Interplay between the RNA interference machinery and HIV-1
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ONE

General introduction
1. HUMAN IMMUNODEFICIENCY VIRUS TYPE 1

The virus that causes the acquired immunodeficiency syndrome (AIDS) was first identified in 1983 and later named human immunodeficiency virus type 1 (HIV-1) (4,32). HIV-1 was classified as a retrovirus belonging to the subfamily of lentiviruses. Over the past 3 decades it is estimated that more than 60 million individuals have been infected with HIV-1 and over 30 million have died of AIDS. In 2009, approximately 33 million adults and children were living with HIV-1 worldwide, 2.6 million became newly infected and 1.8 million deaths occurred due to AIDS. The most affected countries are located in sub-Saharan Africa (http://www.unaids.org). Mainly due to limited access to the antiretroviral drugs in these countries, HIV-1 infection has reduced life expectancy by more than 20 years and slowed down economic growth.

Although significant efforts have been made to develop a vaccine or cure for HIV-1 infection, none have been successful so far. Antiretroviral drugs were successfully developed that inhibit HIV-1 replication. Initially, patients were treated with a single drug, which eventually resulted in the emergence of drug-resistant virus variants. When more drugs reached the clinic, patients could be treated with a combination of antiretroviral drugs also known as highly active antiretroviral therapy (HAART), which effectively suppresses HIV-1 replication and prevents the evolution of drug-resistant virus variants (7,22). However, HAART remains expensive and adherence to the drug regimen is an important prerequisite for the success of HAART by preventing the selection of drug-resistant virus variants and avoiding treatment failure. Therapy adherence is sometimes difficult due to severe side effects of the drugs (18). Thus, there is a need for novel therapeutic approaches against HIV-1. The discovery of the RNA interference (RNAi) mechanism almost fifteen years ago provided a new strategy to potently inhibit invading pathogens in a sequence-specific manner (17,26,46). In this thesis, I will describe the development of alternative therapeutic strategies to inhibit HIV-1 replication. These antiviral approaches are based on the RNAi mechanism, but were also tested in combination with antiretroviral drugs. Furthermore, the relationship between the RNAi machinery of the host cell, HIV-1 infection and the small noncoding RNA molecules expressed in the infected cell are investigated in more detail. This introduction will provide an overview of the basic features of the HIV-1 replication cycle and the cellular RNAi mechanism.

1.1 HIV-1 genome

The viral RNA genome present in HIV-1 particles is composed of two identical positively stranded RNA molecules of 9.8 kb. The HIV-1 proviral DNA genome is depicted in Figure 1A. The protein-coding sequences are flanked by non-coding sequences that include the long terminal repeats (5’ and 3’ LTR), which consist of three domains designated U3, which is uniquely present at the 3’ end of the transcribed RNA, R (repeat region) and U5 (unique at 5’ end of the RNA). These non-coding regions contain the transcriptional promoter and several structural/sequence motifs that are important for viral replication. Viral transcription starts at the first residue of R in the 5’LTR and the transcript is polyadenylated at the last residue of R in the 3’LTR (11,33). The three structural genes gag, pol, and env are translated as polyproteins, which are further processed and cleaved into functional proteins (81,98). The Gag polyprotein precursor is cleaved in nucleocapsid (NC), capsid (CA), and matrix (MA). The components of the viral core ensure packaging of the two RNA strands (Fig. 1B). Protease (PR), reverse transcriptase
(RT) and integrase (IN) are translated from a polyprotein encoded by the pol gene (29). The env gene encodes the envelope of the virus, which is expressed as a gp160 precursor protein that is cleaved into a gp41 transmembrane domain and the gp120 outer surface domain (105). The envelope protein is incorporated in the lipid membrane derived from the infected host cell that encloses the viral core during budding of virion particles. Tat and Rev are both essential regulatory proteins. The Tat protein is a transcriptional activator that selectively activates HIV-1 transcription. The Rev protein is required for nuclear export of the unspliced and single spliced HIV-1 transcripts (31,47). The accessory proteins Vif, Vpr, Vpu, and Nef are not strictly required for viral replication, but facilitate virus replication in certain cell types and contribute to virus replication in vivo (21,93).

1.2 The HIV-1 replication cycle
HIV-1 replication primarily occurs in CD4-positive T lymphocytes, but monocytes, macrophages, and dendritic cells are also susceptible to infection. The primary CD4 receptor and a chemokine co-receptor, CXCR4 or CCR5, are required for HIV-1 entry into the host cell target (Fig. 2)
HIV-1 attaches to the target cell via its envelope protein gp120 that first binds to CD4, followed by interaction with one of the co-receptors. This elicits a conformational change within the envelope protein that drives fusion of the viral and cellular membrane, triggering release of the viral core into the cytoplasm where it is partially uncoated. Upon infection of a host cell, the RNA is copied into double-stranded DNA within the virion core by the viral RT enzyme, a process that is initiated from a cellular tRNALys3 molecule that is used as specific primer for reverse transcription. The core is subsequently transported to the nucleus where the DNA is inserted into the cellular genome by the viral integrase enzyme (1). The integrated DNA state is called the provirus. Integration occurs at a random chromosomal location and some infected cells may harbor more than one provirus in their genome (29). The proviral DNA serves as a template for the production of new viral RNA and proteins, virus assembly, budding and maturation of the virion particle.

Figure 2. The HIV-1 replication cycle. Important steps in the HIV-1 replication cycle include virus entry, reverse transcription of the viral RNA genome into dsDNA, integration of the viral DNA into the host genome, production of new viral RNA and proteins, virus assembly, budding and maturation of the virion particle.
1.3 Highly active antiretroviral therapy against HIV-1 infection

HIV-1 infection is associated with a progressive loss of CD4+ T cells and a steady increase in viral load. This triggers the gradual collapse of the immune system, resulting for instance in opportunistic infections that define the onset of AIDS and may ultimately result in death. Viral replication can be inhibited with antiretroviral drugs, but there is currently no cure available that can eliminate the virus. A variety of antiretroviral drugs are available that target distinct steps in the viral life cycle: nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors (PI), integrase inhibitors and entry inhibitors (78). NRTIs interfere with HIV replication by competitive binding to the active site of the RT enzyme and act as chain-terminator during reverse transcription, thereby preventing the virus from copying its RNA genome into DNA. NNRTIs bind to the RT enzyme at a domain that is distant from the active site, but binding results in a conformational change and inhibition of enzyme function (87). PIs bind to the active site of the PR enzyme, thereby preventing cleavage and maturation of the viral polyproteins (27). New classes of inhibitors include the entry inhibitors that interfere with binding and/or fusion of HIV-1 with the host cell and integrase inhibitors that block the IN enzyme, thereby preventing insertion of the viral DNA genome into the chromosome of the infected cells.

Anti-HIV chemotherapy has been very successful over the past 25 years from the first approved anti-HIV agent in 1987 to the triple drug cocktails that are in use today as HAART. Despite the success of HAART in decreasing AIDS-related mortality, a substantial fraction of HIV-infected patients experience therapy failure accompanied by the emergence of drug-resistant viruses (54,74). In addition, a lifelong regimen is required to suppress the virus and patients may suffer from severe side effects. Thus, there is a need for alternative therapeutic approaches. One such strategy is an RNAi-based gene therapy to deliver antiviral genes that interfere with viral replication in order to make cells resistant to HIV-1. A durable anti-HIV gene therapy has the potential to significantly improve the quality of life of HIV-1 infected individuals.

2. RNA interference mechanism

RNAi is an evolutionarily conserved post-transcriptional gene silencing mechanism in eukaryotes that is triggered by double stranded RNA (dsRNA) (26,67). The key players in the RNAi mechanism are small noncoding dsRNAs of 20 to 30 basepairs (bp) in length, with a 5′ monophosphate, a 3′ hydroxyl group and 3′ dinucleotide overhang (12,69). Several classes of these small noncoding RNAs exist, including microRNAs (miRNAs), small interfering RNAs (siRNAs) and Piwi-interacting RNAs (piRNAs). These RNAs associate with a specific member of the Argonaute protein family (AGO) and the combination of small RNA and AGO protein determines the biological function. The biological role of RNAi in eukaryotes includes regulation of gene expression by promoting degradation or translational repression of target mRNAs, modification of chromosome structure and protection from mobile genetic elements (50).

Mature miRNAs are transcribed in the nucleus as primary miRNAs (pri-miRNAs) by RNA polymerase (Pol II, occasionally Pol III) and are subsequently processed by the Microprocessor complex, comprising ribonuclease (RNase) III Drosha and subunit DCGR8, into ~70 nucleotide (nt) pre-miRNAs (23,36) (Fig. 3). Pre-miRNAs fold a hairpin structure with a terminal loop and usually have interspersed mismatches or bulges along the RNA duplex. The pre-miRNA associates with Exportin 5, which recognizes the 2-nt 3′ overhang (characteristic of RNase III
Figure 3. The mammalian RNAi interference pathway. Host pri-miRNAs and exogenous shRNAs are transcribed in the nucleus, processed by Drosha and DGCR8 and subsequently transported by Exportin 5 to the cytoplasm. Further processing takes place by Dicer, eventually leading to RISC programming to induce gene silencing by translational repression or cleavage of the mRNA. The position where long dsRNA and siRNAs enter the RNAi pathway is also indicated, bypassing several processing steps in the pathway. See text for further details.
mediated cleavage) and facilitates the export to the cytoplasm (109). The cytoplasmic complex that contains RNase III Dicer, TAR RNA-binding protein (TRBP) and PACT processes pre-miRNAs into the mature miRNA duplexes of 20-24 bp. The miRNA duplex associates with an AGO protein within the precursor RNAi-induced silencing complex (pre-RISC) (63,79). Either miRNA strand can mediate gene silencing, but many miRNAs show asymmetry, preferentially loading one of the strands (guide strand) into RISC. Thermodynamic properties largely determine which strand of the duplex will be incorporated as guide into RISC (51,84). The remaining strand of the duplex (passenger strand) is cleaved and degraded. The mature RISC contains a single-stranded small RNA, which guides the RISC complex to a complementary target mRNA for post-transcriptional gene silencing (PTGS). Watson-Crick base pairing between miRNA and target mRNA is usually partial, but with high complementarity at nt 2-8 from the 3' end of the miRNA, which is known as the seed region (55,110). The expression of miRNAs is often tissue specific and developmentally regulated, and this regulation occurs both transcriptionally and post-transcriptionally. Regulated miRNA expression is important and altered miRNA expression has been linked to human diseases. miRNAs are predicted to regulate up to 30% of the protein-coding mRNAs, implicating them in almost all cellular processes including cell differentiation, proliferation and apoptosis (3,5,6).

Apart from cellular miRNAs, long dsRNA virus replication intermediates or transgene expressed short hairpin RNAs (shRNAs) of exogenous origin are processed in a similar manner by the RNAi machinery into siRNAs that are generally fully complementary to their target mRNA (Fig. 3), triggering sequence-specific target cleavage (41). Long dsRNA usually originates from infections with RNA viruses, inverted repeat containing transgenes or aberrant transcription products. The RNAi pathway can also be induced by artificial substrates, transiently by siRNA and stably by an expressed shRNA precursor (15,24). siRNAs can also be of endogenous origin, known as endo-siRNAs. In Drosophila Melanogaster endo-siRNAs originate from convergent transcription units or from annealing of sense and antisense RNAs from unlinked loci (34,70). The only endo-siRNAs of mammalian origin have been detected in mouse oocytes and embryonic stem cells and were derived from retrotransposons or pseudogenes (89,101,102).

2.1 The importance of the miRNA and shRNAs terminal loop
The sequence and structural configuration are important features of miRNAs and shRNAs and have been shown to influence their RNAi activity (64). The loop sequence is likely to have an effect on Dicer recognition or processing of the shRNA (44,64). Recognition elements or sequences within the miRNAs or shRNAs that determine the processing efficiency have not been examined in detail. The loop of a hairpin may function as binding site for a co-factor that determines Dicer specificity (96). It was recently shown that a class of miRNAs is recognized by KSRP (KH-type splicing regulatory protein) through high affinity binding to loop sequences, which facilitates both nuclear Drosha- and cytoplasmic Dicer-mediated processing (91). For pre-miRNAs, mutations of the loop sequences affected or even completely abrogated miRNA processing (58,65). The hnRNP A1 protein that has been implicated in many aspects of RNA biosynthesis and processing was also reported to interact with a miRNA cluster to specifically promote the production of the pri-miR18a unit (37). This ubiquitously expressed protein was shown to act as an auxiliary factor at the level of Drosha processing. Subsequent studies indicated that hnRNP A1 binds to the loop of pri-miR-18a to induce a relaxation of the
stem, thereby creating a more favorable template for Drosha cleavage (65). Interestingly, the same study argued that the high sequence conservation of certain miRNA loop sequences throughout evolution suggests a requirement for other not yet identified auxiliary factors to facilitate miRNA processing.

2.2 The Argonaute proteins

One key component of all RNAi related pathways is the AGO protein. Human cells have four AGO genes, AGO1-AGO4. In mammals, Dicer assorts the siRNAs and miRNAs among the different AGO proteins. The AGO proteins exhibit different affinities for certain RNA molecules, leading to either PTGS or transcriptional gene silencing (TGS). This sorting is influenced by Dicer processing, the structure of the small RNA duplex (thermodynamic properties) and its terminal nucleotides (28,61,100). For instance, miRNAs derived from imperfectly basepaired hairpin structures are preferentially loaded into AGO1 containing RISC complexes, whereas siRNAs combine with AGO2 complexes. Only the AGO2 protein is known to have “slicer” activity, executed by a catalytically active RNaseH-like domain that can cleave the target mRNA (25). Endonucleolytic cleavage of the target mRNA occurs opposite nucleotide position 10 and 11 of the annealed guide strand of the siRNA. The products are released from RISC and subsequently degraded by the endonuclease complex (59).

It was recently discovered that there are some distinct exceptions to the regular RNAi processing pathways. Several studies showed that specific pre-miRNA hairpins can be loaded directly into RISC, without a requirement for initial Dicer-mediated processing. This alternative miRNA processing pathway is thus independent of Dicer, but requires AGO2 catalytic activity (19,20,107). The slicer activity of AGO2 cleaves the passenger strand of these duplexes, after which the cleaved products dissociate from RISC.

3. INTERACTION BETWEEN HIV-1 AND THE RNAi PATHWAY

RNAi is an attractive means to inhibit viruses in a sequence-specific manner (13,40,46). Transient siRNA treatment can be developed for acute infections with e.g. influenza virus A and respiratory syncytial virus. But for chronic infections with HIV-1, hepatitis B (HBV) and hepatitis C virus (HCV) a constant supply of siRNAs would seem required. A gene therapy with shRNA expression vectors can provide a constant supply of antiviral siRNAs (39,90).

Accumulating evidence suggests that RNAi plays a role as antiviral defense mechanism in mammalian cells, as it does in plants. Mammalian viruses closely interact with the host miRNA pathway to modulate the course of virus infection. Several early studies suggested that virus infection in mammalian cells can be influenced or counteracted by cellular miRNAs (53,72,103). Recently, evidence emerged that a set of cellular miRNAs, which are enriched in primary resting CD4+ T lymphocytes, contribute to proviral latency via interactions with the 3’ untranslated region (3’UTR) of the HIV-1 mRNAs (45). Other reports also hinted at a suppressive role of the cellular RNAi machinery during HIV-1 replication. Inhibition of miRNA-29a led to enhanced HIV-1 production in human T-lymphocytes and HIV-1 replication was enhanced when miRNA production was impaired by knockdown of Drosha or Dicer (68,92). The cellular miR-17/92 cluster was actively suppressed upon HIV-1 infection in order to facilitate virus replication. The mRNA for histone acetylase PCAF has four potential targets in the 3’UTR for miR-17-5p and miR-20a
that are encoded by the miR-17/92 cluster. PCAF is an important cofactor for Tat transcriptional activation of an integrated HIV-1 provirus. Suppression of miR-17-5p/miR-20 leads to enhanced PCAF expression that triggers HIV-1 production (92). Finally, viruses may require a specific cellular miRNA for their replication cycle, exemplified by the liver-specific miRNA-122 that is critical for HCV replication by boosting the translation of viral mRNAs (43,48,49). These findings together may indicate a defensive role for miRNAs against viral infections in mammalian cells.

3.1 Virus encoded microRNAs: vmiRNA
Apart from cellular miRNAs, the virus itself can produce small RNA molecules to reprogram the cellular RNAi pathway. Herpesviruses encode more than 140 miRNAs in their large DNA genome (14). Epstein–Barr virus, cytomegalovirus, rhesus rhadinovirus and Kaposi’s sarcoma-associated herpesvirus were subsequently shown to express virus encoded miRNAs (vmiRNAs). With respect to gene organization, the vmiRNA genes resemble their cellular counterparts by being expressed either as a single gene or in clusters that allow coordinated expression (16,76,77,82). These vmiRNAs regulate fundamental processes by targeting both host and virus genes involved in antiviral immunity, apoptosis, viral latency and reactivation and other key steps of the virus replication cycle (14,88,94). Some vmiRNA sequences have been conserved during virus evolution, which suggests an important role in viral replication (62,86).

Several groups recently proposed that HIV-1 also encodes vmiRNAs. The structured trans-activation responsive (TAR) element of the HIV-1 RNA genome is a strong vmiRNA candidate (73). A TAR-derived vmiRNA was shown to regulate viral gene expression through chromatin remodeling of the viral LTR promoter. The TAR hairpin is present at the extreme 5’ end of all HIV-1 transcripts and forms a substrate for Dicer processing into small fragments of miRNA size (52,73,108). Candidate guide strand RNAs originate almost exclusively from the 3’ side of the TAR hairpin. These guides were predicted to target apoptosis related mRNAs, suggesting that TAR-derived vmiRNAs may prevent apoptosis of the infected cell in order to prolong virus production. A computational study was performed to predict five putative vmiRNAs encoded by HIV-1, including the TAR vmiRNA (10).

3.2 Virus derived small interfering RNAs: vsiRNAs
Virus infection of plants, insects, nematodes and fungi results in the production of virus-derived small interfering RNAs (vsiRNA) that are processed from dsRNA replication intermediates. These vsiRNAs instruct the RNAi pathway for sequence-specific cleavage of the perfectly complementary viral transcripts (85,97,99,104,106). Although the RNAi machinery is well conserved in mammals, similar vsiRNAs could not easily be detected in early studies with virus-infected cells of mammalian origin. Recent deep sequencing studies do however indicate that such vsiRNAs do arise in virus-infected mammalian cells. Small virus-derived RNAs have been detected in cells infected with Dengue virus, vesicular stomatitis virus, polio virus, HCV and West Nile virus (75). The 454-sequencing technology also revealed that transposon-specific siRNAs are produced in mouse oocytes to block transposon activity (102). Because retroviruses such as HIV-1 are related to retrotransposons in terms of integration of the proviral DNA genome in the host cell DNA, it is not unlikely that HIV-1-specific vsiRNAs can be produced in infected cells to regulate and possibly down-modulate virus replication. A recent study has reported the accumulation of small RNAs in HIV-1 infected cells (108). The exact biological function of
these vsiRNA has yet to be determined, as are the possible routes for production of these virus derived vsiRNA (66) (75).

3.3 Virus encoded RNAi suppressors
Like plant viruses, mammalian viruses also encode viral factors with RNAi suppressive activity, suggesting that RNAi may have an antiviral function in animals that needs to be counteracted by the invading viruses (2,9,38,57). Virus-encoded RNA silencing suppressors (RSS) are well known to block the antiviral RNAi activity in plants. Some RSS factors interfere with the RNAi pathway by binding siRNAs or cellular miRNAs, others prevent RNAi by binding to pre-Dicer substrates (53,56,57). The VP35 protein of Ebola virus is a RSS that suppresses RNAi by binding to dsRNAs, thus inhibiting Dicer activity. A very different RSS is encoded by the human adenovirus. The abundant and highly structured virus associated (VA) RNAs inhibit RNAi via Dicer and RISC saturation during virus infection (2). HIV-1 has also been reported to encodes RSS activity in the form of the Tat protein (9,83). Apart from the function of Tat to activate HIV-1 transcription from the viral LTR, it has been suggested that Tat inhibits Dicer activity by binding dsRNAs (8).

4. SCOPE OF THIS THESIS
RNAi can be used to inhibit HIV-1 replication by targeting the viral RNA genome. However, HIV-1 can generate RNAi-resistant variants with specific mutations in the target sequence. For durable inhibition of HIV-1 replication the emergence of such escape viruses must be controlled. In chapter 2 we present a strategy that anticipates HIV-1 escape by designing 2nd generation shRNAs that form a complete match with the viral escape sequences. By this approach popular viral escape routes can be blocked effectively, but virus evolution cannot be stopped. As a consequence viral evolution was skewed towards new escape routes. In chapter 3 we investigate an alternative strategy combining the activity of a protease inhibitor (PI) with 2nd generation shRNAs designed to specifically block the emergence of PI-resistant HIV-1 variants. We demonstrate that dominant viral escape routes can be blocked by 2nd generation shRNAs and that virus evolution can thus be halted or redirected towards second best solutions with virus variants that have a reduced fitness. In chapter 4 we investigated the development of more potent anti-HIV shRNAs and tested the impact of different hairpin loop sequences, varying in size and sequence. We were able to transform weak inhibitors into intermediate or even strong shRNA inhibitors by replacement of the loop sequence and demonstrate that the efficacy of these optimized shRNA inhibitors is improved significantly in diverse cell types due to increased siRNA production. Further analyses on additional shRNA constructs that differ in stem length and loop size were performed in chapter 5. We show that the stem length of the shRNAs is critical for the activation of a novel shRNA processing mechanism. A specific shRNA design was identified that triggers this alternative RNAi-processing mechanism, which is Dicer-independent but AGO2-dependent. Alternatively processed shRNAs yield only a single siRNA strand that shows potent inhibition, whereas conventional shRNA processing produces an siRNA duplex from which both strands can theoretically trigger RNAi.

A complex interplay exists between human viruses and components of the cellular RNAi machinery. The role of RNAi as an antiviral defence mechanism in mammalian cells has been under debate for some time. Although vsiRNAs from different mammalian viruses have recently been
identified, their functions and possible impact on viral replication remain unknown. In chapter 6 we study HIV-1 derived small RNAs. Specifically, we used deep sequencing to analyse vsiRNA accumulation in HIV-1 infected T-lymphocytes. We identify numerous small RNAs that correspond to the HIV-1 RNA genome and found evidence to suggest that HIV-1 infection triggers the antiviral RNAi defence mechanism by the production of vsiRNAs. In addition, we show that HIV-1 encodes candidate miRNAs that could modulate cellular and/or viral gene expression. In chapter 7 we report that deep sequencing approaches are particularly prone to misannotations and that two recently proposed miRNA genes are in fact endogenous retroviral elements. Furthermore, we propose that the small RNA fragments that led to the description of these miRNA candidates are likely derived from tRNA processing. In chapter 8 we determined the relative concentration of cellular miRNAs in a T cell line, before and after HIV-1 infection, and compared those values to miRNA concentrations in the produced virion particles. A small subset of the cellular miRNAs is dramatically concentrated in the virions, suggesting a biological function in HIV-1 replication.

REFERENCE LIST


