteins by facilitating transport of their mRNA from nucleus to cytoplasm (36, 87). HIV-1 Rev protein carries two functional domains: the N-terminal domain contains an arginine-rich sequence that serves as its own nuclear localization signal (NLS), and an RNA-binding domain that is required for binding to the RRE present in all unspliced viral transcripts of structural proteins (13, 85, 88). The C-terminus of Rev protein also carries a nuclear export signal (NES) (61, 85, 144). The combination of an NLS and an NES appears to enable Rev to actively shuttle between the nucleus and cytoplasm (87, 95). In 1994, the Malim group showed that mutations in the NES domain (LQLPPLERLTLD), specifically mutations in the leucine residues, prevent transport of the unspliced viral RNA from the nucleus to the cytoplasm (87, 95). In 1990, the Parslow group

showed that mutations in the first exon of *rev* can inhibit nuclear localization and transactivation (91). In our study, 3 significant amino acid substitutions due to A-to-G mutations in exon 1 of *rev* were observed. ADAR1 induced mutations were also observed in the RRE binding domain of *rev*, which also contains a NLS. Therefore it is highly possible that the ADAR1-induced mutations in the NLS/RRE binding region and in the first exon of *rev* cumulatively affect shuttling of Rev protein between nucleus and cytoplasm and thereby affect transport of singly and multiply spliced 4kb and 9kb mRNA from nucleus to cytoplasm. In fact Malim, *et al.* and Emerman *et al.* showed that mutations in the RRE and NES regions cause accumulation of unspliced HIV-1 mRNA in the cell nucleus that encodes viral structural proteins (34, 87). In addition to mutations in *rev* RNA, we have also demonstrated ADAR1 mutations in the RRE region of *env* RNA that probably further attenuated synthesis of Env due to its inability to bind to the RRE of Rev. This supports our observation that ADAR1 inhibits synthesis of structural proteins without inhibiting synthesis of their mRNAs. In addition to mutations in the RRE region of *env* RNA, ADAR1 also induced mutations in the V2 and V4 coding regions of *env* (72). These mutations may have resulted in production of non-infectious virions due to defective Env in virions. This is supported by the observation that virions produced in the presence of ADAR1 had significantly less infectivity.

In summary, our data demonstrate that ADAR1 is a restriction cellular factor that inhibits HIV-1 replication and probably infectivity by editing the viral *rev* and *env* genes. Evaluating the RNA editing effects of ADAR1 during HIV infection and understanding its mechanism(s) may provide further insight into the host cell-pathogen interaction and reveal an important mediator of antiretroviral innate immunity.

4.0 CHAPTER TWO. MECHANISM OF ADAR1 MEDIATED HIV-1 INHIBITION: FUNCTUNAL CONSEQUENCES OF HIV-1 REV AND ENV RNA MUTATIONS

4.1 PREFACE

This chapter is adapted from a manuscript submitted to *Virology* (Nabanita Biswas [1], Tianyi Wang[1], Ming Ding[1], Ashwin Tumne[1], Yue Chen[1], Qingde Wang[2], Phalguni Gupta[1])

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This fulfills Aims 2 and 3 of this dissertation.

All these experiments in this chapter were performed by Nabanita Biswas except the Real-time PCR. Real-time PCR was done by Ming Ding.

4.2   ABSTRACT

ADAR1 catalyzes the conversion of adenosine to inosine in dsRNA by hydrolytic deamination of the purine at the C-6 position. Our previous results indicated that ADAR1 inhibits HIV-1 replication and infectivity at a post-transcriptional level. Furthermore mutational analysis showed that ADAR1 introduced most of the A-to-G mutations in the first exon of *rev* at positions 5998, 6011, 6017, 6036, RRE (positions 8413 and 8438) of *rev* RNA, and *env*. Here, we observed that some A-to-G mutations in *rev* had significantly negative effect on the expression of Rev, while other A-to-G mutations in *rev* did not have any effect on its expression. However, all mutations could be complemented by wild type Rev. A-to-G mutations in the RRE of *rev* also inhibited the binding of *rev* to the RRE in the *env* and inhibited the transport of primary transcripts like *gag, pol* and *env* from nucleus to cytoplasm. Site- directed mutagenesis studies showed that A-to-G mutations at positions 6036, 8413 and 8438 in *rev* of an infectious proviral clone could completely abolish the replication capacity of HIV-1. Together these data show that ADAR1-induced A-to-G mutations in *rev* are responsible for its inhibitory effect on the replication of virus.

4.3   INTRODUCTION

ADAR1 is an RNA editing enzyme which converts adenosine (A) residues to inosine (I) specifically on double-stranded RNA or RNA molecules which contain secondary structures in their genome (120). This has a number of effects. The A-to-I substitution destabilizes the RNA structures. The A-to-G mutation affects pre mRNA splicing by changing the splicing site and it also decreases the binding of RNA to protein thus leading to functional changes in the protein (138). During mRNA translation I is recognized as G and therefore it changes one codon resulting in changes in the amino acid sequence. Previously it has been shown that ADAR1 introduces A-G mutation in HCV replicon cell line resulting in inhibition of HCV replication upon IFNα treatment (132).

HIV-1 Rev protein is a 116- amino acid regulatory protein which regulator of viral RNA transport and splicing. Rev gets expressed from the fully spliced 2kb RNA and it acts at the post transcriptional level. Rev exports partially spliced (4kb) and unspliced (9kb) mRNA from cytoplasm to nucleus (87).

Previously we have shown that ADAR1 introduces A-to-G mutations in *rev* and *env* region and inhibits HIV-1 replication and infectivity. Here we determined the mechanism by which ADAR1 inhibits HIV-1 replication and infectivity. We found that some A-to-G mutations introduced by ADAR1 decreased the expression of Rev protein and inhibited its binding to the RRE binding region. Further experiments showed that such mutations in the *rev* region inhibited the transport of primary transcripts *gag, pol* and *env* from the nucleus to cytoplasm. Site directed mutagenesis in the proviral DNA confirmed that A-to-G mutations in Rev are responsible for the inhibition of HIV-1 replication.

4.4 MATERIALS AND METHODS

4.4.1 Cell Lines and DNA constructs:

293T (ATCC CRL-11268), TZM-bl (NIH AIDS research and reagent program Catalog number 8129) cell lines were cultured in DMEM (Invitrogen Life Technologies) supplemented with 10% FBS (HyClone), 1% penicillin, and 10μg/ml streptomycin.

Initial experiments were done using ADAR1 and ADAR1 mutant constructs as described (77). Subsequently a new ADAR1 construct made in the pcDNA 3.1 vector was used. The sequence of the ADAR1 was confirmed by sequencing and found to be identical to that described originally by Nishilura group (77). ADAR1 ΔC is a deletion mutant construct where the catalytic domain (nucleotide 839bp to 1222bp of ADAR1) is deleted. In case of ΔM ADAR1, the double stranded RNA binding domain (from nucleotide 1689bp to 2376bp of ADAR1) was deleted. In the E-to-A mutant construct, a single point mutation in the catalytic domain at the position 917, from glutamic acid to alanine was made which abolishes the catalytic activity of ADAR1 and was used for the experiment. Renilla Luciferase (RLuc) plasmid (Promega) was used as a transfection control (90). pRev(-) construct was obtained from NIH AIDS Research and Reagent Program (catalog number 2086). pDM-128 and control plasmids were obtained from Dr. Thomas Hope (Hope et al., 1990)

4.4.2 Tranfection and P24 ELISA

For transfection in 293T cells, media containing plasmids (*rev* mutant constructs or control RLuc DNAs) and Lipofectamine 2000 (2x of the amount of plasmid) were incubated together at room

temperature for 15-20 min, and added slowly to the wells containing 1×10^6 million 293T cells. After 4 h the media was replaced with fresh media containing 10% fetal bovine serum. Virus-containing supernatants were collected ~48 h post-transfection, and virus production was quantified by analyzing p24 production using ELISA (Perklin Elmer p24 ELISA kit). To evaluate the Renilla luciferase activity, cells were centrifuged at 5000 x g for 10 min and lysed by incubating with 100µl 1X lysis buffer (Promega Duel luciferase reporter assay kit, E1910) for 30min. Thirty microliters of cell lysate was mixed with 100µl Stop and Glo buffer and the luciferase activity was measured using Turner BioSystems Veritas™ Microplate Luminometer. To determine the intracellular p24 Gag production, 48 h post-transfection, the cell pellet was washed with PBS twice, resuspended in PBS containing 0.5% NP-40 and was incubated in ice for 10min. The mixture was centrifuged and supernatant used for p24 ELISA.

4.4.3 Infectivity Assay:

TZM-bl (JC53-bl) is a genetically engineered HeLa cell line that expresses CD4, CXCR4 and CCR5 and contains Tat-inducible Luc reporter gene(NIH AIDS research and reagent program Catalog number 8129). Equal amounts of viruses (as determined by p24[Gag] ELISA) obtained from 293T cells in the absence or presence of ADAR1, were used to infect TZM-bl cell line. 12 h post-infection, the cells were washed with PBS and replaced with DMEM media supplemented with 10%FBS, Pen Strep. Cells were lysed 48 hours after infection with lysis buffer (Promega) and the productive infection was measured as the induction of luciferase activity using LARII buffer (firefly luciferase) and the color was measured using the Turner BioSystems Veritas™ Microplate Luminometer (29, 111).

4.4.4 Western Blot:

Cell lysates were prepared by lysing 293T cells with a lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P40, 50 mM NaF, 1 mM Na_3VO_4, 5 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) supplemented with a protease inhibitor mixture (Sigma) on ice. Lysates were clarified by centrifuging at 14,000 x g for 20 min and subjected to Western blot analysis using Rev antibody (1:1000 dilutions), anti-actin antibody (Sigma, 1:1000 dilution) and anti Histone H3 antibody (1:500 dilutions).

4.4.5 CAT assay:

293T cells were grown to 90% confluency and were transfected with 0.9 ug pDM-128 (62) construct and different *rev* mutant construct (4ug and 8ug). Cells were lysed, and the amount of CAT protein was measured using a CAT ELISA (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions.

4.4.6 Site directed mutagenesis:

Single nucleotide substitutions (A-to-G) were introduced into *rev* region of pNL4.3 using the Stratagene quick-change site directed mutagenesis kit in pNL4.3 proviral backbone. Single A-to-G mutations were introduced at positions 6036, 8413 and 8438 and double mutations were introduced at position 8413 and 8438. A-to-G mutations were also introduced simultaneously in these three positions. After site-directed mutagenesis the PCR products were digested with

EcoRI and XhoI and re-cloned it in pNL4.3 backbone. Clones were sequence and the correct clones were used for further experiments.

4.4.7 RNA extraction and real-time RT-PCR:

293T cells were co-transfected with 0.1ug of pNL4.3 and 0.5ug pc DNA or 0.5ug ADAR1 plasmid. After 48 hours, cells were washed and the PARIS kit (Ambion) was used to isolate RNA and protein from cytoplasm and nucleus. To check whether there is any contamination of cytoplasmic RNA in nucleus or vice versa, Western blot was performed to check for the presence of cytoplasmic protein in nucleus and the nuclear protein in cytoplasm. β-actin (found only in the cytoplasm) and Histone 3 (found only in the nucleus) served as loading controls and to check for purity of the fractions.

RNA was also isolated from cells and virus pellets using RNA Bee (TEL-TEST, INC) according to the manufacturer's instructions. The extracted RNA was treated with RNase free DNase (Roche applied science) for 30 minutes followed by heat inactivation and subjected to RTPCR. The cDNA was synthesized using SuperscriptII reverse transcriptase (Invitrogen), random hexamar (IDT) and RNase inhibitor (Roche Applied Science). 20ng of RNA was applied for reverse transcription using TaqMan® Reverse Transcription Reagents (Applied Biosystems) according to the manufacture's protocol. A TaqMan® PCR was performed in a 30ul reaction mixture consisting of 5μl cDNA with TaqMan® Universal PCR Master Mix (Applied Biosystems), 900nM each of forward and reverse primers and 250nM FAM/TAMRA or FAM/MGB labeled probe. Real-Time RT-PCR for the endogenous gene was performed using eukaryotic 18S rRNA Endogenous Control Reagents (Applied Biosystems). An ABI Prism 7000 Sequence Detection System was used to carry out Real-Time PCR using the following cycling

condition: 50 ºC for 2 min, 95 ºC for 10 min, 45 cycles of 95 ºC for 15 sec and 60 ºC for 1 min. The primers and probes used for the Real-Time PCR were based on HIV-1 pNL4-3 sequence (gene bank accession number m19921) encoding the *pol, gag and env* regions respectively. (Table 1). A serial dilution of pNL4-3 plasmid DNA ranging from 10 to 10^6 copies was applied to each PCR assay serving as HIV standard curve. An endogenous gene standard curve containing serial diluted cellular RNA ranging from 10^0 to 10^5 pg was included in each endogenous gene test.   Both no RT controls and no template controls were included in each assay to monitor the genomic DNA contamination and guard against PCR cross contamination, respectively.   Each sample was run in triplicate. ABS Prism 7000 SDS Software (Applied Biosystems) was used for PCR data analysis and copy number estimation. To rule out the sample input and amplification variations, HIV quantity in each sample was normalized by the endogenous control, and the final result was expressed as HIV-1 RNA copies per microgram of total RNA.

Table 3: HIV-1 real-time PCR primers and probes.

Region		Sequence	HXB2 location
pol	Forward	5'-TGG GTT ATG AAC TCC ATC CTG AT-3'	3241→3260
	Reverse	5'-TGT CAT TGA CAG TCC AGC TGT CT-3'	3296←3315
	Probe	5'-FAM-AAT GGA CAG TAC AGC CTA TAG TGC TGC CAG AA-TAMRA-3'	3265→3293
gag	Forward	5'-CCCATGTTTTCAGCATTATCAGAA-3'	1297→1320
	Reverse	5'-CCACTGTGTTTAGCATGGTGTTTAA-3'	1339←1363
	Probe	5'-FAM-AGCCACCCCACAAGA-MGB-3'	1323→1337
env	Forward	5'-TTCATCAAATATTACAGGGCTGCTA-3'	7559→7583
	Reverse	5'-CAGGTCTGAAGATCTCGGACTCA-3'	7613←7635
	Probe	5'-FAM-AGATGGTGGTAATAGCA-MGB-3'	7592→7608

4.5   RESULTS

4.5.1   A-to-G mutation in *rev* affects the expression of Rev protein

From our previous studies, significant mutations were observed in the *rev* and *env* regions of HIV-1 in the presence of ADAR1 expression. We first determined whether these mutations had an effect on Rev expression. Mutant 1 has mutations at amino acid positions 23, 41, 49, 53 and 74 in Rev. Mutant 2 had mutations in the positions 23, 41, 49 in Rev. Mutant 3 had mutation at position 15 and 74 in Rev. Mutant 4 has mutation at position 17 in Rev. The Rev mutants displayed varying levels of protein expression (Figure 21A and B). The expression of Rev in mutant clones were either similar to the wild type clone (mutants 1 and 2) or 1.5-fold (mutant 4) to 5-fold (mutant 3) less than the expression of wild type Rev. This observation was confirmed by Western blot using three different anti-Rev monoclonal antibodies (data not shown). However, because of mutation in *rev*, Rev inhibited viral replication in 293Tcells in presence of ADAR1, regardless of their expression levels. Therefore the level of Rev expression was not associated with its antiviral activity. These results suggest that it is the functionality rather than expression level of Rev which may be important for its antiviral activity.
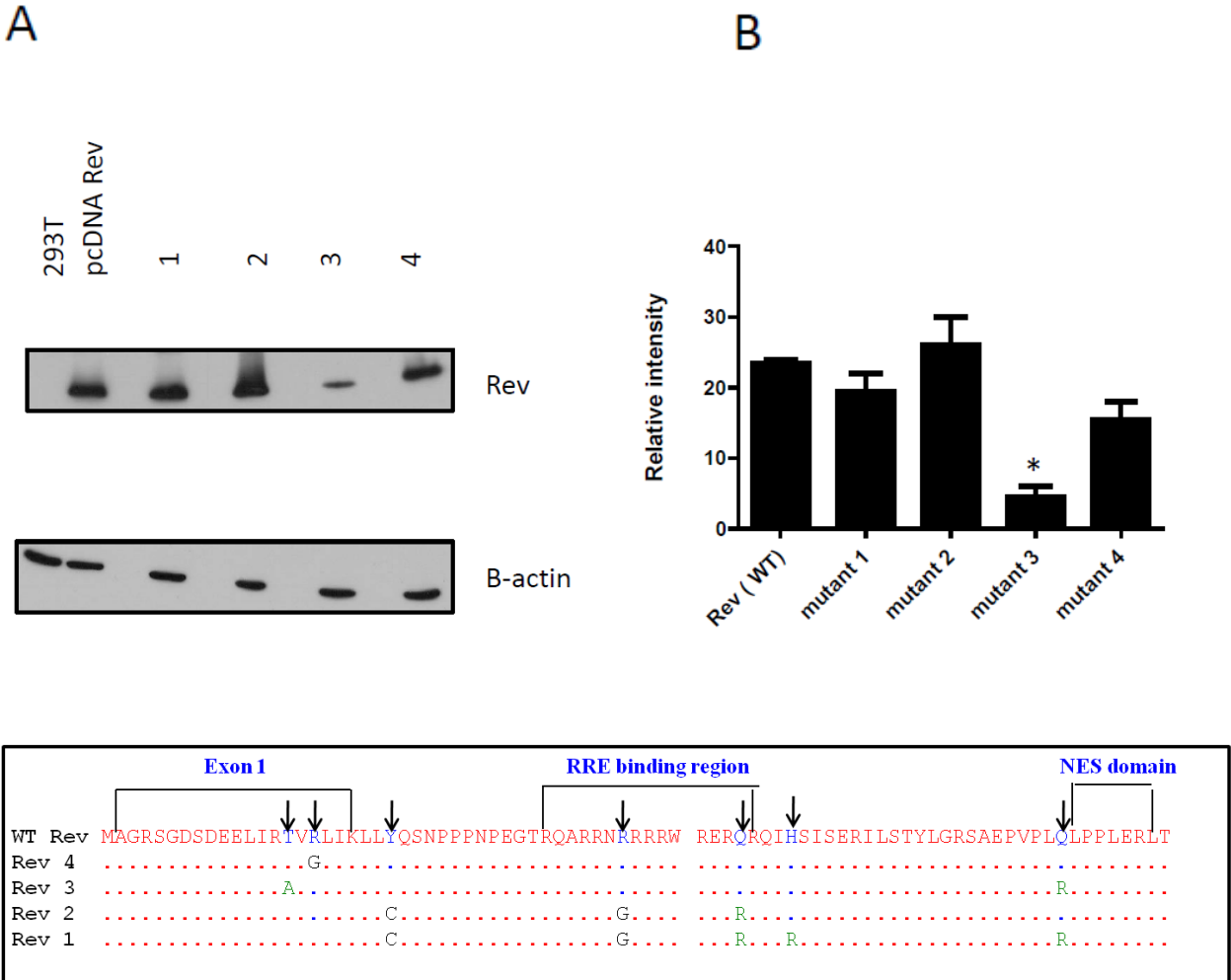
Figure 21: A-to-G mutation in *rev* affects the expression of Rev protein.

293T cells were co-transfected with WT Rev and mutant clones 1, 2, 3 and 4. Cell lysate prepared from the transfected cells were subjected to Western blot analysis using monoclonal Rev antibody. Western blot of Rev and β-actin (A). Relative Rev expression (normalized with β-actin expression) levels based on band density in Western Blot (B). * indicates there is a significant difference (P<0.05) compared to the respective control.

4.5.2 Rev mutants to rescue replication of HIV-1 Δ *rev*

To determine whether the Rev mutants were functional, we examined the capacity of each mutant to recue Rev function in a *rev* deleted HIV-1 (pMrev(-)). The Rev mutant construct pMrev(-) was derived from an infectious proviral clone of HIV-1$_{IIIB}$ in which Rev is not synthesized but in which functionally active Tat is expressed (119). For this purpose, 293T cells were co-transfected with 0.2μg pMrev(-) and 0.3μg wild type *rev*- or mutant *rev*- expressing plasmid DNA. RLuc was used as a transfection control. Virus-containing supernatants were collected approximately 48 h after transfection, and virus production was quantified by p24 production in cell culture supernatant (Figure 22). As expected, in the absence of Rev, low level viral replication was observed which increased by 8-fold in the presence of wild type Rev. Mutant constructs 3 and 4 also rescued virus replication to wild type levels. However, mutant constructs 1 and 2 did not rescue virus replication as compared to wild type Rev. Mutant 1 and 2 had mutations at position 6036 and in the RRE binding region of Rev, respectively. Thus, these mutations in *rev* that were induced by ADAR1 expression decreased Rev function.
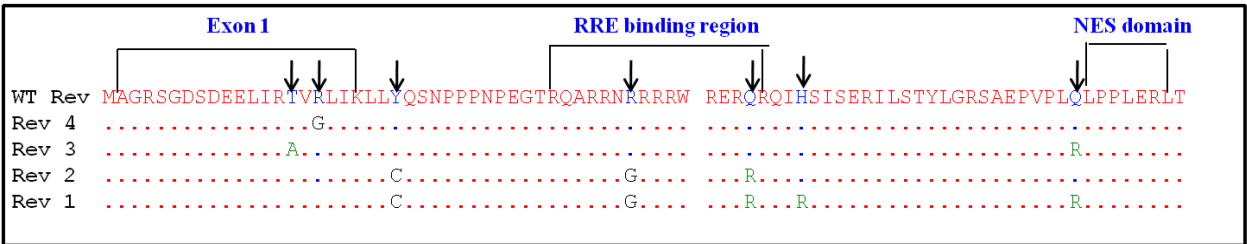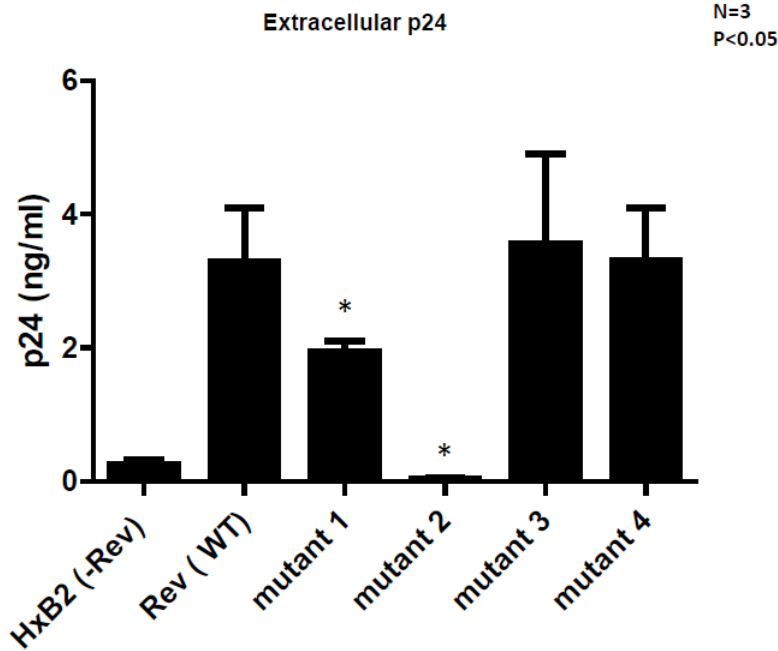
Figure 22: Rev mutants for rescue of HIV-1 Δ *rev* replication.

293T cells were transfected with 0.2μg pMrev(-) construct along with 0.3ug wild type *rev* and *rev* mutant constructs 1, 2, 3 and 4. Virus-containing supernatants were collected 48 h post transfection, and virus production was quantified by analyzing p24 production by ELISA; the mean +s.d. values for three independent experiments are shown.

4.5.3   A-to-G mutation in RRE region in *env* affects the binding of *rev*

Because ADAR1 induced specific A-to-G mutations were observed in the RRE binding region of *rev* we examined whether mutations in the RRE have any effect in the binding of Rev to *env* RRE. For this purpose, we used pDM128, a reporter plasmid derived from the *env* region of HIV-1$_{SF2}$. The transcripts produced by pDM128 harbor a single intron containing the CAT coding sequence, which is excised when the RNA is spliced. Cells transfected with pDM128 alone express only spliced viral transcripts in the cytoplasm and, thus produce only trace levels of CAT enzyme activity. Co-transfection with a plasmid encoding wild type Rev, however, permits unspliced transcripts to enter the cytoplasm, thereby increasing CAT activity. 293T cells were co-transfected with 0.6 µg pDM128 construct and 8µg wild type Rev or Rev mutant plasmids. After 48 h post-transfection, cells were lysed and a CAT assay was performed on cell lysates. (Figure 23). There was a 45% inhibition in CAT activity in the case of mutants 1 and 2 compare to wild type. Expression of mutants 3 and 4 led to similar levels of CAT activity as compared to wild type Rev expression. The binding activity of Rev to the *env* RRE region are consistent with rescue activity of the mutants, suggesting that A-to G mutations in *rev* RRE present in mutants 1 and 2 decreased the binding of Rev to RRE, resulting in a defect in functional activity of Rev.
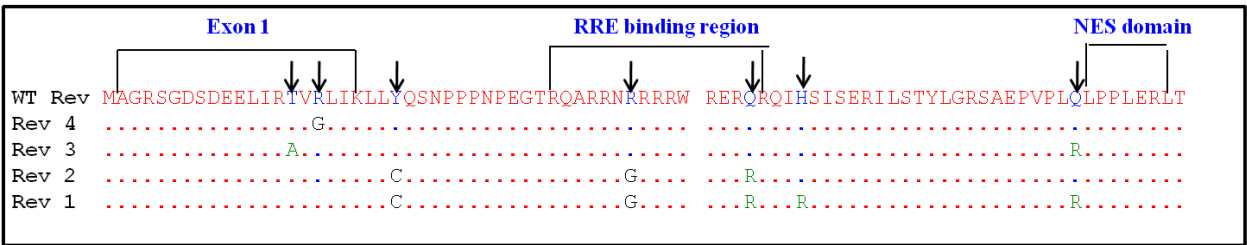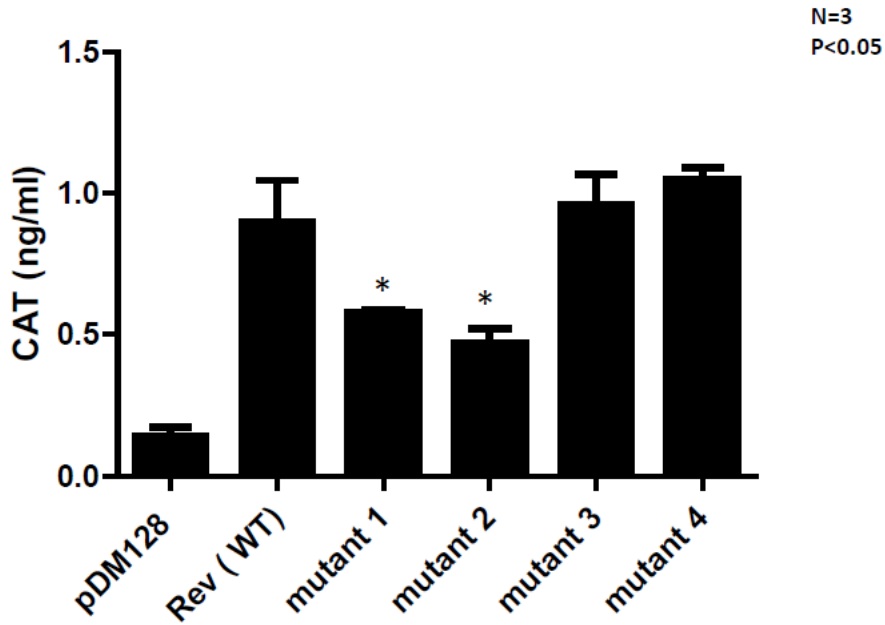
Figure 23: A-to-G mutation in RRE region in *env* affects the binding of Rev.

293T cells were transfected with 0.6ug pM128 construct with 8ug wild type *rev* or mutants *rev* constructs 1, 2, 3 and 4. Cells were lysed after 48 hours and CAT ELISA assay was performed. Renilla luciferase plasmid was used to normalize the internal transfection efficiency.

4.5.4 A-to-G mutations introduced by ADAR1 affect the export of *gag*, *pol* and *env* RNA transcripts from the nucleus to the cytoplasm

Rev is involved in regulating synthesis of the viral proteins Gag, Pol and Env by facilitating transport of their partially and fully spliced messages into the cytoplasm (36, 87). We examined whether ADAR1-induced A-to-G mutations in *rev* inhibited transport of partially spliced (4kb) and unspliced (9kb) RNA from the nucleus to the cytoplasm. 293T cells were co-transfected with 0.1μg of pNL4.3 and 0.5μg control pcDNA or 0.5μg ADAR1 plasmid. After 48 h of transfection, RNA and protein from the cytoplasm and the nuclei were isolated. To determine whether there was any cross-contamination between RNA isolated from the nuclear and cytoplasmic fractions, we examined the presence of β-actin, a cytoplasmic protein, and histone 3, a nuclear protein. Western blot analysis showed no contamination between the nuclear and cytoplasmic fractions (Figure 24A), as β-actin, a cytoplasmic protein was observed only in the cytoplasmic fraction and not in the nuclear fraction, whereas histone 3, a nuclear protein was expressed only in the nuclear fraction and not in the cytoplasmic fraction.

Real-time RT-PCR of *gag, pol* and *env* RNA was performed using RNA isolated from both the cytoplasmic and nuclear fractions. The ratio of nuclear RNA: cytoplasmic RNA was calculated for cells transfected with pcDNA control or ADAR1 plasmids. In general, in the presence of ADAR1 there were more *gag* (approximately 3-fold), *pol* (approximately 4-fold), and *env* (approximately 4-fold) RNA copies present in the nucleus to those measured in the cytoplasm (Figure 24B). These data together indicate that ADAR1-induced mutations in *rev* cause inefficient binding of *rev* to the RRE region of HIV-1 which leads to reduced transport of HIV-1 RNA from the nucleus to the cytoplasm, resulting in inhibition of viral protein synthesis without any effect on viral RNA synthesis.

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A

0.5ug PC DNA cytoplasm
0.5ug ADAR1 cytoplasm
0.5ug PC DNA nucleus
0.5ug ADAR1 nucleus

β-actin

Histone 3B

B

gag RNA
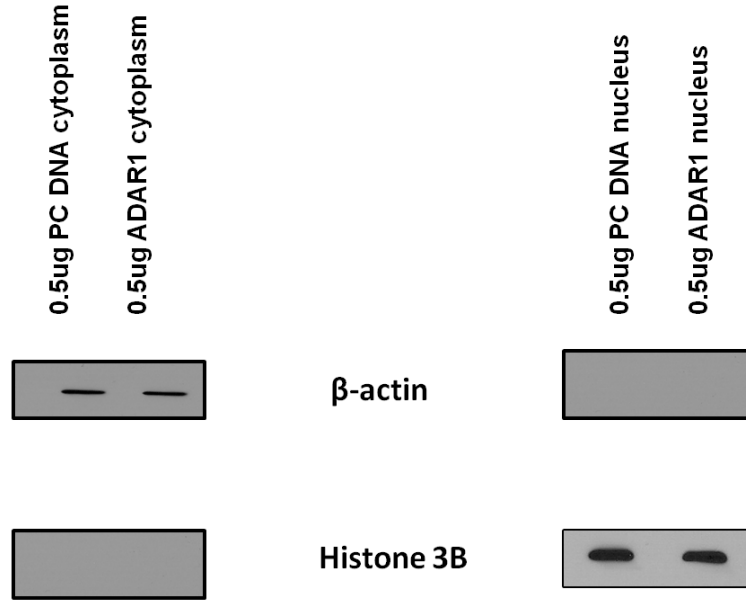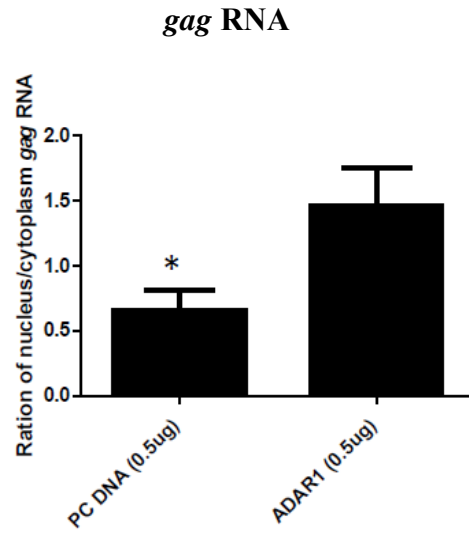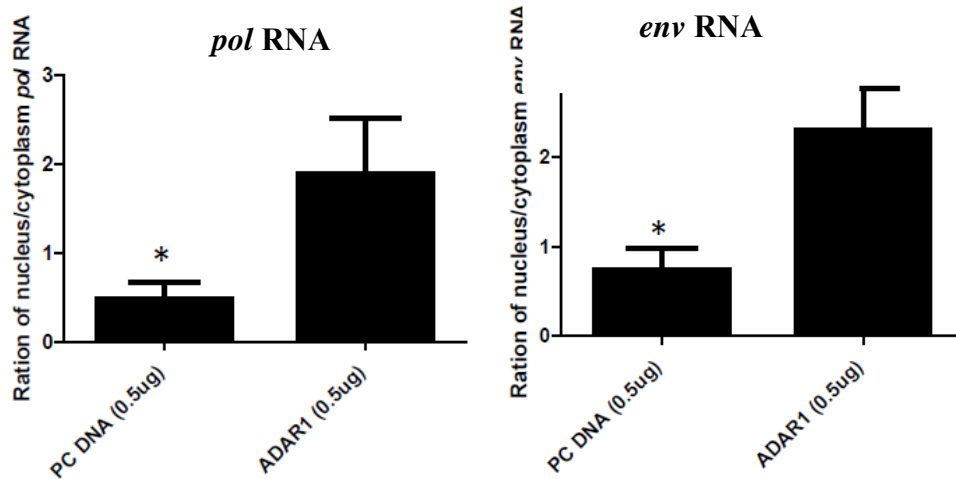
Figure 24: Expression of cytoplasmic protein in nucleus and nuclear protein in cytoplasm and A-to-G mutations introduced by ADAR1 affects the export of *gag*, *pol* and *env* RNA transcripts from nucleus to cytoplasm.

293T cells were co-transfected with 0.1ug of pNL4.3 and 0.5ug pc DNA or 0.5ug ADAR1. After 48 hours PARIS kit (Ambion) was used to isolate RNA and protein from cytoplasm and nucleus. Western Blot was performed to check the presence of β –actin protein in nucleus and Histone 3 protein in cytoplasm (A). 293T cells were co-transfected with 0.1ug of pNL4.3 and 0.5ug control pc DNA or 0.5ug ADAR1. After 48 hours PARIS kit (Ambion) was used to isolate RNA and protein from cytoplasm and nucleus of transfected cells. Real-time PCR of *gag*, *pol* and *env* RNA was performed using RNA isolated from cytoplasm and nucleus fraction. The ratio of nuclear RNA/cytoplasmic RNA of five independent experiments was plotted for both control pcDNA and ADAR1 (B).

Table 4 shows position ADAR1 induced mutation in *rev* RNA and its consequence on RRE binding capacity, rescue capacity of HIV-1 Δrev and expression. A-to-G mutations at position 6036, 8413 and 8438 have the maximum effect in the Rev functionality which is manifested in its RRE binding capacity and the rescue capacity of HIV-1 Δrev. In order to validate whether the mutations at position 6036, 8413, 8438 have any effect on HIV-1 replication and infectivity, A-to-G mutations were introduced in pNL4-3 proviral DNA at the same positions by site directed mutagenesis.

Table 4: Summary of the *rev* mutant constructs and their functional consequences.

	Rev Exon 1			RRE binding region/ NOS domain		Multimerization Domain 2	Before NES domain	Expression	Rescue capacity Of HIV-1 Δ*rev*	RRE binding activity
AA position	15	17	23	41	49	53	74			
	5998	6011	6036	8413	8438	8450	8513			
WT	−	−	−	−	−	−	−	100%	100%	100%
4	−	+	−	−	−	−	−	100%	100%	100%
3	+	−	−	−	−	−	+	20%	100%	100%
2	−	−	+	+	+	−	−	100%	2%	50%
1	−	−	+	+	+	+	+	80%	30%	50%

4.5.5 A-to-G mutations in the proviral genome at position 6036, 8413 and 8438 affects the replication of the virus

To confirm that A-to-G mutations at positions 6036, 8413 and 8438 in the RRE binding region of *rev* are responsible for inhibition of viral protein synthesis and not viral RNA synthesis, we introduced these mutations individually or together in the *rev* gene of an infectious proviral construct (pNL4-3) by site directed mutagenesis and measured their effect on HIV-1 replication. As shown in Figure 25A, a single A-to-G mutation at positions 6036, 8413 and 8438 inhibited p24 production by 50%, 80% and 45%, respectively, as compared to wild type HIV-1. A-to-G

mutations at both positions 8413 and 8438 inhibited virus production by 75% as compared to wild type virus. All three mutations together caused virus replication to be inhibited 98%. Similarly, intracellular HIV-1 p24 was also inhibited to similar levels in these Rev mutants (Figure 25B). However, there was no change in intracellular viral *gag* RNA levels in the cells transfected with any of these mutants (Figure 25C).
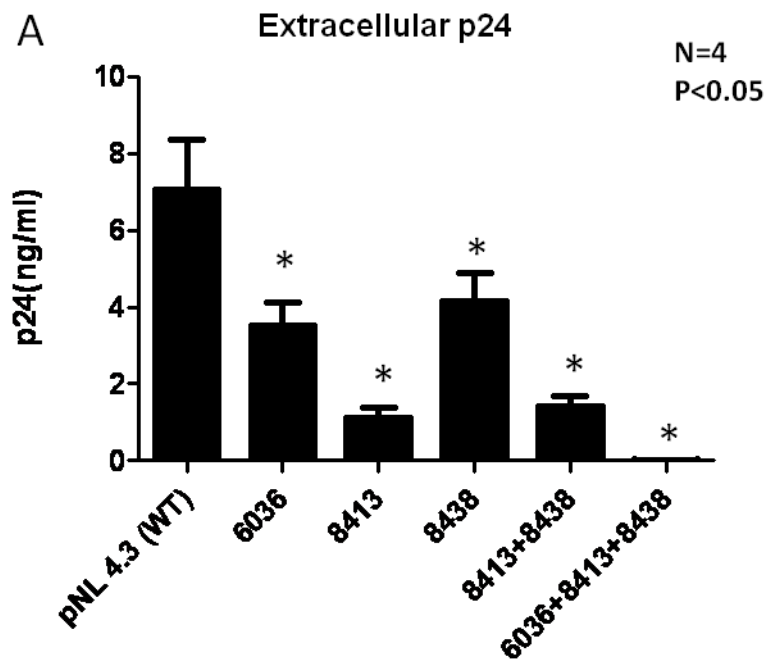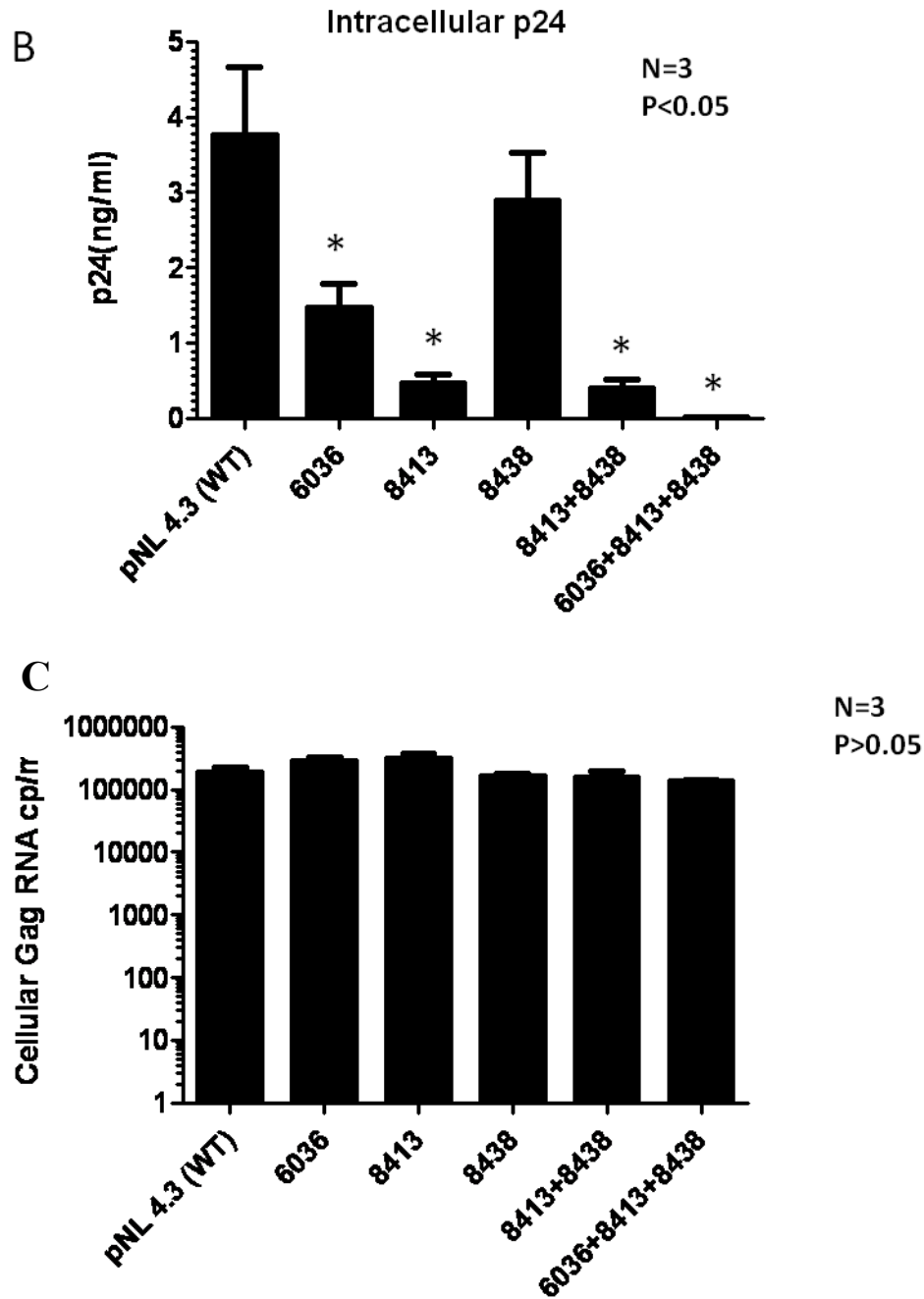
Figure 25: A-to-G mutation in the proviral genome at position 6036, 8413 and 8438 affects the replication of the virus.

A-to-G mutations were introduced at position 6036, 8413 and 8438 (single, double and triple) using site directed mutagenesis kit (Stratagene). 293T cells were transfected with 0.1 μg mutant constructs and Renilla Luciferase plasmid. Virus replication was monitored by measuring HIV p24 in culture supernatant (A) and intracellular HIV p24 production (B). Viral *gag* RNA was quantitated in cells transfected with mutant constructs by Real-time RT-PCR (C).

4.5.6   A catalytic domain of ADAR1 is critical for inhibition of HIV-1 replication and production of infectious virus.

Human ADAR1 has a Z-DNA binding domain, three dsRNA binding domains and a conserved deaminase domain (33, 118). Figure 26A shows a schematic diagram of ADAR1 and ADAR1 mutant constructs used to determine which domains of ADAR1 are critical for antiviral activity of the protein (Figure 26A). Figure 26B shows the expression of full length ADAR1, ADAR1ΔC (catalytic domain deletion mutant), and ADAR1 E->A (single amino acid change from glutamic acid to alanine in the catalytic domain). ADAR1ΔM construct (double stranded RNA binding domain deletion) did not show expression, as the ADAR1 antibody targeted the DRBM (double stranded RNA binding domain) domain. To determine which domains are involved in inhibiting viral replication and infectivity, 293T cells were co-transfected with either full length ADAR1 or ADAR1 deletion constructs in the presence of pNL4.3 DNA. Cell supernatants were collected 48 h post-transfection and virus production was quantified. ADAR1 with a deletion in the deaminase domain (ADAR1ΔC) or a catalytic domain mutation (ADAR1 E-to-A) significantly diminished inhibtion of extracellular p24 production (Figure 26C), intracellular p24 levels (Figure 26D), and infectivity of HIV-1 produced from the cells (Figure 26E). In contrast, ADAR1 ΔM, which has a deletion in double stranded RNA binding domain, showed inhibition of extracellular (Figure 26C) and intracellular (Figure 26D) p24 production as well as reduced infectivity of HIV-1 produced from the cells (Figure 26E). This mutant behaved similar to wild type ADAR1.

From these experiments, conclude that the catalytic motif of the ADAR1 deaminase domain, but not the dsRNA binding domain, is critical in mediating inhibition of HIV-1 replication and production of infectious virus.

Because the catalytic domain of ADAR1 appears to be involved in inhibition of virus replication, we investigated whether this domain is also responsible for A-to-G mutations in *rev*. Therefore, we analyzed mutations in *rev* RNA isolated from cells transfected with wild type ADAR1 or the various deletion constructs described in Figure 26A. No mutations were observed when ADAR1ΔC, ADAR1 E->A, or the control was expressed. However, mutations were observed in the presence of wild type ADAR1 or ADAR1ΔM expression, presumably due to the presence of a functional catalytic domain in both. (Figure 26F).
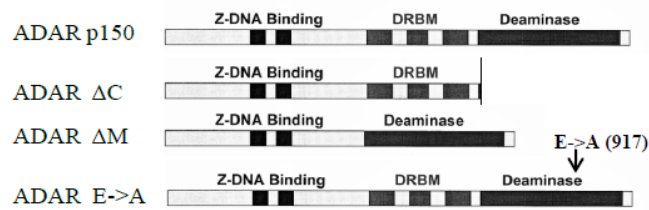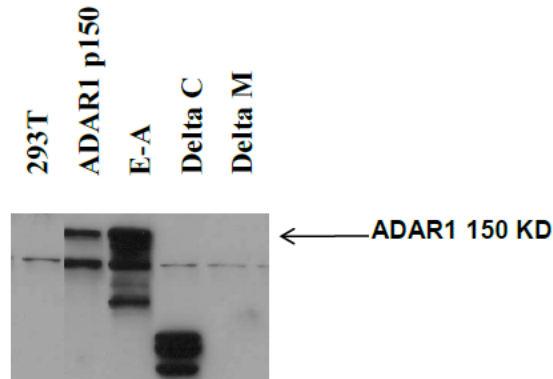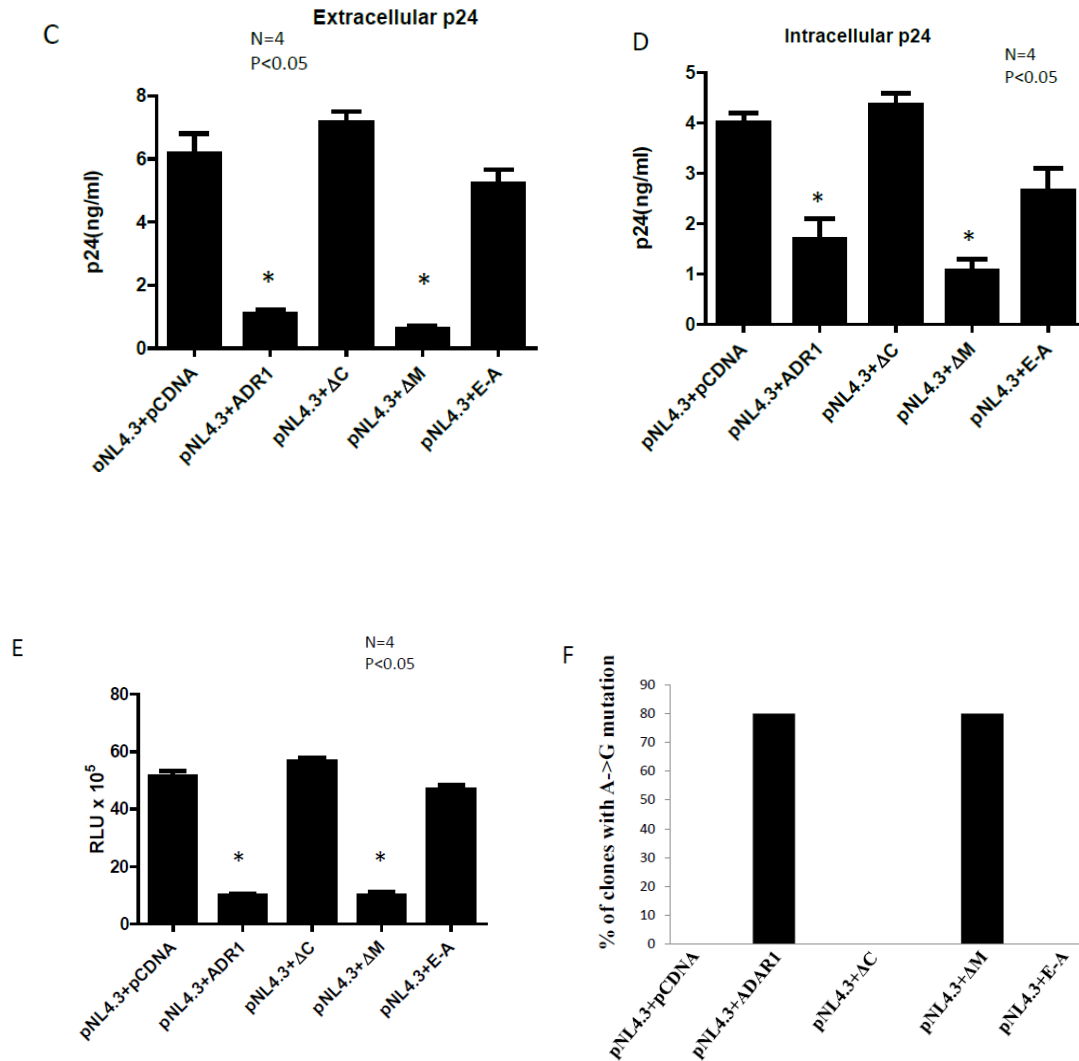
Figure 26: Evaluation of domain of ADAR1 critical for inhibition of HIV-1 replication and production of infectious virus

Schematic representation of full length human ADAR1 and ADAR1 mutant constructs (A) and their expression (B). 0.1 µg of pNL4-3 plasmid was co-transfected with 1 µg of ADAR1 and different domain mutant constructs of ADAR1. The Renilla Luciferase expression was used as transfection normalization control (data not shown). Extracellular p24 production in presence and absence of ADAR1 and its mutant constructs (C). Synthesis of intracellular HIV-1 p24 in presence and absence of ADAR1 and its mutant constructs (D). Measurement of infectivity of extracellular virus in presence and absence of ADAR1 and its mutant constructs (E). Mutational analysis of the *rev* region of HIV-1 in presence of wild type and ADAR1 mutant constructs (F).

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4.5.7 Evaluation of domain of ADAR1 critical for inhibition of HIV-1 *gag*, *pol* and *env* RNA synthesis

To examine the effect of these ADAR1 deletions on viral RNA synthesis, intracellular levels of HIV-1 *gag, pol* and *env* RNA were measured in cells transfected with pNL4.3 and ADAR1, ADAR1ΔC, ADAR1ΔM, ADAR1 E->A DNA or control plasmid by the real-time RT-PCR. No significant changes in the level of *gag, pol* or *env* RNA were observed in the presence of ADAR1 or the mutant constructs, as compared to the pcDNA vector control (Figure 27). These results support our previous data that ADAR1 acts at a post-transcriptional step of viral replication.
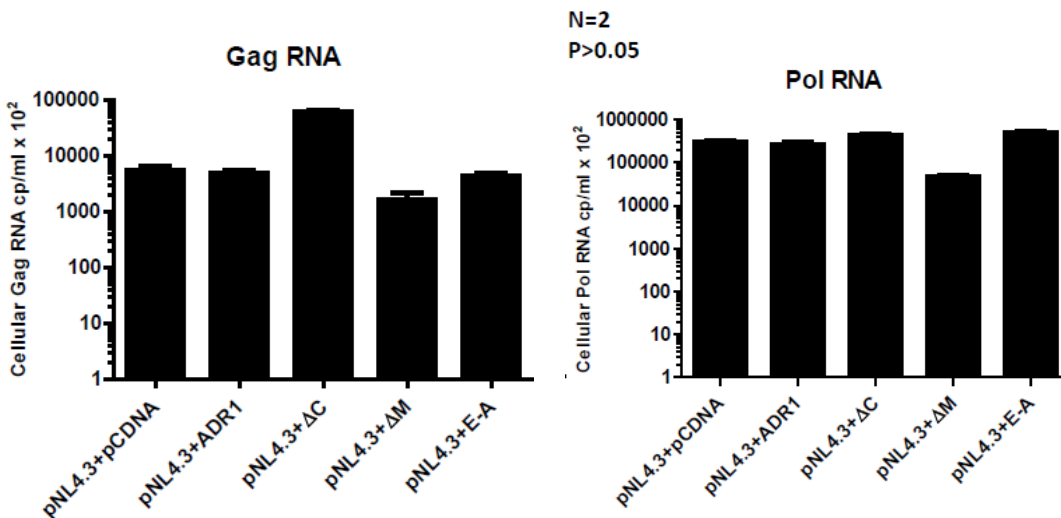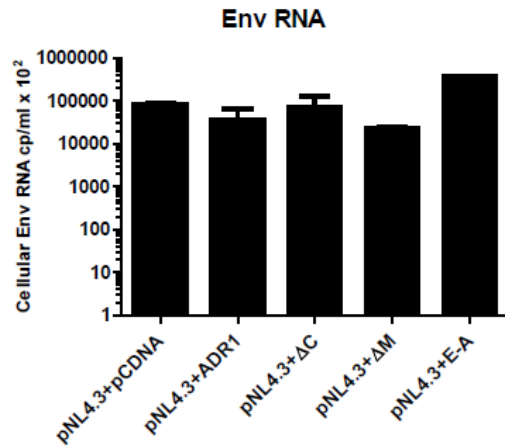
Figure 27: Evaluation of domain of ADAR1 critical for inhibition of HIV-1 *gag*, *pol* and *env* RNA synthesis

Level of HIV-1 *gag, pol,* and *env* RNA in presence and absence of ADAR1 and its mutant constructs was determined by the real-time RT-PCR assay.

Table 5 shows the domains of ADAR1 critical for HIV-1 replication, infectivity and A-to-G editing activity of ADAR1. No changes were observed in extracellular and intracellular virus production, infectivity of the virus, viral RNA production and A-to-G mutations in the *rev* region in presence of control DNA (pcDNA), ΔC and E-A mutant. Inhibition of extracellular and intracellular virus production, infectivity of the virus and A-G mutations were observed in presence of full length ADAR1 and ΔM constructs.

Table 5: Summary of the domains of ADAR1 critical for the inhibitory effect during HIV-1 replication.

	Extracellular Virus	Intracellular Virus	Infectivity	Intracellular gag/pol/env RNA	A-G mutation in *rev* region
pc DNA	100%	100%	100%	100%	-
ADAR1	17%	37.5%	20%	100%	+
Δ C	100%	100%	100%	100%	-
Δ M	18%	25%	21%	100%	+
E-A	100%	65%	90%	100%	-

4.6   DISCUSSION

Our previous data showed that over-expression of ADAR1 inhibits replication and infectivity of HIV-1 by introducing A-to-G mutations in *rev* and *env* RNA. Here, we determined the mechanism by which ADAR1-induced mutations inhibited HIV-1 replication.

HIV-1 Rev is a 116 kD protein that is involved in the transport of viral mRNAs, affecting the expression of unspliced or partially spliced mRNA. Felber, *et al.* showed that Rev affects the stability and transport of single spliced (4kB) and unspliced (9kB) mRNA but not fully spliced (2kB) mRNA (87). They also determined that Rev affects the half-life of singly spliced and unspliced mRNA but not fully spliced mRNA and decreased the amount of mRNA targeted for splicing. Malim *et al.* also proposed that Rev is required for the transport rather than splicing of unspliced or singly spliced mRNA from the cytoplasm to nucleus (91). In the same paper they also stated that in order to transport the unspliced or singly spliced RNA, Rev binds to a special secondary structure in *env*, called the RRE (Rev Response Element).

Rev carries two functional domains: the N terminal domain contains an arginine-rich sequence that serves as an NLS, and the RNA binding domain is required for binding to the RRE, which is present in all unspliced viral transcripts of viral proteins (13, 85, 88). Rev binds to the RRE region in *env* and transports the unspliced and singly spliced mRNA from the nucleus to the cytoplasm. The C-terminus of Rev also carries an NES domain (61, 85, 144). The combination of an NLS and an NES appears to enable the Rev protein to actively shuttle between the nucleus and the cytoplasm. Here we showed that A-to-G mutation in *rev* inhibited the transport activity of Rev, resulting in accumulation *of gag, pol* and *env* in the nucleus and thereby inhibiting translation of structural proteins Gag, Pol and Env. Such inhibitions of HIV-1 structural proteins were responsible for inhibiting viral replication.

In 1994 Meyer and Malim's group showed that mutation in the NES domain (LQLPPLERLTLD), specifically, mutations in the leucine residues, prevent transport of the unspliced viral RNA from nucleus to cytoplasm (87, 95). In 1990 Parslow's group showed that mutation in the first exon of *rev* can inhibit nuclear localization and transactivation (91). In our current study amino acid changes were observed due to A-to-G mutations in exon 1 of *rev*. ADAR1 induced mutations were also observed in the RRE binding domain of *rev*, which also contains a nuclear/nucleolar localization signal (NLS). Malim M.H *et al* (1989) and Emerman M *et al* (1989) showed that mutations in RRE and NES regions caused accumulation of unspliced HIV-1 mRNA that encode viral structural proteins(34, 87). Here we have showed that the A-to-G mutation in RRE binding region of *rev* inhibited the binding of Rev to the RRE region of *env* which eventually decreased the transport of unspliced and singly spliced mRNA from cytoplasm to nucleus. Additionally, we have also showed that, both the RRE binding region and the first exon of Rev is important for the replication of virus. A-to-G mutation at position 6036 completely abolished the rescue capacity of HIV-1 Δ*rev* virus. Site directed A-to-G mutation in the first exon (position 6036) and in RRE binding region (position 8413, 8438) inhibited the viral replication and infectivity without affecting the production of viral RNA. Mutational analyses of the various domains of ADAR1 showed that the catalytic domain of ADAR1 is important for the inhibition of replication and infectivity of the virus. The catalytic domain was also important for the editing activity of ADAR1. The double stranded RNA binding domain (DRBM) recognizes specific moieties of the RNA and interacts with the RNA duplex. However our data revealed that ADAR1 was able to inhibit replication and infectivity in the absence of DRBM domain. No band was observed in the western blot for the delta M construct (which lacks the RNA binding domain) as the antibody was directed against the DRBM (Figure 26B). Despite the lack of

DRBM, the ability of ADAR1 to catalyze the deamination reaction on the mRNA species might indicate a role for host cellular factors for bringing the enzyme closer to the RNA molecule. Over all our data shows that deletion of the catalytic domain abolishes the anti HIV-1 effect of ADAR1.

5.0   OVERALL DISCUSSION

The innate immune system is the first line of host defense against invading pathogens. Viruses have developed a number of strategies to circumvent the host's immune response to infections. Cellular antiviral restriction factors have evolved due to the selective pressure exerted by repeated viral infections. One of these host cellular restriction factors is ADAR1. ADAR1 is an RNA editing enzyme that acts on complete or partially double-stranded RNAs, and deaminates the adenine base of nucleotides. Double stranded RNA species are common intermediate products of replication of many viruses, including HIV-1. In human cells, two immunologically related forms of ADAR1 are found: ADAR1 p150 which is present in both the cytoplasm and the nucleus, and p110, which is present only in the nucleus. ADAR1 p150 is induced by both Type I and Type II interferons, such as IFNα and IFNγ. Although it is known that ADAR1 is present in many organisms ranging from unicellular protozoa to humans, the expression level of ADAR1 in different cell lines and in human immune cells is unknown. ADAR1 is known to have inhibitory effects on the replication of many viruses including HCV, measles virus, human parainfluenza virus, respiratory syncytial virus, influenza virus, lymphocytic choriomeningitis virus, Rift Valley fever virus, mumps virus and hepatitis C virus However, the effect of ADAR1 on HIV-1 is also unknown.

In our current study, we provide evidence for the inhibitory effect of ADAR1 on HIV-1 replication and infectivity. Our data show that ADAR1 inhibits HIV-1 replication irrespective of

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viral tropism or clade. ADAR1-mediated HIV-1 inhibition was observed in the cell lines, 293T, HeLa and Jurkat cells, and in primary CD4+ T cells isolated from peripheral blood. We have shown that in cells expressing ADAR1, inhibition of HIV-1 replication can be observed regardless of whether the cells are transfected with a molecular clone of HIV, or infected with a primary isolate of HIV. Furthermore, inhibition of HIV-1 replication could be observed in Jurkat T cells by endogenous ADAR1 induced by IFNα treatment. Additionally, we have shown that antiviral activity of ADAR1 can be blocked by shRNA against ADAR1 p150, demonstrating specificity of ADAR1 induced inhibition of HIV-1 replication. Our results indicate that ADAR1 mediated inhibition of viral replication occurs at a post-transcriptional step of viral replication, namely at the step of nuclear export of viral *gag*, *pol* and *env* mRNA. This effect of ADAR1 on the nuclear export was seen to be due to ADAR1-induced mutations on *rev* and RRE region on *env*. These ADAR1 induced A-to-G mutations on HIV-1 *rev* and *env* mRNA correlated with inhibition of virus replication, production and infectivity. No ADAR1-induced mutations were observed in the LTR, *vif* and *tat* regions, indicating a differential susceptibility of mRNA species to the enzymatic action of ADAR1.

The enzymatic action of ADAR1 depends on certain secondary of the RNA. Therefore, differential susceptibility of specific regions for mutation could be due to structural specificity of the RNA. ADAR1 is known to nonselectively edit RNA molecules that are longer that 50 base pairs and hence, more thermodynamically stable (79). Shorter RNA molecules are edited more selectively and in fewer sites compared to long dsRNA. Furthermore, it has been shown that ADAR1 is more selective in editing the structure of dsRNA rather than RNA sequence (79). For example, an RNA molecule with many loops or bulges likely will not be edited selectively by ADAR1. The presence of internal loops in a long dsRNA molecule can be identified by ADAR1

as a series of short dsRNA segments that can be edited more selectively. We have shown that HIV-1 *env* and *rev* are deaminated in the presence of ADAR1. It is possible that because of the RNA sequences in some internal loops of these two genes may be the target for ADAR1 to selectively deaminate these RNA species. A lack of ADAR1-induced mutations in LTR and *tat* is consistent with our observation of no change in viral RNA production in the presence of ADAR1. However, it is not clear why the sequences in *tat* that overlap with *rev* did not have mutations. It is possible the overall secondary structure of *tat* is different from the secondary structure of *rev* as *rev* has more bulge and loop structure that *tat* has and ADAR1 is known to have structural specificity than sequence specificity.

Since ADAR1 inhibits HIV-1 replication and concomitantly induces mutations in viral *rev* RNA, we examined the mechanisms by which these mutations could perturb viral replication. In mammalian cells, export of mRNA does not take place, unless the mRNA is fully spliced. Unspliced viral mRNA thus need a separate process for their export and cannot depend wholly upon the cellular nuclear export machinery. Fully spliced HIV-1 mRNA encode the viral regulatory proteins Tat, Rev and Nef. Partially spliced (4kb) or unspliced (9kb) mRNA encode viral Gag, Pol and Env proteins (49, 113). The synthesis of Gag, Pol and Env proteins is regulated by Rev, which facilitates the transport of *gag*, *pol* and *env* mRNA from nucleus to cytoplasm (36, 87). HIV-1 Rev has an RNA binding domain required for binding to the RRE region present in all unspliced viral transcripts and such a binding enables rev to export unspliced viral mRNA species from nucleus to cytoplasm (61, 85, 144).

We have shown that ADAR1-induced mutations in the first exon and the NLS/RRE binding region of *rev* resulted in a decrease in binding of Rev to the RRE and reduced transport of singly and multiply spliced HIV-1 *gag*, *pol* and *env* mRNA from the nucleus to the cytoplasm. The

importance of these mutations have been further demonstrated by their introduction into an infectious molecular clone of HIV-1 DNA, resulting in a loss of HIV-1 replication, a decrease in viral protein synthesis, and normal levels of viral RNA synthesis. These data together indicate that ADAR1-induced mutations in *rev* cause inefficient binding of Rev to the RRE region of HIV-1 which leads to reduced transport of unspliced and single spliced HIV-1 mRNA from nucleus to cytoplasm.

ADAR1 induced mutations in the RRE region of *env* RNA further attenuated binding of Rev to *env* message resulting in decrease in transport of *env* message from nucleus to cytoplasm and subsequent inhibition of Env protein synthesis.

In addition to mutations in the RRE region of *env* RNA, we also observed ADAR1-induced mutations in the sequence encoding the V2 and V4 regions of gp120. It is known that constant loop 4 (C4) and variable loop 3 (V3 loop) along with V2 loop are important for viral entry process and therefore infectivity of the virus (72). These ADAR induced mutations in HIV-1 *env* probably resulted in production of virions with defective Env proteins, which decreased the infectivity of the virions. This supports our observation that virions secreted in the presence of ADAR1 were non-infectious.

Our results contradict previous studies on the effect of ADAR1 on HIV-1. Two reports showed that ADAR1 enhanced the expression of HIV-1 proteins (32) (108). Phuphuakrat *et al.* found increased p24 production from infected cells in the presence of ADAR1, although they also observed that the infectivity of progeny virions was decreased. Doria *et al.* also showed an increase in p24 production in the presence of ADAR1, although they found an ADAR1 – mediated enhancement of infectious virus production. Recently, Clerzius *et al.* (23) reported that

ADAR1 inhibited interferon-induced protein kinase during HIV-1 infection and increased viral replication

Phuphuakrat *et al* (108) and Doria *et al* (32) did not provide any adequate explanation for the observed ADAR1 induced enhancement of viral replication. Phuphuakrat *et al* (108) did not explain clearly the reason of the enhancement of replication of virus. They stated that a decrease in unspliced RNA in the nucleus may have resulted in increased nuclear RNA splicing. Furthermore, they commented that sequencing of viral RNA in the RRE region in cells overexpressing ADAR1 showed some site-specific A-to-G editing at nucleotides 8164 to 8166, which could provide an advantage in viral protein expression via an unknown *cis*-acting element. However, they did not provide evidence in support of these hypotheses. Doria *et al* (32) explained their data on the observed enhancement of viral replication in the presence of ADAR1 by citing other reports indicating that ADAR1 inhibits PKR kinase. Doria et al hypothesized that inhibition of PKR kinase could suppress viral protein synthesis by phosphorylating the transcription elongation factor, eIF-2a (23). If that were indeed the case, we would have expected increased synthesis of all viral proteins not only gp120, p24 and Nef proteins. However, our data shows that ADAR1 did not affect Nef or Tat protein synthesis.

The antiviral effect of ADAR1, on the other hand, makes sense from a biological and a mechanistic point of view. ADAR1 is an interferon-inducible protein, known to be involved in antiviral activity against a number of RNA viruses (58, 98, 104, 112), such as measles virus, human parainfluenza virus, respiratory syncytial virus, influenza virus, lymphocytic choriomeningitis virus, Rift Valley fever virus, mumps virus and hepatitis C virus (106). Viperin, another interferon-inducible protein, has been shown to have anti-viral activity against HCV and influenza virus (56, 59, 66). Viperin inhibits influenza virus budding and release by disrupting

lipid rafts. Viperin inhibits the replication of HCV by binding to lipid droplets, small organelles involved in lipid homeostasis and which are essential for HCV replication. (63, 106, 112, 122).

Thus, it can be expected that ADAR1 could have similar antiviral properties against HIV-1.

The expression of ADAR1 is low in T cells, B cells, DCs and other immune cells. Thus, perhaps HIV-1 can overcome the effect of ADAR1 when the expression of ADAR1 is limited to its baseline level. However, we have seen that the expression of ADAR1 could be increased by stimulating with IFNα, which is one of the key cytokines expressed soon after infection, by plasmacytoid DCs. Being a host cellular factor, ADAR1 atleast at the level induced by interferon is probably not toxic to cells. Thus, ADAR1 might well play an important role in control of virus replication in vivo.

In summary, our data demonstrate that ADAR1 is an important cellular protein that inhibits HIV-1 replication by editing viral *rev* RNA, thereby disabling transport of unspliced and singly spliced viral mRNA from the nucleus to the cytoplasm. Therefore, ADAR1 constitutes a novel multi-targeted cellular protein against HIV-1 which can have therapeutic implications.

6.0   FUTURE DIRECTIONS

6.1   SITE DIRECTED MUTAGENESIS TO INTRODUCE ADAR1-INDUCED MUTATIONS IN *ENV.*

Our results clearly show that that ADAR1 introduces A-to-G mutations in *rev* RNA, which contributes to the ADAR-1 mediated inhibition of HIV-1 replication. Additional mutations were also observed in RRE and V2 region of *env*. These mutations may also result in reduction of viral infectivity. Further experiments are required to verify whether A-to-G mutations in RRE and V2 region of *env* have any effect in the infectivity of the virus. To verify this, site-directed mutagenesis should be performed to introduce the ADAR1-induced A-to-G mutations observed in the *env*, specifically in the RRE region and in the V2 region of *env* in the proviral clone. Mutant clones would be transfected into 293T cells and the amount of virus produced will be estimated by p24 ELISA. The infectivity of the mutant viruses will be evaluated by standard TZM-bl infectivity assay.

6.2   TANDEM PULL DOWN ASSAY TO IDENTIFY CELLULAR PROTEINS INTERACTING WITH ADAR1.

The double stranded RNA binding (DRBM) domain of ADAR1 binds to the RNA and mediates its editing activity.

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We have previously shown that DRBM domain deletion mutant of ADAR1 was able to inhibit HIV-1 replication and infectivity. We have also shown that in the absence of DRBM, ADAR1 was able to introduce A-to-G mutations in the *rev* region of HIV-1 genome. These results indicate that other cellular partners may be involved in ADAR1 editing of HIV-1 viral RNA. The ADAR1 interacting cellular proteins may bind to HIV-1RNA and contribute to ADAR1 specificity. To identify the cellular proteins interacting with ADAR1, tandem pull down co-immunoprecipitation assay will be performed using HA and FLAG antibody. Briefly, dually tagged ADAR1 with HA and FLAG tags will be expressed and a sequential pull down will be performed to isolate interacting cellular partners. The dual tags will help to minimize non specific proteins pulled down in the elute. The eluted interacting partners will be identified by standard mass spectrometry based assays.

6.3   THE EFFECT OF ADAR2 AND ADAR3 IN THE REPLICATION AND INFECTIVITY OF HIV-1 VIRUS.

So far we have evaluated the inhibitory effect of ADAR1 on HIV-1 replication and infectivity. ADAR2 is also an RNA editing enzyme which causes A-to-G mutations in the DNA. Here we would test whether ADAR2 and ADAR3 can cause inhibit the replication and infectivity of HIV-1. Cells would be transfected with ADAR2 or ADAR3 expressing DNA and pNL43, and the replication of HIV-1 would be monitored by p24 ELISA assay. Equal amount of viruses would be added to TZM-bl cells to determine the infectivity of the virus. The editing effect of ADAR2 and 3 would be verified by analyzing A-to-G mutations of the different HIV-1 genome in cells transfected with pNl4-3 and ADAR2 or ADAR3.

6.4   THE EXPRESSION OF ADAR1 IS HIV-1 INFECTED PATIENCE AND THE REPLICATION OF HIV-1

The expression of ADAR1 will be first analyzed in PBMC from the healthy donors to establish the expression pattern in the absence of HIV-1 infection. Following this, the expression of ADAR1 in HIV-1 infected patient will be evaluated to identify inter patient variations in ADAR1 expression level. Finally, the expression profile of ADAR1will be evaluated from patients who are in the acute symptomatic phase and also from those who are in chronic or asymptomatic phase to identify differences in the expression pattern of ADAR1 at different stages of HIV-1 infection. For this CD4+ enriched T cells from these patients will be lysed and Western blot analysis will be performed to check the endogenous expression of ADAR1 in these patients.

6.5   ADAR1 BASED GENE THERAPY FOR HIV INFECTED PATIENTS.

Our results clearly demonstrate the ability of ADAR1 to inhibit HIV infection and replication and establishes ADAR1 as a novel host cellular restriction factor. This exciting finding opens new avenues for development of novel therapeutics. Our study confirms that ADAR1 is induced by interferon alpha and contributes to HIV restriction. Even though interferon has been evaluated as a therapeutic modality, the adverse effect associated with interferon treatment discourages patient compliance. Additionally, multiple pathways are modulated by interferon making it a less specific approach. This mandates the need for alternative approaches. Adenoviruses have been classically used as viral vectors for transient expression of proteins and have been exploited as vaccine therapeutics, it is possible to use adenovirus based vectors to have transient expression

of ADAR1 during acute phase of HIV infection or in latent HIV reservoirs where HIV LTR based promoters can induce expression of ADAR1 specifically in virus infected cells can aid in elimination of infectious virus reservoirs. Additionally the ability of ADAR1 to hypermutate viral mRNA, can generate mutant viral proteins that can behave as effective immunogens and can potentiate the immune response against the patient specific HIV virus serotype. This can boost the immune response and help in effective control of HIV infection.

Recent advances in stem cell based therapeutics are promising. It was recently reported that human hematopoietic stem/progenitor cells modified by zinc finger nuclease targeted to CCR5 control HIV-1 in vivo. Such an approach makes it safe to apply gene therapy approach to stably express ADAR1 in hematopoietic progenitor cells and use these cells as an alternative approach to reduce the viral load and eliminate infectious virus load.

7.0   PUBLIC HEALTH SIGNIFICANCE

HIV infection is a worldwide public health problem. Antiretroviral drugs are being used successfully to curtail progression of the disease and HIV-1 transmission. However, antiretroviral therapy has toxic side effects. Furthermore, development of drug resistant variants is a major problem. Therefore, scientists all over the world are looking for alternative ways to control HIV infection. One of the alternative way is to use host cellular factors that have anti-HIV activity. These antiviral cellular proteins are so attractive because being host proteins they are likely to be less toxic. We investigated one such cellular factor ADAR1. Our results clearly show that ADAR1 is a HIV restriction factor. In elucidating the mechanism of action we found that ADAR causes viral inhibition by inducing mutation in the RRE binding region of HIV-1 rev RNA. ADAR1 also induces mutation in viral *env* gene required for viral infectivity. Thus ADAR1 is a novel multi-targeted anti-HIV-1 protein.

These results have number of Public Health implications. First, Understanding the various mechanism(s) involved in ADAR1 mediated restriction of HIV virus will help us designing new strategy for inhibition of viral replication. For example, our results indicate that ADAR1 induced mutation in RRE binding domain of Rev inhibits the synthesis of major viral structural proteins and hence viral replication. Identification of agents that can inhibit RRE binding domain in Rev with RRE region of viral messages can inhibit viral replication.Second,

ADAR1 can be delivered to immune cells through viral vectors, such as adenovirus, which augments the expression of ADAR1 in the immune cells and thereby block viral replication.

Third, the ability of interferon to induce ADAR1 expression is well established but the role of ADAR1 during acute infection is not known. If interferon levels vary during different stages of HIV infection, it is plausible that the ADAR1 expression also varies in different stages of disease. Analyzing the relationship of ADAR1 during different stages of HIV infection as proposed in the future direction will help us evaluate the role of ADAR1 in control of early infection. If ADAR1 is able to restrict HIV replication during the early stages of acute infection, it will reduce the virus set point. Additionally, if ADAR1 has a role in later stages of infection, by its ability to restrict HIV replication, ADAR1 will help to delay the progression of AIDS.

Fourth, Since ADAR1 inhibits HIV and HCV, overexpression of ADAR1 by viral vectors can block replication of these two viruses which are often found in HIV infected subjects and have deleterious effect on the progression of the disease.

Therefore ADAR1 may be an important weapon in battle against HIV infection. ADAR1 constitutes a novel multi-targeted cellular protein against HIV-1 which could have therapeutic implication.

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