THE PKR/PACT RESPONSE PATHWAY IS ALTERED DURING HIV-1 INFECTION

Presented by

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ABSTRACT

The interferon response pathway is an important antiviral mechanism. Protein Kinase RNA-activated (PKR) is an Interferon-Stimulated Gene (ISG) activated by double-stranded RNA (dsRNA), such as the TAR RNA structure from HIV. PKR is one of the most studied ISGs and an efficient HIV suppressor. PKR phosphorylates the translation initiation factor eIF2α, resulting in the inhibition of cellular and viral protein synthesis and a block in viral replication. During HIV infection, our lab has shown that PKR is activated at the beginning of infection in Jurkat cells followed by a deactivation when the virus replicates. This is in part attributed to the inhibition of PKR by dsRNA-binding proteins (dsRBP) such as the TAR RNA binding protein (TRBP) and Adenosine deaminase acting on RNA (ADAR1). In addition, we recently found that another dsRBP and a PKR activator, PACT, becomes a PKR inhibitor in HIV-infected cells.

Our central hypothesis in the laboratory is that the regulation of PKR activation by dsRNA-binding proteins is critical for HIV to evade the innate immune response. Our objectives for this project were to answer these questions: 1) What is the activation status of PKR in HIV-infected Peripheral Blood Mononuclear Cells (PBMCs)? and 2) What changes PACT from a PKR activator to a PKR inhibitor during HIV infection?

1) PKR activation status in HIV-infected PBMCs: We observed a PKR induction and activation at the beginning of infection, followed by a deactivation when HIV replication increased. When IFN α/β was added to HIV-infected PBMCs, PKR activation was restored. This suggests that cells are able to activate PKR but this activation is blocked during HIV infection. Furthermore, PKR activation was moderately increased in HIV-infected untreated patients compared to HIV-

infected successfully treated patients. This further increases the importance of PKR during HIV-1 infection

2) Mechanism that alters PACT's function in HIV-infected cells: In contrast to its previously described function, when PACT was overexpressed in HIV-transfected HEK293T cells, PKR activation was inhibited and HIV expression was increased. We found that PACT-mediated increase in HIV-1 expression and production required the presence of PKR. Furthermore, PACT overexpression led to its incorporation in HIV-1 virions and altered Gag processing and HIV Env protein expression. In addition, we tested HIV-1 infectivity in TZM-bl cells and observed a decrease of infectivity in HIV-1 virions produced from PACT and HIV-1 transfected HEK293T cells. In a proteomic screen, we found that HIV-1 expression led to a complete change of PACT interactome when compared to PACT- interacting partners in mock transfected HEK293T cells.

In conclusion, the PKR/PACT response pathway is targeted during HIV-1 infection. PKR activation is not sustained and is inhibited by PACT, a PKR activator that switches its function to a PKR inhibitor during HIV-1 infection. In addition, HIV-1 induces a complete change in PACT interactome which might explain PACT's change of function. Understanding PKR regulation by HIV could aid in understanding viral persistence.

RÉSUMÉ

La voie de l'interféron est un mécanisme antiviral important. La protéine kinase activée par l'ARN, PKR, fait partie du groupe des gènes stimulés par l'interféron ou ISG et est activée par l'ARN double brin tel que la structure de l'ARN TAR du virus de l'immunodéficience humaine (VIH). PKR est une des ISG les plus étudiés et représente un suppresseur efficace du VIH. L'activité de PKR est de phosphoryler la sous-unité alpha du factor d'initiation de la traduction, eIF2α. Cela résulte en un arrêt de toute synthèse protéique dans la cellule et constitue un blocage dans la réplication virale. Notre laboratoire a démontré que PKR est activée au début de l'infection virale dans des cellules lymphocytes Jurkat suivi par la suite, d'une désactivation lorsque le virus se réplique activement. Cette inhibition de PKR est attribuable à l'action d'un groupe de protéines se liant à l' ARN double brin dont font partie la protéine qui se lie à l'ARN TAR, TRBP et l'adénosine déaminase agissant sur l'ARN, ADAR1. De plus, nous avons identifié une autre protéine se liant à l'ARN double brin et activateur de PKR, PACT, qui devient un inhibiteur de PKR dans des cellules infectées par le VIH. Notre hypothèse principale dans le laboratoire est que la régulation de l'activation de PKR par les protéines se liant à l'ARN double brin est primordiale pour le VIH pour évader le système immunitaire inné. Nos objectifs pour ce projet étaient d'adresser ces questions : 1) Quel est le statut d'activation de PKR dans les cellules mononucléées du sang périphérique infectées (PBMCs) par le VIH? et 2) Quel est le mécanisme qui change PACT d'activateur de PKR en inhibiteur de PKR durant l'infection par le VIH?

1. Le statut d'activation de PKR dans les PBMCs infectées par le VIH : Nous avons observé une induction de l'activation et l'expression de PKR au début de l'infection suivie d'une désactivation lorsque le virus se réplique activement. Lorsque nous avons traité les PBMCs infectées avec de l'interféron, l'activation de PKR a repris. Cela indique que les cellules sont

capables d'activer PKR lors d'une infection par le VIH mais que cette activation est bloquée par un mécanisme induit par le VIH. De plus, lors d'un profilage d'expression protéique dans les patients infectés, nous avons observé une activation modérée de PKR seulement dans les patients infectés par le VIH non-traités comparés aux patients traités avec succès. Ces observations augmentent l'importance de PKR durant l'infection par le VIH.

2. Le mécanisme changeant PACT d'activateur à inhibiteur de PKR : À l'inverse de sa fonction initiale comme activateur de PKR, la surexpression de PACT dans les cellules HEK293T exprimant le VIH inhibe l'activation de PKR et augmente l'expression et la production du VIH dans ces cellules. Nous avons démontré que cette augmentation de l'expression et de la production virale par PACT nécessitait la présence de PKR. De plus, la surexpression de PACT engendre son incorporation dans les virions du VIH et modifie la maturation de Gag et l'expression de l'enveloppe virale. Par la suite, nous avons testé l'infectivité du VIH dans les cellules TZM-bl. Nous avons observé une diminution de l'infectivité des virions produits dans les cellules transfectées avec le VIH en combinaison avec PACT. Nous avons procédé à un criblage protéomique pour identifier les partenaires de PACT qui pourraient jouer un rôle dans l'inhibition de PKR et nous avons trouvé que l'expression virale change presque complètement l'interactome de PACT.

En conclusion, la réponse de PKR/PACT est ciblée lors de l'infection par le VIH-1. L'activation de PKR est temporaire et est inhibée par PACT, un activateur de PKR qui devient un inhibiteur de PKR durant l'infection par le VIH-1. De plus, l'expression virale engendre un changement complet de l'interactome de PACT, ce qui pourrait expliquer son changement de fonction. Comprendre la régulation de PKR par le VIH-1 aiderait à comprendre la persistance virale.

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CONTRIBUTION OF AUTHORS

This thesis was written by me under the supervision of Dr. Gatignol. The introduction sections 1.5 to 1.6 were written by me with help from Dr. Gatignol and included in a review: “HIV-1 translation and its regulation by cellular factors PKR and PACT” by Burugu et al., published in *Virus Research* in November 2014. The results from Figure 4.1.1 and Figure 4.2.1 B (Left) are part of a manuscript “The PKR activator, PACT, becomes a PKR inhibitor during HIV-1 replication” by Clerzius et al., published in the *Retrovirology* in September 2013. Figure 4.1.1 was done by me whereas Figure 4.2.1 B (Left) was done by Dr. Guerline Clerzius.

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LIST OF ABBREVIATIONS

2-5A: 2’-5’ oligo adenylates

ABCE1: ATP binding cassette E1

ADAR1: Adenosine Deaminase acting on RNA1

AIDS: Acquired ImmunoDeficiency Syndrome

APOBEC3: Apolipoprotein B mRNA editing enzyme

ATP: Adenosine triphosphate

CA: HIV capsid

CypA: Cyclophilin A

dsRBD: Double stranded RNA binding domain

DV: Dengue Virus

FANCC: Fanconi Anemia complementation group C (FANCC)

GDP: Guanosine diphosphate

GTP: Guanosine triphosphate

HAART: Highly active antiretroviral therapy

HCV: Hepatitis C virus

HIV: Human immunodeficiency virus

HP68: Host protein 68

HSC: Hematopoietic stem cells

HSP: Heat shock proteins

HSV: Herpes Simplex Virus

IFN: Interferon

IFNAR: Interferon α-receptor

IKK: Inhibitor of NF-κB kinase

IL-2: Interleukin-2

ILF-3: Interleukin enhancer binding factor 3

IN: HIV integrase

IP: Immunoprecipitation

IRES: Internal ribosomal entry site

IRF3: Interferon regulatory factor 3

IRF7: Interferon regulatory factor 7

JAK: Janus kinase

JEV: Japanese encephalitis virus

LPS: Lipopolysaccharide

MA: HIV Matrix

MAPK: Mitogen activated protein kinase

MEK: MAPK kinase

MK2: MAPK activated kinase 2

MK3: MAPK activated kinase 3

NC: HIV Nucleocapsid

Nef: HIV Negative regulatory factor

NF90: Nuclear factor 90

NF-κB: Nuclear factor kappa-light chain enhancer of activated B cells

NNRTI: Non nucleoside reverse transcriptase inhibitor

NRTI: Nucleoside reverse transcriptase inhibitor

OAS: Oligoadenylate synthetase

PACT: PKR activator

PAMP: Pathogen associated molecular patterns

PBMC: Peripheral blood mononuclear cells

PBS: Phosphate buffer saline

pDC: Plasmacytoid dendritic cells

PIC: HIV preintegration complex

PKR: Protein Kinase RNA-activated

PP1α: Protein phosphatase 1α

PR: HIV protease

PRR: Pathogen recognition receptor

PV: Poliovirus

RAX: PKR activator mouse homolog

REV: HIV Regulator of viral expression

RIG-I: Retinoic acid-inducible gene

RLR: RIG-I like receptors

RNAi: RNA interference

RRE: HIV Rev-responsive element

RT: HIV reverse transcriptase

RVFV: Rift valley fever virus

siRNA: Small interfering RNA

SIV: Simian immunodeficiency virus

Slfn: Schlafen

STAT: Signal transducer and activator of transcription

TAT: Trans-activator of transcription

TBK1: TRAF associated NF-κB activator-binding kinase 1

TBS-T: Tris buffer saline containing 0.1 % tween20

TOSV: Toscana virus

TRAF: Tumor necrosis factor (TNF) receptor associated factor

TRIM: Tripartite motif

VHS : HSV Virion host shutoff protein

Vif: HIV viral infectivity factor

VLP : Virion-like particles

Vpr : HIV viral protein R

Vpu : HIV-1 viral protein U

Vpx : HIV-2 viral protein X

VV : Vaccinia Virus

ZBD :Z-DNA binding domain.

Chapter 1. Introduction

1.1 Human immunodeficiency Virus

1981 was the year when a new disease, the Acquired Immunodeficiency Syndrome (AIDS), was described in patients who exhibited various clinical pathologies such as lymphopenia and skin sarcomas[(1)]. After extensive research in Europe and US, a virus was isolated and classified as a separate virus from Human T Lymphoma Virus (HTLV) and was called by different names such as Lymphadenopathy-Associated Virus (LAV) or HTLV-III or AIDS-associated virus[(2, 3),(4)]. In 1986, a subcommittee of viral taxonomists recommended that the virus be renamed the Human Immunodeficiency Virus (HIV) [(5)]. Three decades after, HIV has infected more than 65 million individuals worldwide and more than half of those have died of AIDS according to UNAIDS.

1.1.1 Origins

Evolution studies identified the Simian Immunodeficiency Virus (SIV) infecting chimpanzees *pan troglodytes* (SIV_{chmpz}) as the source of HIV [(6-8)]. Indeed, SIV-infected chimpanzees develop pathologies similar to AIDS in contrast to gorillas and monkeys who do not develop AIDS [(8)]. By collecting fecal samples and analyzing the presence of SIV, SIV reservoirs were found in specific chimpanzee communities in West, Central and East Africa[(8)]. Those studies identified as Cameroon the plausible place of the first HIV infection. SIV transmission to human had probably occurred during a hunt in rural regions of Cameroon. The further risk of transmission between individuals depends on a number of factors such as the infectivity of the virus (i.e. biology of virus), the type of exposure (i.e. body fluids or blood) and the host immune system.

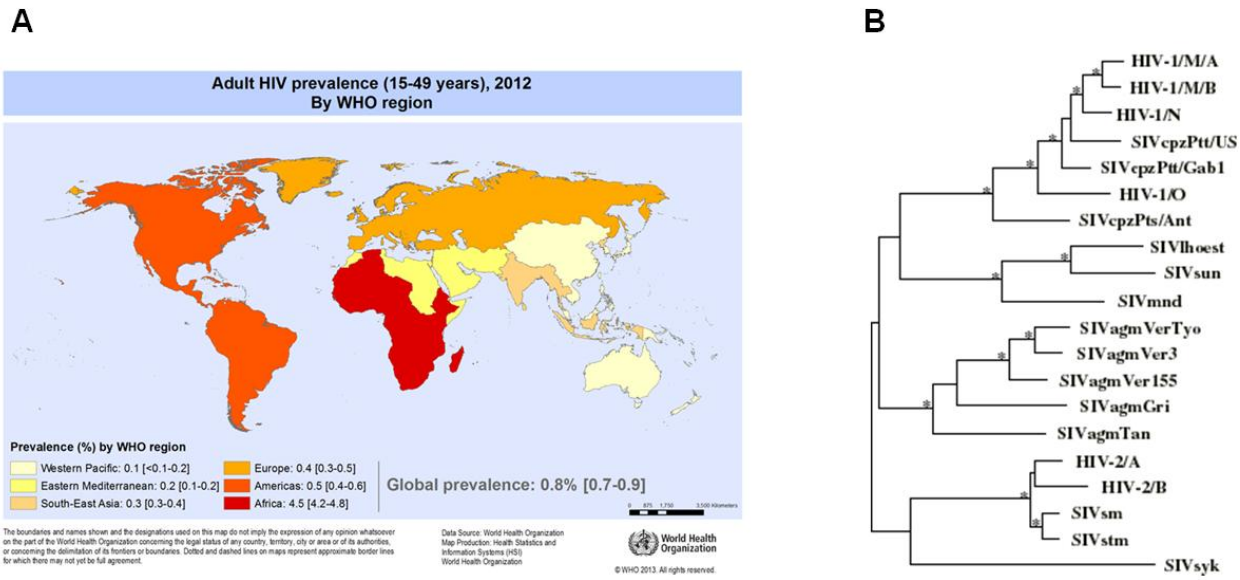

Figure 1.1 HIV prevalence and evolutionary tree. A, Heat map of HIV prevalence in the world. Warmer color indicates the highest prevalence and is mostly located in Sub-Saharan Africa. HIV prevalence in other regions of the world is also indicated on the map. Data obtained from UNAIDS.org website **B,** SIV and HIV evolutionary tree illustrates how HIV-1 is closely related to SIV from chimpanzees (SIVcpz) whereas HIV-2 is closely related to SIV infecting sooty mangabeys (SIVsm). Adapted from Beer B. E., et al, Diversity and evolution of primate lentiviruses, hiv.lanl.gov

To date, there are two types of HIV: HIV-1, predominant and most virulent, and HIV-2, less predominant and less pathogenic[(9)]. In each type of HIV, there are 4 and 8 different groups for HIV-1 and HIV-2, respectively. HIV-1 M is the major group, responsible for the pandemic; it is comprised of 10 subtypes of which B is mainly found in North America[(9)]. In addition, there are also Circulating Recombinant Forms (CRFs) which are viruses that emerged through recombination events upon dual infection of a single cell by various subtypes[(9)],[(10)]. CRF18 represents one of the most complex CRF as it comprises parts derived from 8 different subtypes [(11)].

1.1.2 HIV structure and genome

According to the taxonomy of viruses, HIV belongs to the lentivirus species, part of the *Retroviridae* family of viruses[(8)] . These viruses have in common a reverse transcription step in

their replication cycle, mediated by a viral enzyme called reverse transcriptase (RT) [8]. HIV genome, composed of two ssRNA copies, is contained in a capsid encompassed by an envelope which classifies HIV as part of the enveloped virus group[12].

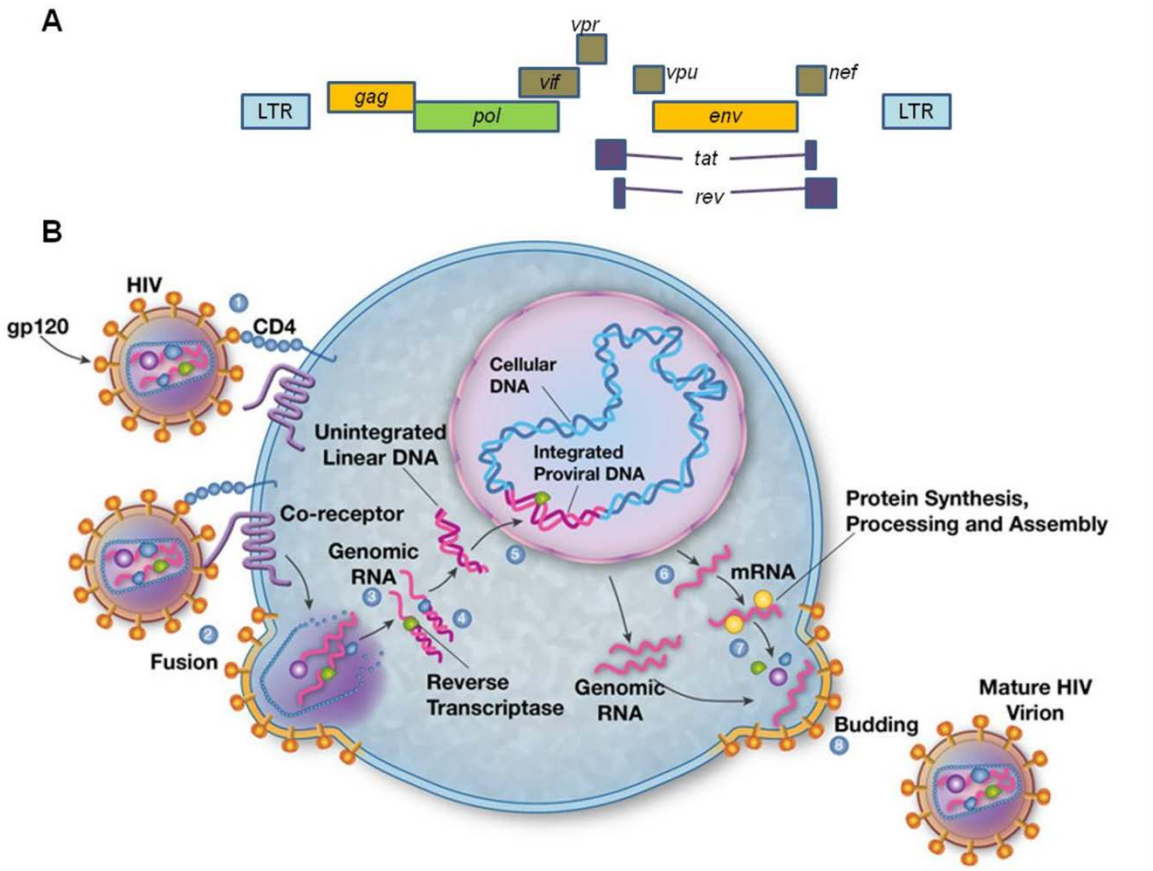

Figure 1.2 HIV-1 genome and replication cycle. A, HIV-1 genome codes for 9 genes: *gag, pol, vif, vpr, vpu, nef, env, tat* and *rev*. The Long Terminal Repeats (LTRs) contains elements required for HIV-1 transcription and are located in the 5' and 3' end of the HIV-1 genome. **B,** HIV-1 replication cycle steps are shown in the figure and include: 1) HIV-1 cell entry using the CD4 receptor and CCR5 or CXCR4 co-receptor ; 2) HIV-1 fusion to the cell membrane; 3) and 4) HIV-1 uncoating and reverse transcription; 5) HIV-1 DNA integration; 6) and 7) HIV-1 mRNA transcription and translation, 8) HIV-1 virion assembly and maturation. Adapted from www.vsbassociates.com

HIV-1 and HIV-2 genomes have 9 genes coding for a total of 15 proteins. Based on their functions, HIV proteins are divided into structural (Gag polyprotein, Pol polyprotein and Env),

regulatory (Tat and Rev) and accessory proteins (Nef, Vif, Vpr, Vpu of HIV-1, Vpx of HIV-2) [13].

1.2 HIV-1 replication cycle

The steps implicated in the replication cycle are crucial for viruses to spread successfully. Each step of the replication cycle is regulated, involves several host factors and begins with the virus entry.

1.2.1 Target cell

$CD4^+$ T lymphocytes are the main targets of HIV. This is explained by the presence of a CD4 receptor on $CD4^+$ T cell membranes required for HIV entry in the cell (Figure 1.2). Furthermore, CXCR4 and CCR5 are chemokine receptors that serve as the main HIV co-receptors to facilitate its binding and stability to the cell membrane[14, 15]. Other HIV-1 and 2 co-receptors have been also identified such as CCR2b and CCR3 [11].

The use of one co-receptor versus another depends on the virus strain. X4 strains bind to the CXCR4 co-receptor and R5 strain bind to the CCR5 co-receptor[16]. A switch to CXCR4 usage correlates with a rapid progression to an advance stage of the disease [16]. This switch might be initiated by an initial X4 and R5 co-infection but the mechanism is not clearly understood[17]. R5 strains are most predominant and this is explained by their ability to : 1) produce more infectious virions, 2) infect a larger pool of cells such as macrophages, non-activated and activated cells and 3) use CCR5 expressing cells known to evade immune recognition by Cytotoxic T lymphocytes[11] .

Furthermore, CCR5 raised a lot of interest as individuals who have a natural homozygous Δ32 mutation in *CCR5* gene are completely resistant to HIV infection and the mutation does not

affect their global health[18, 19]. In addition, the importance of CCR5Δ32 mutation in HIV resistance was illustrated in a case where an HIV-infected individual who had leukemia was cured of HIV after a stem-cell transplant from a CCR5Δ32 donor[20]. There are extensive studies focused on reproducing these results by finding tools to mutate or decrease the expression of CCR5 in HIV-infected individuals such as gene therapy[16].

1.2.2 Fusion and entry in the cell

After binding to the CD4 receptor and CCR5 or CXCR4 co-receptor, HIV enters the cell by fusion with the cell membrane or through endocytic pathways [21, 22]. Following HIV entry in the cell, the coated viral core enters the cytoplasm where the uncoating process, i.e. dissolution of capsid core, begins (Figure 1.2). The precise time where the viral core is uncoated is still in debate [23]. Some studies have proposed that the uncoating happens before the reverse transcription step, other groups suggested that during the transport to the nucleus, the viral core is gradually uncoated in response to several molecular changes in the cells including reverse transcription and finally a third model suggests that the uncoating of the viral core only happens when the reverse transcription step has terminated[23, 24]. The detection of capsid proteins are the main reasons for these discrepancies.

1.2.3 Reverse transcription

The process of reverse transcription is mediated by RT that converts viral RNA into DNA (Figure 1.2). RT is an enzyme with two subunits. The catalytic subunit (p66) has different roles such as a polymerase activity and an RNase activity to degrade viral RNA template while the viral DNA is being synthesized[25] . Reverse transcription is also regulated by cellular proteins. It

uses the tRNA lys as a primer forming then a multiprotein complex called the Reverse Transcription Complex [26]. Upon production of the viral DNA, the RT complex converges into the pre-integration complex to initiate integration of viral DNA into the host genome [24, 26].

1.2.4 Integration in the host genome

HIV integrase (IN) is the critical component of the pre-integration complex (PIC) (Figure 1.2). In addition, other PIC components include HIV Vpr [27] and cellular factors such as the Barrier to autointegration factor, the high mobility group chromosomal protein A1 and the lens epithelium-derived growth factor[28]. These viral and cellular components modulate different stages of the integration process such as the transport of HIV DNA into the nucleus via nucleopores. Once in the nucleus, HIV IN mediates the nucleophilic attack of the target DNAand DNA repair is accomplished by the non homologous end joining process[25,26]. The site of HIV DNA integration is not random[29]. Indeed, HIV prefers sites of integration, which are common to other lentiviruses, that include transcriptionnally active sites and dense gene regions[29, 30].

1.2.5 HIV-1 transcription and translation

HIV transcription by the cellular polymerase, Pol II, occurs after chromatin remodeling by cellular factors [31](Figure 1.2). At the early stage, HIV transcription is at basal levels and HIV full length RNA (9 kb) is doubly spliced into RNAs (2 kb) encoding 3 HIV proteins: The Trans-activator protein (Tat), the Regulator of viral expression (Rev) and the Negative regulatory Factor (Nef)[31-34]. Tat and Rev enter the nucleus while Nef remains in the cytoplasm. In the nucleus, Tat binds to the TAR RNA structure present in the 5'UTR of all HIV mRNA transcripts and increases HIV transcription by hundred folds[33, 35, 36]. This crucial transactivation

mechanism involves the recruitment by Tat of a transcription complex composed of Cyclin-dependent Kinase 9 and Cyclin T leading to phosphorylation of the carboxy-terminal domain of the RNA Pol II and subsequent increase in efficient elongation[31, 33, 35, 36]. Nuclear export of HIV mRNAs is mediated by Rev that targets Rev-responsive element (RRE) present in HIV mRNAs such as the unspliced 9 kb RNAs coding for Gag and Gag-Pol and singly spliced RNAs (4 kb) that code for Env, Vif, Vpr and Vpu[32, 34, 37]. Translation of HIV mRNAs is ensured by the cellular machinery and produces the remaining proteins: Gag polyprotein (containing the capsid (CA), the matrix (MA), the nucleocapsid (NC), p6), Gag Pol polyprotein (RT, IN and PR) and Env (gp 120, gp41), Vif, Vpr and Vpu[38-40].

1.2.6 Assembly and maturation

During virus assembly, Virion-like particles (VLP) are formed at the host cell membrane (Figure 1.2). This process involves the recruitment of viral components containing the packaging signal (or psi signal) that include two copies of full length HIV RNA and viral enzymes (RT, IN and protease (PR)) and the process is mediated by Gag [41]. In this process, various cellular proteins have been shown to be incorporated into VLPs[42] . After HIV virions have assembled at the cell membrane, they move out of the cell by budding. This process is responsible for the phospholipid bilayer present on the surface of HIV virions. At this stage, HIV virions, composed of viral proteins including uncleaved Gag polyprotein and viral RNA, are non-infectious and are termed immature [43]. The maturation process involves a cleavage of Gag and Gag-Pol by PR and restructuration of the matrix and capsid [43]. The mature virions are then able to perpetuate the replication cycle by infecting a new cell.

1.3 Clinical features

1.3.1 AIDS

During HIV infection, the innate and adaptive immune systems are in constant battle against HIV. Eventually, without treatment, HIV takes over and wins this battle in a majority of individuals. These individuals progress to an association of various pathologies and opportunistic infections known as AIDS[44, 45]. These HIV-associated pathologies target various organs such as the brain, the kidneys, the skin and the respiratory system [45-48].The progression time to AIDS varies between individuals and depends on various factors such as the treatment initiation time and the individual immune system.

At the beginning of HIV infection, known as early phase, the individual $CD4^+$ counts drop significantly and have a viral load of about 20000 copies/ml of blood[11, 49]. At that point, HIV-infected individuals who keep undetectable virus in the absence of therapy, i.e. less than 50 RNA copies/ml of blood, are called elite controllers[50, 51]. The immune system of elite controllers has been studied extensively in order to find new tools to fight HIV pathogenesis[52-56]

1.3.2 Therapies

Current therapies used to treat HIV-infected individuals consist of a cocktail of drugs targeting different steps of the virus replication cycle such as RT inhibitors, IN inhibitors, PR inhibitors and entry inhibitors (Table 1). It is known as Highly Active AntiRetroviral Therapy (HAART). The success of this therapy is illustrated by an increase of life expectancy of HIV-infected individuals to nearly normal levels but has been slowed down by the emergence of resistance to some of the drugs therefore, decreasing the drug efficacy[57, 58].

Table 1. List of FDA approved drugs for treatment of HIV infection.

Class of drugs	Drugs
Multi-class combination products	Atripla (BMS and GS), Complera(GS), Stribild (GS)
Nucleoside Reverse Transcriptase Inhibitors (NRTIs)	Combivir (GSK), Emtriva (GS), Epivir (GSK), Epzicom (GSK), Hivid (HLR), Retrovir (GSK), Trizivir (GSK), Truvada (GS), Videx EC (BMS), Videx (BMS), Viread (GS), Zerit (BMS), Ziagen (GSK)
Non-Nucleoside Reverse Transcriptase Inhibitors(NNRTIs)	Edurant (TT), Intelence (TT), Rescriptor (Pfizer), Sustiva (BMS), Viramune (BI)
Protease Inhibitors	Agenerase (GSK), Aptivus (BI), Crixivan (Merck), Fortovase (HLR), Invirase (HLR), Kaletra (AL), Lexiva (GSK), Norvir (AL), Prezista (TT), Reyataz (BMS), Viracept (AP)
Fusion Inhibitor	Fuzeon (HLR and Trimeris)
Entry inhibitor: CCR5 co-receptor antagonist	Selzentry (Pfizer)
HIV Integrase strand transfer inhibitor	Isentress (Merck), Tivicay (GSK)

Table illustrating all the FDA drugs approved for treatment of HIV infection. The manufacturers are indicated in brackets by their acronyms: Bristol-Myers Squibb (BMC), Gilead Science (GS), GlaxoSmithKline (GSK), Hoffmann La Roche (HLR), Tibotec Therapeutics (TT), Boehringer Ingelheim (BI), Abbott Laboratories (AL), Agouron Pharmaceuticals (AP). Information obtained from fda.gov website.

1.4 Innate immune responses to viruses

Antiviral mechanisms have evolved in complexity in concordance with pathogens. Whereas plants and invertebrates antiviral strategies mainly rely on suppression of viral genomic RNA through the RNA interference (RNAi) pathway, vertebrates produce viral interfering molecules known as Interferons (IFN)[(59)]. IFN is a component of the innate immunity and serves as the main antiviral tool. There are 3 types of IFN: Type I to III. Type I (α/β) and III IFNs are mainly produced in plasmacytoid dendritic cells (pDC) and macrophages whereas Type II IFNs are mainly induced by T cells and natural killer cells. The antiviral IFN response mediated by Type I IFN α/β will be discussed here.

The upstream signals leading to cellular production of IFN consist of interactions between pathogen-associated molecular patterns (PAMPs) and pathogen recognition receptor (PRRs) followed by a signalling cascade associated with numerous proteins. PAMPs such as viral RNA, DNA and proteins are recognized by PRRs such as Toll-like receptors (TLR) and Retinoic acid-Inducible Gene 1(RIG-I) like receptors (RLR)[60,61]. TLRs are located in endosomal compartments and on cellular membranes whereas RLRs are located in the cytoplasm and thus, these various locations enhance the virus surveillance system [60, 61] (Figure 1.3).

1.4.1 IFN production and activity during HIV-1 infection

HIV entry in the cell is sensed by different PRRs. Some PRRs that recognize HIV than bypass have direct antiviral effects. These PRRs, the apolipoprotein B mRNA editing enzyme (APOBEC3), Tetherin and TRIM5α, restrict HIV-1 replication in different manners[59]. Indeed, APOBEC3G, a member of the APOBEC3 proteins, diminishes HIV-1 replication by inducing cytidine deamination in the HIV-1 genome, Tetherin inhibits virion release from the infected cell, whereas TRIM5α blocks HIV-1 post-entry replication step through multiple mechanisms that involve TRIM5α-cyclophilin A interactions[61-65]. Furthermore, viral reverse transcription intermediates (ssRNA, RNA-DNA hybrid and ssDNA/dsDNA) are able to activate various PRRs such as TLR7 in pDCs and Trex-1, an endonuclease that cleaves DNA[66]. In addition, HIV-1 RNA activates RIG-I, part of the RLRs. This activation is inhibited by HIV protease action which targets RIG-I to the lysosome[67].

TLR and RLR activation lead to a signalling cascade, mediated by the adaptor protein Myd88, which results in the activation of IFNα/β gene expression by a transcription factor (Figure 1.3).

The IFNα gene is transcribed following the activation of the transcription factor Interferon Regulatory Factor (IRF) 7 whereas IRF3 induces IFNβ gene transcription. In addition, IFNβ gene expression can also be induced by the activation of the Nuclear factor of kappa light chain enhancer of activated B cells (NF-κB)[64].

IRF3 is widely expressed in most cells whereas IRF7 is mostly restricted to pDCs[68]. HIV disrupts IRF3 signalling by inducing IRF3 degradation, and as a consequence less IFNβ is produced in the infected cell[68, 69]. Depletion of IRF3 contributes to the diminished efficacy of the innate immune response observed during HIV infection[69]. HIV-mediated IRF3 degradation was attributed to Vpu whereas another group showed that Vpu inhibits NF-κB activation[69-71]. Nevertheless, both studies show that IFNβ production is altered in HIV-1 infected macrophages. In contrast, high levels of IFNα are observed in the serum of chronically HIV-infected individuals and are thought to serve as an indicator for AIDS progression [72-74]. This elevated IFN response seems to be more detrimental to the host as it creates a chronic immune activation leading to depletion of $CD4^+$ T cells but the correlation between IFN and HIV pathogenicity remains unclear[75, 76].

During a viral infection, macrophages and pDCs secrete IFNα/β that act on interferon α/β receptors (IFNAR) located on membranes of neighbouring non-infected cells to set an antiviral state[60](Figure 1.3). In addition, they act in an autocrine fashion to inhibit virus spread. Signalling through IFNAR1/2 activates the JAK-STAT pathway leading to the transcription of more than 300 Interferon Stimulated Genes (ISG) part of the IFN response. ISGs include Adenosine Deaminase Acting on RNA (ADAR1), 2'-5' oligoadenylate synthetase and RNAse L, Protein Kinase RNA-activated (PKR) and Mx GTPase (Figure 1.3). A general down regulation

of ISGs is observed during HIV infection in macrophages which contributes to HIV activity to circumvent the IFN response[77].

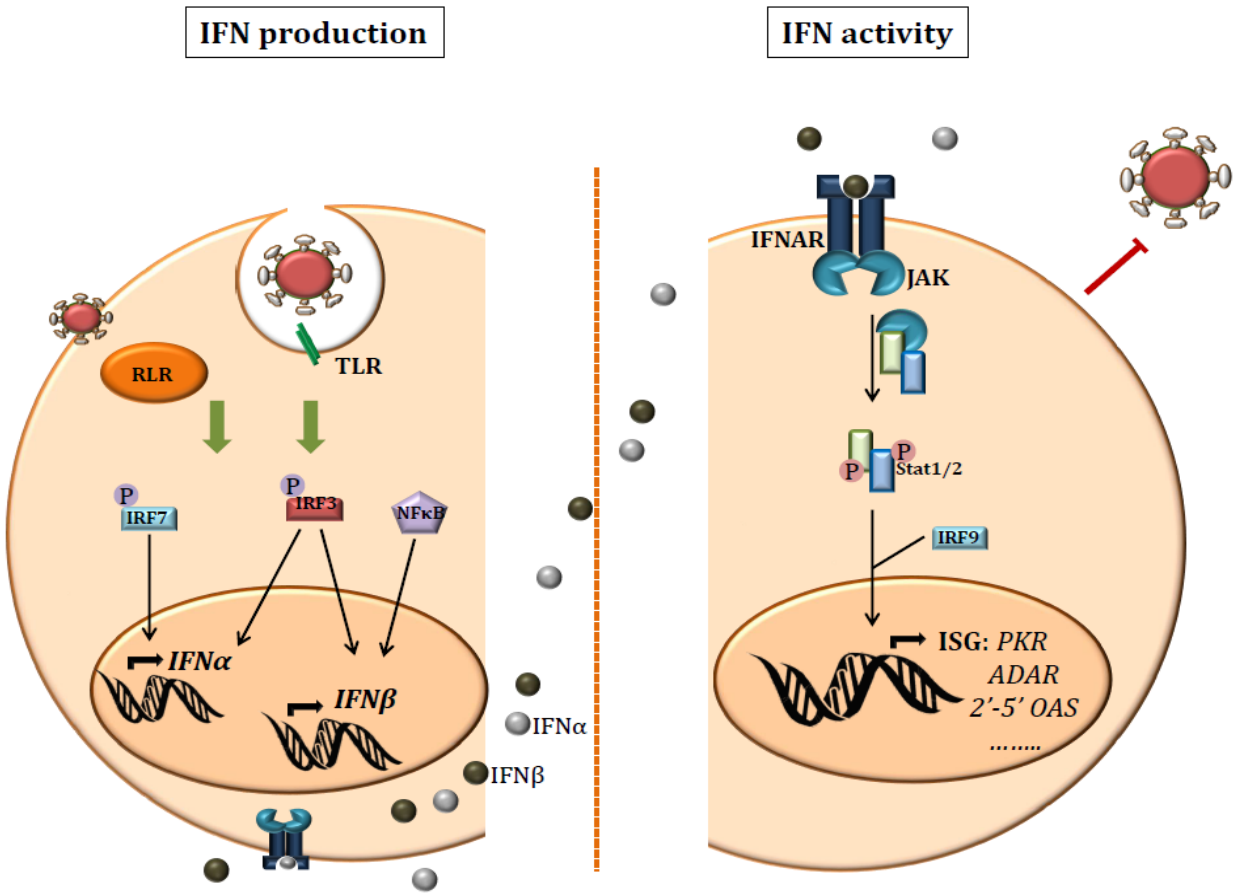

Figure 1.3 IFN production and activity. (Left) IFN production in a cell infected by a virus. The viral RNA is recognized by one or more PRRs, such as RLRs and TLRs. Activation of PRRs by the virus leads to a signalling cascade that activates the transcription of type I IFN genes (IFNα, IFNβ) by three activated transcription factors (IRF3, IRF7 and NF-κB). IFNα/β is secreted from the infected cell. **(Right)** IFN activity. IFNα/β binds to the IFN alpha-receptor (IFNAR) on the surface of non-infected cells and infected cells. IFNAR-IFNα/β interaction leads to activation of JAK-STAT signalling pathway and formation of a trimer with the transcription factor IRF9. This trimer translocates in to the nucleus and binds to the ISRE DNA on responsive promoters, inducing the production of more than 300 ISGs.

1.4.2 ISGs acting on HIV-1 translation

Due to the broad and diverse antiviral mechanisms exerted by ISGs, we will focus on those involved in HIV-1 viral mRNA translation.

1.4.2.1 2'-5' oligoadenylate synthetase (2'-5' OAS) and RNAse L

2'-5' OAS is activated by dsRNA and produces 2'-5'oligo adenylates (2-5A) from cellular ATP. 2-5A activates RNase L, a protein that degrades cellular and viral RNAs and serves as an efficient antiviral tool by preventing viral protein expression[78, 79]. 2'-5' OAS and RNAse L are active against many RNA and DNA viruses[79]. In several cases, the different forms of the OAS protein exert various antiviral effects[80-85]. Viruses have also evolved mechanisms to regulate the activity of either 2'-5' OAS or RNAse L activity through the expression of viral components or by the induction of cellular inhibitors[86-90]. In addition, the RNase L inhibitor (also called HP68 or ABCE1) induced during HIV-1 replication also has a post-translational role by regulating HIV-1 capsid assembly[79, 89, 91].

1.4.2.2 ADAR1

ADAR1 mediates the Adenosine to Inosine (A-to-I) editing on dsRNAs, including mRNAs, small RNAs and viral RNAs. Inosines are recognized as Guanosines by the translational machinery leading to A-to-G mutations and alterations in translation. ADAR1 contains two N-terminal Z-DNA binding domains (ZBD), three double stranded RNA binding domains (dsRBDs) and a catalytic domain mediating the editing function[92-95]. Viral edited transcripts from different viruses have been observed in infected cells but surprisingly, the function of this editing is an enhancement of viral replication[96, 97]. In HIV-infected cells, ADAR1 editing is

observed in the 5'UTR, in the *tat*, *rev* and *env* genes. In addition, ADAR1 stimulates virion release and increases virion infectivity. Overall these modified sequences increase viral replication, but the exact mechanism is not understood[98, 99]. Similarly, ADAR2 also edits HIV-1 RNA, increases viral production and virion release, but does not affect viral infectivity [100]. In contrast, in one study, A-to-G mutations by ADAR1 editing affected Rev-RRE binding and RNA transport resulting in decreased viral replication[101]. The difference between these studies is not currently understood but may be due to different experimental settings. Furthermore, ADAR1 has an additional function by binding to PKR and inhibiting its activation, which results in enhanced HIV-1 translation[98, 102].

1.4.2.3 Schlafen 11 (slfn11)

The Schlafen (slfn) family of proteins are involved in the control of cell proliferation, T cell development as well as the regulation of bacterial and viral infections[103]. Several members of the murine and human Slfn proteins are ISGs. Slfn11 has been recently shown to restrict HIV-1 replication. Slfn11 has an RNA helicase domain and inhibits HIV-1 protein synthesis via a viral codon bias usage. Indeed, HIV-1 uses a relatively different synonymous codon usage (also known as the codon bias) than the one used by the host[104, 105]. It was shown that Slfn11 selectively inhibited the expression of an HIV-1 Gag vector that used a codon usage similar to HIV while it did not affect a similar HIV-1 Gag vector that was codon-optimized for human cell expression[92]. How HIV-1 counteracts this effect remains unknown[106, 107].

1.5 PKR a central immune response pathway

Viruses have evolved strategies to regulate the IFN response. Targeting PKR is among those strategies.

1.5.1 PKR response pathway

PKR was initially discovered in viral infected cells as an antiviral protein induced by IFN treatment[108, 109]. PKR is also implicated in the cellular response to stress such as regulation of certain components of the mitogen-activated protein kinases (MAPK) pathway and cellular response to cytokines[110].

1.5.1.1 Structure

PKR is a 551 amino acids long protein and is composed of two double-stranded RNA binding domains (dsRBD) in the N-terminus and one kinase catalytic domain in the C-terminus. PKR activation can be RNA-dependent by binding to viral RNA species through its dsRBD or RNA-independent through interaction with cellular factors[111](Figure 1.4).

1.5.1.2 PKR activators

To activate PKR in a RNA-dependent manner, the two dsRBDs bind to RNA species of a minimum of 15bp length and subsequent activation requires at least 30bp long RNAs[112]. The RNA structure, not the nucleotide sequence, is required for PKR activation. Moreover, this specificity of RNA structure allows the selection of dsRNA and not ssRNA or dsDNA. Thus, certain classes or categories of viruses can potently activate PKR, whereas others do not elicit a PKR response[112]. In that sense, PKR is activated in HTLV-1 and 2 infected cells but not by Sendai virus RNA products[112, 113].

The PKR ACTivator protein (PACT) was the first protein identified as a PKR activator in the absence of RNA [114]. PACT is activated by stress and its mechanism of action is further discussed in the next section. Additional cellular products that activate PKR in a RNA-

independent manner include ISG15, the IFNγ-mRNA 5'UTR pseudoknot, the tumor suppressor melanoma-associated gene 7(mda-7) and the transcription factor E2F-1[115, 116].

Following RNA-dependent or independent stimuli, PKR kinase activity leads to the dimerization of PKR molecules on the 3rd basic region and subsequent autophosphorylation on threonine 446 and threonine 551 on the C-terminus[110](Figure 1.4).

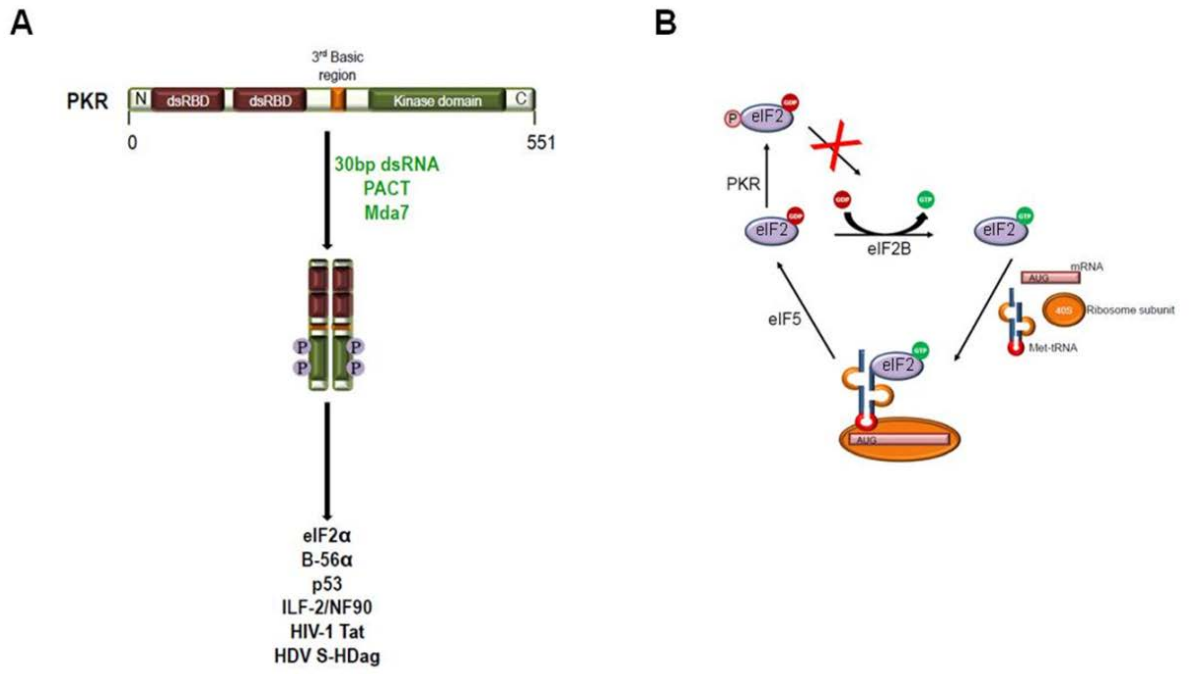

Figure 1.4 PKR activation. A, PKR protein domains are composed of two dsRBDs, a third basic region and a kinase domain on the C-terminus. Upon activation by 30bp dsRNA, PACT or MDA-7, PKR molecules dimerize through their 3rd basic region and induce a conformational change that activates the kinase domain. PKR molecules trans-autophosphorylate at threonine 446 and 551. Activated PKR phosphorylates its substrates, eIF2α, B56α, the tumour suppressor p53, ILF2-NF90, the HIV-1 Tat and the S-HDag of HDV. **B,** The initiation of mRNA translation is mediated by eIF2, a heterotrimeric complex composed of α, β and γ subunits. Activation of the initiation of mRNA translation is accomplished by the exchange of GDP bound to eIF2 to GTP by the guanine nucleotide exchange factor eIF2B. This enables the formation of a quaternary complex composed of eIF2-GTP, the mRNA to be translated, the methionine transfer RNA (Met-tRNA) and the 40S ribosomal subunit that initiates mRNA translation. eIF2 GTP to GDP state is mediated by eIF5, a GTPase-activating protein. In stress and viral conditions, eIF2α subunit is phosphorylated by PKR which blocks the GDP-GTP exchange required for the initiation of mRNA translation.

1.5.1.3 PKR targets

Once activated as a kinase, PKR phosphorylates the serine 51 of the translation initiation factor, eIF2α. This phosphorylation blocks the GDP-GTP exchange necessary for the recruitment of the ternary initiation complex Met tRNA-GTP-eIF2 of mRNA translation[(116)]. The resulting inhibition of the synthesis of eIF2α–dependent proteins affects a large number of cellular and viral proteins and thus participates to the antiviral activity linked to PKR.

The list of PKR cellular and viral targets is expanding as more targets are discovered. The current list includes B56αa (subunit of the phosphatase protein PP2A), the tumor suppressor protein p53, ILF-3 or NF90 (a subunit of a transcription factor called Nuclear Factor of activated T-cells), mitogen-activated protein kinase (MAPK) kinase 6 , Insulin Receptor Substrate 1(IRS-1), Cell division cycle 2 protein and HIV tat protein [(117,118)]. In addition, PKR is involved in other pathways such as NF-κB activation through association with TNF receptor associated factor (TRAF) in the NF-κB inhibitor kinase kinase (IKK) complex, cJun, p38 phosphorylation and was recently found to have a role in inflammasome activation [(119,120)].

1.5.2. PKR regulation by viral and cellular factors

Given that PKR activation is involved in diverse cellular pathways, PKR is regulated by various mechanisms. PKR is a target for many viral and cellular proteins, the latter being induced or not by viruses. These inhibitors are categorized by their mode of inhibition on PKR (Figure 1.5).

1.5.2.1 RNA sequestration

As mentioned above, dsRNA is a strong activator of PKR activity. Viruses employ different viral proteins to inhibit this step in the PKR pathway. Some of those viral proteins include the virion

host shutoff (VHS) RNase protein from herpes simplex virus (HSV) that degrades viral and cellular RNA structure that could potentially activate PKR[121]. Furthermore, VHS RNase activity is dependent on MAPK Kinase (MEK) expression. Indeed, PKR activation was restored only in ΔVHS virus-infected MEK deficient HT1080 cells, which is in concordance with a previous study showing PKR inhibition by MEK during HSV replication[121, 122]. Another strategy used by viruses to prevent PKR activation is to act on the production of dsRNA molecules. Indeed, K1 protein from Vaccinia virus (VV) inhibits PKR activation in VV-infected HeLa cells by diminishing the production of intermediate gene transcripts that have the potential to activate PKR[123]. The mechanism of action is unclear but it was ruled out that it is through dsRNA-K1 interactions as co-immunoprecipitation (IP) experiments showed no interactions[123, 124]. E3L protein from poxviruses is known to inhibit PKR by sequestering dsRNA and by directly binding to PKR[125]. E3L dsRBD, present in the C-terminus, sequesters dsRNA and heterodimerizes with the dsRBDs of PKR whereas the Zα domain of E3L N-terminus inhibits PKR activity by binding to PKR kinase domain[126].

1.5.2.2 Inhibitory binding to PKR

Cellular factors can be employed by viruses to counteract the innate immune response. The Influenza A virus NP protein counteracts PKR activation by using $p58^{IPK}$, a PKR cellular inhibitor. This mechanism of action involves the release of $p58^{IPK}$ from Heat shock protein 40 (Hsp40) binding, thus, leading to PKR inhibition by $p58^{IPK}$ [127]. In addition, Influenza virus induces inhibition of PKR kinase activity by forming complexes with p88rIPK and $p58^{IPK}$ upon MK2 and MK3 activation by p38 kinase [128]. Other cellular factors interacting with PKR and resulting in PKR inhibition include Hsp90 and Hsp70 complexed with Fanconi Anemia

complementation group C (FANCC). Hsp70 can be activated by dengue virus (DV) infection. In DV-infected cells, Hsp70 inhibition by siRNAs, correlates with an increase in PKR expression [(129)]. Furthermore, Hsp90 is necessary for replication of a variety of viruses and in many cases, Hsp90 uses Hsp70 as a co-factor [(129-131)]. This suggests that viruses might use this pathway to inhibit PKR activation.

Additional strategies employed by viruses to evade PKR activation are the use of cap-independent translation such as the Internal Ribosomal Entry Site (IRES). The domain II of Hepatitis C Virus (HCV) IRES activates PKR *in vitro*, whereas, in cell lines, HCV IRES competitively binds to PKR and inhibits its activation[(119,132)]. In addition, HCV outer protein, E2, is normally located in the ER lumen but unglycosylated E2 is in cytosol where it interacts with PKR and inhibits PKR activation[(133, 134)]. HCV NS5A protein also binds to PKR in the region between 244aa-296aa comprised in the domain responsible for PKR dimerization. As a consequence, there is an inhibition of PKR activation[(116)]. It was recently proposed that HCV NS5A-mediated PKR inhibition was through the interaction with HCV IRES domain II [(119)] . This further adds to the strategies employed by HCV to regulate PKR activation.

Furthermore, another study showed that a related *flavivirus*, the Japanese Encephalitis Virus (JEV), actively suppresses PKR activation and it was mediated by NS2A protein[(135)]. Indeed, at early time course of infection, NS2A binds to PKR and inhibits its activation possibly through inhibition of PKR dimerization like NS5 protein from HCV. In contrast, at later time course of infection, JEV NS2A was not able to inhibit PKR activation which indicates that PKR might be an antiviral factor in the beginning of infection and a proviral factor at later time course of infection[(135)].

1.5.2.3 Acting on eIF2α

An additional approach used by viruses to bypass PKR activation is to target the downstream effect of the PKR pathway: the phosphorylation of eIF2α. HSV protein γ_1 34.5 is involved in viral DNA synthesis and viral pathogenesis. In addition, γ_1 34.5 is able to inhibit PKR-induced translation inhibition by binding to a phosphatase, PP1α, leading to dephosphorylation of eIF2α[(116, 136)]. γ_1 34.5 also inhibits other members of the IFN response pathway such as TRAF associated NF-κB activator -binding kinase 1 (TBK1) activity[(136, 137)].

K3L is the second PKR inhibitor from poxviruses in addition to E3L viral protein. K3L mimics PKR substrate, eIF2α and gets phosphorylated by activated PKR[(127)]. Furthermore, K3L anti-PKR activity is enhanced when VV is challenged by serial propagation in human cells[(138)].

1.5.2.4 PKR degradation

PKR protein degradation is another measure used by viruses to counteract the inhibition of protein synthesis. Poliovirus (PV) infection elicits a strong PKR activation and eIF2α phosphorylation followed by a degradation of PKR. Despite the presence of PV-encoded proteases, PKR is degraded by cellular proteases and not by viral proteases and PKR-RNA interaction is required for this process[(116, 139)]. How cellular proteases are induced to cause PKR degradation remains unclear.

Another example is the Rift Valley Fever Virus (RVFV) that encodes for Non Structural proteins called NSs. These proteins play a crucial role in RVFV virulence such as suppression of host transcription[(140)]. Furthermore, RVFV bypasses PKR activation by inducing PKR degradation. This mechanism, mediated by NSs proteins, is through a proteasome-dependent pathway and is independent of their suppression of host transcription[(140-142)]. In addition, NSs from another

Bunyaviridae virus, Toscana virus (TOSV), also induces PKR degradation but not suppression of host transcription[(143)].

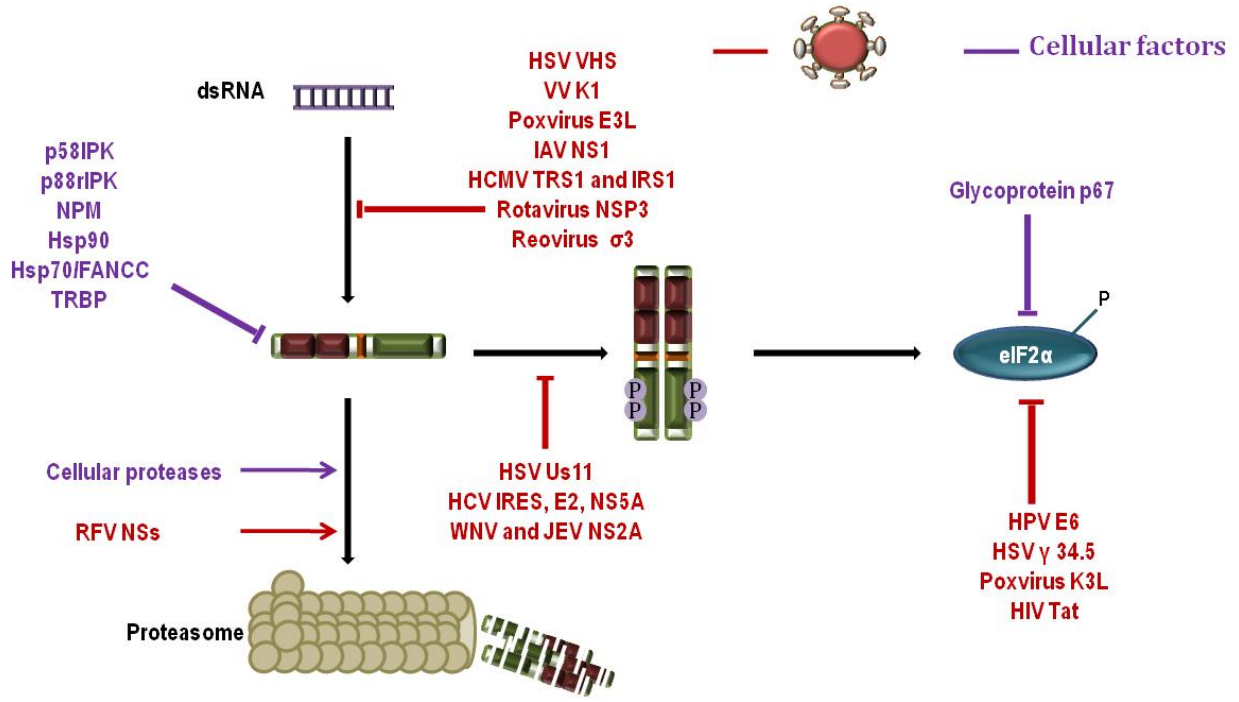

Figure 1.5 PKR inhibition by viral and cellular factors. PKR inhibitory viral factors are represented in red. Cellular inhibitory factors are in purple. Inhibitory mechanisms include: **(1)** limiting the availability of the dsRNA activator; **(2)** prevention of PKR activation by PKR sequestration; **(3)** inhibiting the phosphorylation of the substrate eIF2α; **(4)** targeting PKR for degradation in the proteasome.

1.6. HIV-1 anti-PKR mechanisms

HIV evades the immune system by various strategies. As part of the innate immune system, PKR regulates HIV-1 mRNA translation. HIV infection elicits a PKR response in two phases: an activation phase and an inhibition phase.

1.6.1 PKR activation phase

Following HIV-1 genome integration into the host's genome, HIV RNA transcripts are synthesized, all starting with the TAR RNA stem-loop structure. The TAR RNA is recognized by PKR and plays a dual role in its regulation. At low levels, TAR RNA is a potent PKR activator, whereas high concentrations of TAR inhibit PKR activation because PKR monomers are diluted out and can no longer dimerized [(84, 144, 145)]. Furthermore, TAR dimerization promotes the binding of two PKR molecules, therefore inducing its activation [(112)]. It is possible that other dsRNA structures from the HIV-1 RNA activate PKR, but those have not been studied. PKR is extremely effective in restricting HIV-1 translation and replication in cell culture suggesting that it is effectively activated upon viral expression[(102, 146-151)]. Furthermore, knocking down PKR by small interfering (si) RNAs or expressing a transdominant mutant of PKR increases HIV-1 production[(151)]. Infection of the lymphocytic cell line Jurkat by HIV-1 shows an activation of the already expressed PKR at the beginning of the infection[(102)]. This activation is not sustained throughout HIV replication and PKR is deactivated when the virus replicates at high levels[(102)]. Cumulative data show that viral and cellular factors contribute to PKR deactivation in HIV-1 expressing cells[(111)].

1.6.2 PKR inhibition phase

Although PKR activation is induced by HIV infection, our lab has shown that this activation is not sustained throughout HIV-1 replication in Jurkat cells[(102)]. It could be explained by cellular and viral factors present in HIV-1 infected cells which counteract PKR action. The TAR RNA

binding protein (TRBP), ADAR1 and PACT are among the cellular PKR inhibitors whereas Tat and TAR RNA represent the viral PKR inhibitors.

1.6.2.1 TAR RNA: As mentioned above, high amounts of TAR RNA structures produced during active HIV replication inhibit PKR. This inhibition could be explained by PKR molecules being diluted in comparison to increasing amount of TAR RNA species which will favour a 1:1 ratio of TAR: PKR. This will increase the formation of PKR monomers and decrease PKR dimers required for PKR *trans*-autophosphorylation [(112)].

1.6.2.2 TRBP: TRBP was discovered two decades ago by its ability to bind to HIV TAR RNA[(152, 153)]. TRBP sequesters TAR RNA and decreases the amount of RNA available for PKR activation. Moreover, TRBP directly binds to PKR and further inhibits PKR activation[(154, 155)]. As a result, in HIV infected cells, TRBP inhibits PKR via sequestration of TAR RNA and via physical interaction with PKR.

1.6.2.3 ADAR1: ADAR1 binds to PKR and inhibits its activation in the context of VSV and MV infection[(156,157)] . ADAR1 inhibits the formation of stress granules mediated by PKR activation[(158)]. Our lab has shown that ADAR1 also inhibits PKR activation in HIV-1 infected cells[(102)]. Indeed, ADAR1 interaction with PKR is increased when HIV-1 is replicating actively[(102)]. Knockdown of ADAR1 expression in HIV-transfected HEK293T cells partially restores PKR activation [(148)]. This suggests that additional proteins inhibit PKR and that ADAR1 is part of that PKR inhibitory complex during HIV-1 infection.

1.6.2.4 Tat: Tat is essential for HIV transcription as it increases the level of basal transcription by 100 fold when bound to TAR RNA[31, 35, 36]. Tat binds to PKR and serves as pseudo substrate for PKR phosphorylation, thereby replacing eIF2α [159]. In addition, PKR phosphorylates Tat and results in an increased Tat-TAR interaction [160] .

1.7 PKR inhibition by PACT

1.7.1 PACT

PACT was initially identified in a yeast two hybrid assay that used PKR as bait[114]. Its murine homolog, RAX, was cloned from a library using a kinase deficient PKR[161]. PACT and RAX activate PKR only in the presence of stress[161-163]. PACT expression is regulated by the transcription factor sp1 and is important in the post-natal development[164]. Studies in mice carrying a knock-out RAX have shown a disruption in post-natal development that leads to growth impairments such as a smaller size, low progesterone levels and ear defects[165].

1.7.2 PKR activation by PACT

Activation of PKR by PACT involves PACT's domain 1 and 2 (two dsRBDs) that bind to PKR molecules and a domain 3 (activation region in PACT C-terminus) that induces a conformational change, thereby activating PKR trans-autophosphorylation[166] (Figure 1.4).

Phosphorylation of PACT at two serines, ser246 and ser287, and increased interactions between PACT molecules are additional requirements for PKR activation by PACT [167-169]. This phosphorylation is mediated by a stress activated protein kinase that remains unknown[167]. The stress treatment disrupts a strong TRBP-PACT interaction and enables the phosphorylation of PACT and activation of PKR [163, 167].

PACT-PKR pathway is involved in various cell processes and in host-virus interactions. As an example, PKR is known to phosphorylate and activate the tumor suppressor protein, p53[(116)]. It was found that the PACT-PKR pathway inhibits cellular replenishments of p53 by a combination of p53 sumoylation promoted by PACT and p53 phosphorylation by PKR, consequently leading to cell cycle arrest[(170)]. In addition, PACT-PKR pathway was shown to be induced following LPS treatment in human gingival cells and this pathway activated NF-κB signalling and pro-inflammatory cytokines[(171)].

1.7.3 PACT as a PKR inhibitor during HIV-1 replication

In the context of HIV-1 infection, PACT was found to increase viral replication as shRNAs against PACT decreased HIV-1 production[(172)]. PACT-mediated PKR activation during HIV-1 infection was recently investigated[(148)]. PACT overexpression increased HIV-1 expression and production in HIV-1 expressing cells. Furthermore, instead of activating PKR, PACT decreased PKR and eIF2α phosphorylation that has been induced by HIV-1 expression. In addition, PACT knockdown by shRNA partially restored PKR-mediated inhibition of HIV-1 expression.

To assess the role of TRBP in the observed change of PACT's function, U251MG cells, an astrocytic cell line, were used due to their natural low levels of TRBP and a heightened PKR response[(151, 173, 174)] . A decrease in TRBP's expression did not restore fully PKR activation and eIF2α phosphorylation, therefore TRBP was not solely implicated in this PACT's change of function. Other HIV-1 co-factors such as ADAR1 might be involved as an interaction between PACT and ADAR was identified in HIV-1 expressing HEK293T cells[(148)].

Chapter 2. Hypothesis, research objectives and experimental approaches

IFN response blocks viral replication by a multitude of mechanisms induced by ISGs. PKR response pathway is a component of the IFN response and has been shown to be an efficient antiviral mechanism. PKR inhibits viral replication through eIF2α phosphorylation, which leads to an arrest in cellular and viral mRNA translation. Many viruses evolved mechanisms to target and inhibit PKR activation such as targeting dsRNA activators of PKR. Our laboratory has shown that PKR activation is not sustained and is inhibited in HIV-1 expressing cell lines. ADAR1, TRBP and PACT have been identified as PKR inhibitors during HIV production. These PKR inhibitors increase HIV-1 expression and production and have in common the ability to bind to dsRNA which makes them part of the dsRBP family. Our central hypothesis in the laboratory is that the regulation of PKR activation by dsRBPs is critical for HIV-1 to evade the innate immune response. Our objectives for this project were to answer these questions:

1) What is the activation status of PKR in HIV-1 infected Peripheral Blood Mononuclear Cells (PBMCs)?

2) What changes PACT from a PKR activator to a PKR inhibitor during HIV-1 infection?

Experimental approaches:

For the 1st objective, we infected PBMCs with HIV-1 and assessed PKR activation by western blotting. We also assessed if HIV-1 infected PBMCs were able to respond to IFN treatment in order to look at their IFN response pathway. Furthermore, we conducted a protein expression profile in HIV-1 infected patients and examined PKR activation in those patients.

In the 2nd objective, we looked if PKR was required for PACT-mediated increase in HIV-1 expression and production by silencing PKR expression using siRNAs. Furthermore, we examined if the effect of PACT on HIV-1 production also affected HIV-1 infectivity by infecting an HIV reporter cell line with virions from PACT and HIV-1 transfected cells and also, by examining the quality of the virions produced in PACT and HIV-1 transfected cells. To further elucidate PACT switch of functions, we looked at PACT-interacting partners by mass spectrometry in HIV-1 expressing cells.

Chapter 3. Materials and methods

Plasmids and siRNAs

HIV-1 molecular clone, pNL4-3 was obtained from Dr. M A. Martin and used to transfect HEK293T cells in combination with pCMV2-Flag-PACT and pCMV2-Flag and were previously described[148, 175].

PKR siRNAs (ON-TARGETplus siRNA SMARTpool, Human EIF2AK2 catalog number # L-003527-00-0005) were purchased from Dharmacon as well as the non-target siRNAs (catalog number #D-001810-10-05).

Cells

HEK293T (ATCC CRL-11268) and TZM-bl (NIH AIDS reagent program catalog number #8129) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Hyclone) supplemented with 2mM L-glutamine, 10% Fetal Bovine Serum (FBS) (Hyclone) and 1% penicillin-streptomycin(Life technologies) at 37°C in 5% CO_2.

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors previously selected to be negative for HIV, HTLV-I and II, HCV, CMV and syphilis and from HIV-1 infected individuals. Blood sample collection was approved by the ethics review board of McGill University. PBMCs were maintained in RPMI medium supplemented with 10% heat inactivated FBS, 2 mM L-glutamine, 1% penicillin-streptomycin.

Transfections

HEK293T cells were used for pNL4-3, pCMV2-Flag-PACT and pCMV2-Flag transfections as follow: Cells were plated at 60% 24 hours prior to transfections. Plasmids were transfected into cells using Polyethylenimine (PEI) and left for 4 hours prior to changing media. 48 hours post-transfection, cells were lysed in cold lysis buffer consisting of 50mM Tris HCl at pH 7.4, 150 mM NaCl, 5 mM EDTA at pH 8.0, 1% nonyl phenoxypolyethoxylethanol (NP)-40 (Sigma), 10% Glycerol supplemented with protease and phosphatase inhibitors (Roche). Cell lysates were subjected to Western blot analyses and cellular supernatants were collected for RT assay.

For PKR knockdown, the lipofectamine RNAi Max reagent protocol was followed with modifications: HEK293T cells were plated 7 hours prior to addition of PKR siRNAs and Non Targeting (NT) siRNAs using lipofectamine RNAi Max reagent (Life technologies). The following day, PKR siRNAs and NT siRNAs were added again to the cells and left for 5-6 hours prior to pNL4-3 and pCMV2-Flag-PACT transfections using TransIT-LT1 transfection reagent (Mirus). Cellular supernatants for RT assay and lysates for Western blot analysis were collected 48 hours post-transfection.

Reverse transcriptase assay

Viral production was measured as previously described[*(176)*]. In brief, 5µl supernatants from HIV-1 transfected HEK293T cells or HIV-1 infected PBMCs were collected and added to 25µl of a non-radioactive cocktail (60mM Tris-HCl pH 7.8, 75mM KCl, 5mM $MgCl_2$, 0.1% NP-40, 1.04 mM EDTA). Subsequently, 25 µl of a radioactive RT cocktail (60mM Tris-HCl pH 7.8, 75mM

KCl, 5mM $MgCl_2$, 0.1% Nonidet P-40, 1.04 mM EDTA, 10 µg/mL polyA , 0.33 µg/mL oligo dT) supplemented with 5 µl αP32 radiolabeled TTP (Perkin Elmer) and 4 µl Dithiothreitol (DTT) per 500 µl of RT cocktail and was mixed with the non-radioactive cocktail containing the virions. The mixture (55µL) was incubated for 2 hours at 37°C and 5 µl of the reaction was spotted onto a DEAE paper (Perkin Elmer). The DEAE paper was left to dry for 15 min prior to 4 washes (5min per wash) in 2X SSC buffer (3 M NaCl and 300 mM $Na_3C_6H_5O_7$) and 2 washes (1min per wash) in 95% ethanol and left to dry again. Papers were sealed in a plastic bag (Perkin Elmer) and radioactivity amount, expressed in cpm, were counted on a MicroBeta Jet scintillation counter (Perkin Elmer).

Infectivity assay

TZM-bl cells were plated at 60% 24 hours prior to infection. The following day, supernatants (50 µl) from HIV-1 transfected HEK293T cells supplemented with 50 µl of media were added to the cells. Cells were washed 1X with PBS and were lysed in lysis buffer (Promega) 48 hours post-transfection. Luciferase activity (RLU) was measured in a Promega luminometer (20/20) after adding 10 µl of cellular lysates to 40 µl of luciferase reagent (Promega).

HIV-1 infection in PBMCs

For PBMCs HIV-1 infection, cells were stimulated with 2.5 µg/ml phytohaemagglutinin (Sigma cat. # 12646) for three days in supplemented RPMI (Invitrogen). 24 hours prior to infection, recombinant human interleukin 2 (IL-2) (R&D Systems, cat. # 202-IL) was added to the cells for a final concentration of 10 ng/ml. 6.5×10^7 cells were infected with HIV-1 cell supernatant corresponding to 1.3×10^7 cpm measured by standard RT assay in a final volume of 2.5 ml

supplemented RPMI in polypropylene round-bottom tube, and incubated for 2 hours at 37°C. RPMI supplemented with IL-2 for a final concentration of 10 ng/ml was then added to the cell-virus mixture, transferred to a T25 flask and incubated at 37°C. The cells were fed on average every two days with fresh medium supplemented with IL-2 (10 ng/ml). At each time point, supernatant was collected for RT assay and cells were washed in PBS prior to lysis using cold lysis buffer. Cell lysates were subjected to Western blot analysis.

Isolation of virions

Supernatants from HIV-1 transfected cells were collected 48 hours post-transfection and spun down for 5 minutes at 1500 rpm to remove cellular debris. Afterwards, supernatants were spun down at 18'000 rpm for 1 hour at 4°C, the medium was removed and the pellet was resuspended in cold lysis buffer and subjected to Western blot analysis.

Immunoblotting

Cellular and viral lysates (between 50-120 μg) were boiled for 5 min at 95°C in SDS loading buffer (0.5 M Tris HCl, 25% SDS, 20% Glycerol and 0.01 % Bromophenol blue) prior to loading into a 10% or 7.5% SDS PAGE gel, where indicated. The proteins were then transferred onto a nitrocellulose membrane by semi-dry (Bio-Rad Laboratories) or wet transfer (Bio-Rad Laboratories) for 1 hour at 10V or 100 V, respectively, using a transfer buffer (48mM Tris-HCl, 39mM glycine, 0.375% SDS and 20% ethanol). The membranes were then blocked in 5% milk diluted in Tris saline buffer supplemented with 0.1% Tween20 (TBS-T) for 1 hour and washed 1 time with TBS-T prior to adding primary antibodies overnight at 4°C . The following day, membranes were washed 4 times in TBS-T, and the incubated with TBS-T and secondary

antibodies for 1 hour. Membranes were washed again 4 times in TBS-T and Western lightning solution (GE healthcare life sciences) was added prior to visualisation.

Table 2. Antibodies used for Western Blot and Immunoprecipitation

Antibodies	Company	Dilution	Animal
p-PKR (Phospho T446)	Abcam	1:500	Rabbit monoclonal
p-eIF2α	Invitrogen	1:1000	Rabbit polyclonal
PKR 70-10	Dr.A Hovanessian	1:500	Mouse monoclonal
eIF2α	Cell signaling	1:1000	Rabbit polyclonal
ADAR1	Dr B.L. Bass	1:1000	Rabbit polyclonal
TRBP-JBX	Gatignol's Lab	1:500	Rabbit polyclonal
HIV p24	Dr. M.J. Tremblay	1:1000	Mouse monoclonal
HIV Env	NIH AIDS reagent program	1:1000	Goat polyclonal
RIG-I	Cell signaling	1:1000	Rabbit monoclonal
MDA-5	Cell signaling	1:1000	Rabbit monoclonal
Actin	Chemicon	1:5000	Mouse monoclonal
Flag	Sigma	1:5000	Mouse monoclonal

Flag-PACT immunoprecipitation

The protocol used for IPs was previously described [(102)] and was slightly modified as follows: Protein G agarose fast flow compact beads (Sigma) were blocked for non specific binding using 3% TNET buffer (50 mM Tris pH 7.4 , 100 mM NaCl, 1 mM EDTA, 0.5% NP40 and 3% BSA) prior to adding 2µg of anti-Flag mouse antibody. The beads and antibodies were incubated overnight at 4°C with rotation. The following day, the beads were washed 3 times with cold lysis buffer prior to adding 2.5 mg of proteins and the mixture was incubated overnight at 4°C with rotation. The next day, the beads were washed 2 times with cold lysis buffer and 1 time with PBS before adding SDS loading buffer. The immunoprecipitates were subjected to Western blotting.

Mass spectrometry analysis

Flag-immunoprecipitated samples were sent to Université Laval proteomic services (http://proteomique.crchul.ulaval.ca/fr/services.html) to identify proteins interacting with PACT. Results were analyzed using Scaffold4 software obtained from http://www.proteomesoftware.com/products/scaffold/ . The minimum protein and peptide probability threshold was set to be above 80% and the minimum number of peptide was set to 1.

Chapter 4. Results

4.1 Regulation of PKR expression in HIV-1 infected PBMCs

4.1.1. PKR regulation in PBMCs infected with HIV-1 in vitro

Previous data in our laboratory have shown that PKR activation is not sustained during HIV-1 infection of Jurkat cells [(102)]. This led us to assess if the effects of HIV-1 infection on PKR activation were similar also in primary cells (Figure 4.1.1). PBMCs were infected with HIV-1 throughout a 14 day time course in which supernatants and cell lysates were collected at different time points for analysis.

HIV production was analyzed by RT assay in the supernatants collected and peaked at day 12 post-infection (Figure 4.1.1 A). The expression of various proteins, in the cellular lysates collected throughout the course of infection, was analyzed by immunoblotting (Figure 4.1.1B). PKR expression and activation were detected at the beginning of infection; between day 4 and day 10 post-infection in comparison to the mock-infected PBMCs whose PKR expression was not detected (Figure 4.1.1 B).

The activation was not sustained and by day 12, a pronounced decrease in PKR activation and expression was observed. Active HIV-1 replication, i.e. an increase in HIV-1 production as assessed by RT assay and by an HIV p24 Western Blot, correlated with an upregulation of ADAR1p150 and a slight increase in PACT expression, ADAR1 was previously identified as a PKR inhibitor and the role of PACT was investigated further and found to be also a PKR inhibitor[(102, 148)].

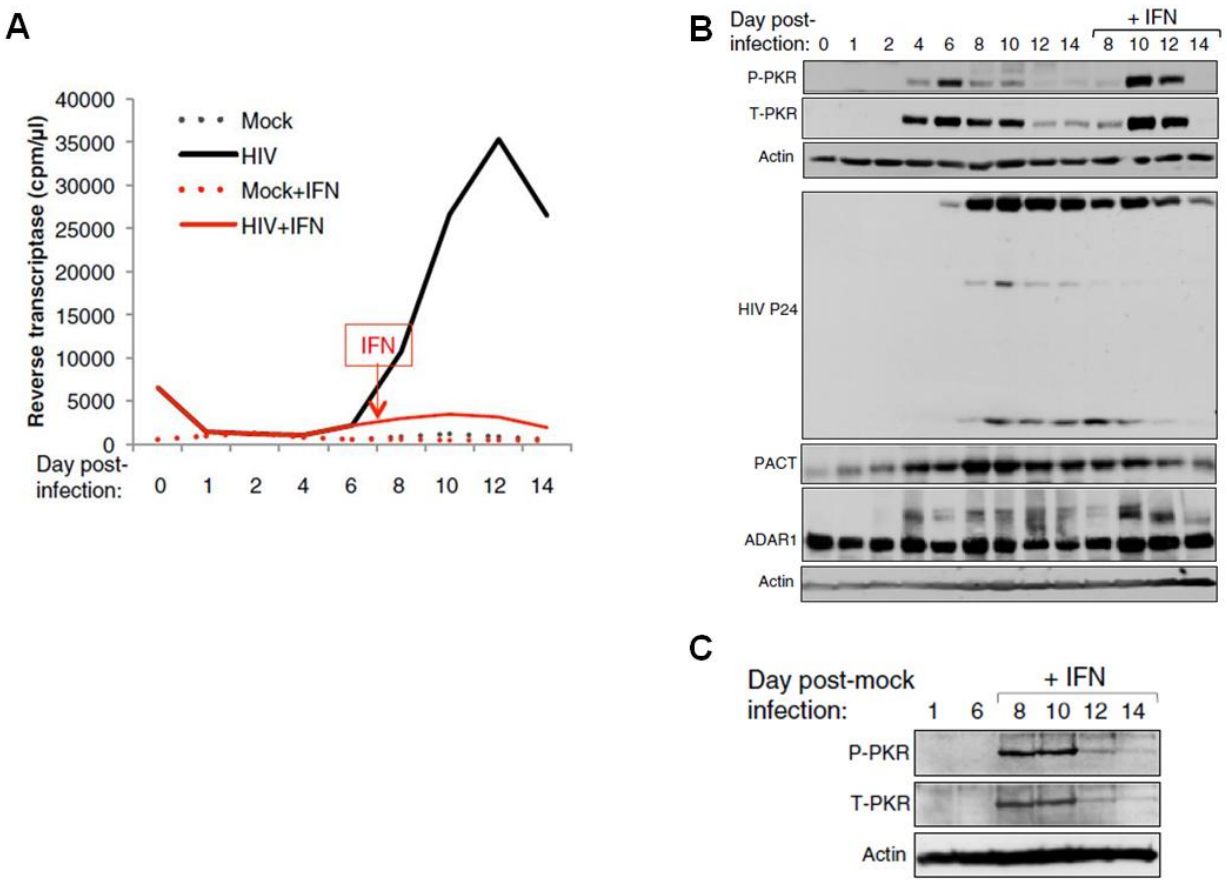

Figure 4.1.1 **PKR activation in HIV-1 infected PBMCs is not sustained but is restored following IFN treatment**. **A,** HIV-1 NL4-3 kinetics from infected PBMCs. 6.5 × 10^7 PBMCs from a healthy donor were infected with HIV-1 NL4-3. At day 7, cells were separated in two flasks and IFN α/β (10000U/mL) was added to the cells in one of them up to day 14. Aliquots of cell supernatant were collected at different times and assayed for RT activity. **B,** Protein expression of pNL4-3-infected PBMCs. 50 µg of whole-cell extracts from NL4-3-infected PBMCs from different harvest times were subjected to a 10% SDS PAGE and blotted with anti-P-PKR, anti-PKR, anti-HIV-p24, anti-PACT, anti-ADAR1 and anti-actin antibodies as indicated. **C,** Protein expression of mock-infected PBMCs. 6.5 × 10^7 PBMCs from the same donor as in B were cultured and passed at the same time as in B. IFN α/β was added similarly from day 7 to 14. 50 µg of whole-cell extracts from mock-infected PBMCs from the indicated times were subjected to a 10% SDS PAGE and blotted with anti-P-PKR, anti-PKR and anti-Actin antibodies as indicated. Published as Figure 1 in Clerzius et al., *Retrovirology*, 2013.

4.1.2. Protein expression profile in HIV-1 infected patients

To investigate PKR regulation in HIV-1 infected patients, a protein expression profile was analyzed by Western blot in HIV-1 infected viremic-untreated and ART-treated patients and compared to healthy subjects (Figure 4.1.2). Patient's PBMCs were obtained from Dr. J-P. Routy at the Royal Victoria Hospital part of McGill Health Centers. The clinical and demographic characteristics of the healthy subjects and patients are illustrated in Figure 4.1.2A. HIV-infected viremic-untreated patients had low $CD4^+$ counts, ranging from 194-370 $CD4^+$ T cells/mL of blood, and high viral loads (between 4.3 and 4.82 log copies of HIV RNA/mL of blood) as measured at the time of sample collection (Figure 4.1.2 A). In contrast, ART-treated individuals had near normal $CD4^+$ counts and undetectable viral load (Figure 4.1.2 A).

The expression of proteins such as phospho and total eIF2α, the pathogen recognition receptors RIG-I and Mda5 and PACT was analyzed (Figure 4.1.2 B and C). However, no correlation between the viremia and their expression could be made. Protein expression profile using PBMCs from patient 1 and 2 was not assessed due to a lack of protein sample. In contrast, the activation of PKR was slightly detected in viremic-untreated patients and not in non-infected subjects or successfully treated patients (Figure 4.1.2.C).The expression of total PKR was not assessed due to a membrane problem and should be done to determine if PKR is both induced and activated.

A

	CD4	CD8	VL (log)	Age	Sex
Healthy Subjects					
1	ND	ND	0	60	F
2	862	609	0	48	F
3	810	1405	0	42	M
Viremic untreated patients					
4	370	851	4,43	37	M
5	195	768	4,3	27	M
6	204	361	4,82	43	M
ART-treated patients					
7	554	288	<1.6	29	F
8	318	431	<1.6	45	M
9	847	944	<1.7	62	M

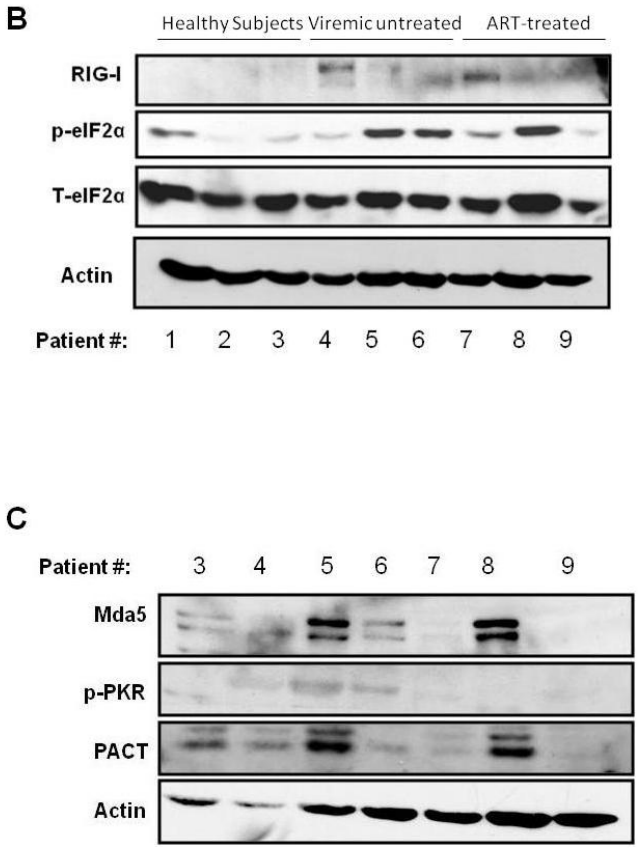

Figure 4.1.2 **Increased p-PKR expression correlates with viral replication in HIV-1 infected patients**. **A**, Patient's clinical and demographic characteristics are displayed in the table and include CD4 and CD8 counts (number of $CD4^+$ or $CD8^+$ cells /mL of blood), viral load (log10 values of HIV RNA copies/mL of blood) and sex (Male or Female). Two categories of patients, viremic-untreated patients and ART-treated patients are indicated in the table. Samples from viremic-untreated patients were collected after diagnosis in HIV-infected patients without previous therapy whereas ART-treated patients are HIV-infected patients who are successfully treated. Data from Dr. J.P Routy at Royal Victoria Hospital. **B** and **C**, 50µg of patients and healthy subjects samples were subjected to a 10% SDS-PAGE in which, RIG-I, p-eIF2α, total eIF2α (T- eIF2α), Mda5, p-PKR, PACT and Actin were analyzed by Western blot.

4.2 PACT/PKR pathway during HIV-1 production

4.2.1 PACT switches from a PKR activator to a PKR inhibitor during HIV-1 production

PACT is known to activate PKR only in the presence of a stress inducer in cell lines[*(102, 162, 163, 168)*] . We looked at PACT-mediated PKR activation by inducing a stress in PBMCs and HEK293T cells (Figure 4.2.1 A). PKR phosphorylation was observed 1h post-treatment when PBMCs were treated with 2mM of sodium arsenite which induces apoptosis by activation p38

MAP kinases and Bcl2 family proteins [177]. HEK293T cells transfected with PACT-expressing plasmid also showed PKR activation upon sodium arsenite treatment (Figure 4.2.1 A).

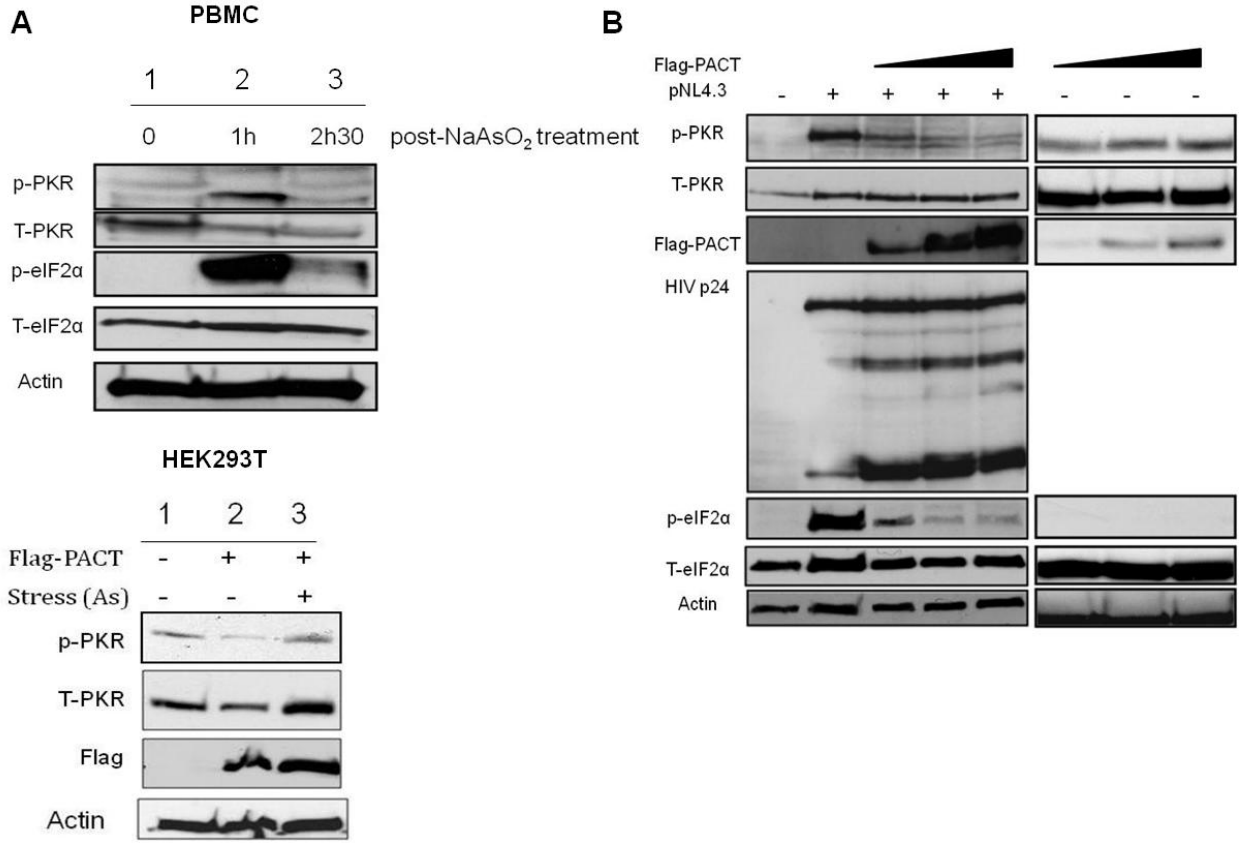

Figure 4.2.1 **PACT activates PKR after a stress but inhibits PKR during HIV-1 production**. **A**, **PACT activates PKR after a stress.** (Top) PBMCs were treated with 2mM $NaAsO_2$ (sodium arsenite) for 0, 1h or 2.5h. 50µg of cell lysates were collected and subjected to a 10% SDS-PAGE gel. p-PKR, T-PKR, p-eIF2α, T-eIF2α and Actin expression were analyzed by Western blot. (Bottom) HEK293T cells were transfected with 2 µg of pCMV2-Flag-PACT or mock-transfected and were treated or not with 2mM of Arsenite for 1h. 15µg of cellular lysates were then were subjected to a 10% SDS-PAGE gel. p-PKR, T-PKR, Flag-PACT, and Actin expression were analyzed by Western blot. **B, PACT becomes a PKR inhibitor in HIV-1 expressing cells.** HEK293T cells were mock-transfected (right) with increasing amount of pCMV2-Flag-PACT (0.5, 1 and 2 µg) or transfected in combination with 2.5 µg of pNL4-3 plasmid (left). pCMV2-Flag-empty was supplemented to ensure that DNA amounts in each well were equal. 50 µg of cellular lysates from transfected cells were ran on a 10% SDS-PAGE gel and p-PKR, T-PKR, p-eIF2α, T-eIF2α, Flag-PACT and Actin expression were analyzed by Western blot. Experiment in B (left) was conducted by Dr. Guerline Clerzius and is included as part of Figure 3 in Clerzius et al., *Retrovirology*, 2013.

Similarly, in PBMCs, eIF2α was largely increased at 1 hour post-stress treatment indicating a strong induction of this pathway. In PBMCs, PKR and eIF2α phosphorylation were no longer

detectable 2h30 post-treatment (Figure 4.2.1 A), which indicates that the stress induced by sodium arsenite treatment is not permanent.

In comparison, in HIV-transfected HEK293T cells, PACT inhibited PKR phosphorylation (Figure 4.2.1 B, left). In addition, PACT overexpression increased HIV p24 expression and decreased eIF2α phosphorylation in PACT and HIV-transfected cells, which illustrates that PACT switched from a PKR activator to an inhibitor during HIV-1 production in HEK293T cells. In contrast, PKR phosphorylation slightly increased and eIF2α phosphorylation was not visible in a similar experiment with no transfected HIV (Figure 4.2.1 B, right).

4.2.2 PKR is required for PACT-mediated increase in HIV-1 expression and production

To verify if the presence of PKR was required for PACT-mediated increase in HIV-1 expression, we silenced PKR expression using siRNAs (Figure 4.2.2). Cellular lysates and supernatants were collected and subjected to Western blot and an RT assay, respectively.

As expected, HIV-1 expression induced PKR phosphorylation but was inhibited when PACT was added (Figure 4.2.2 A). PKR siRNAs induced an increase in HIV-1 expression in comparison to the non-targeted siRNAs, illustrating more the efficient HIV-1 inhibition by PKR. Furthermore, PACT in combination with PKR knockdown in HIV-transfected cells did not induce an increase in Gag and p24 expression.

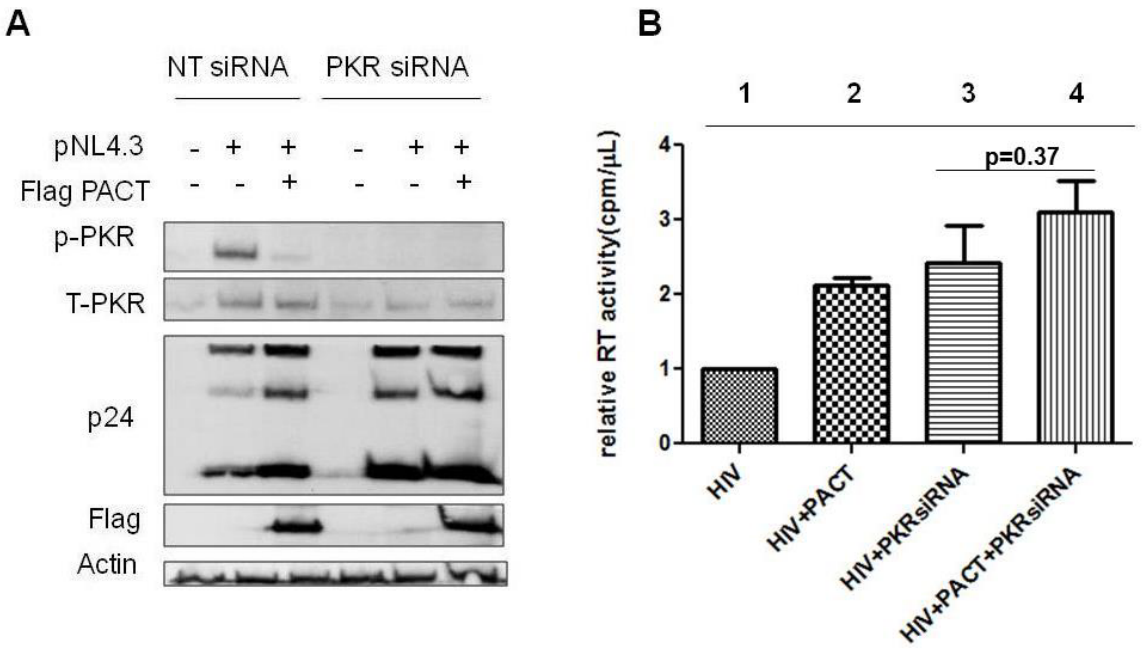

Figure 4.2.2 **PACT-mediated increase in HIV-1 expression and production is PKR dependent. A,** HEK293T cells were transfected with 30 pmol of non-targeting (NT) or PKR siRNAs prior to transfection with 2 µg of pCMV2-Flag-PACT alone or in combination with 2.5 µg of HIV-1 molecular clone, pNL4-3. 120 µg of cellular lysates collected from transfected cells were subjected to a 10% SDS-PAGE gel. p-PKR, T-PKR, Flag-PACT and Actin protein expression were analyzed by Western blot. **B,** 5 µL of supernatants from transfected HEK293T cells were collected and assayed for RT activity. RT values, in cpm/µL, are indicated on the Y axis. They were normalized to HIV-transfected alone. n=4

We next evaluated if the effects were similar during HIV-1 production by conducting an RT assay (Figure 4.2.2 B). HIV-1 production increased significantly when PACT was added to the cells but this increase required PKR expression as PKR knockdown decreased the effect (Bars 1 and 2 in comparison to bars 3 and 4 in Figure 4.2.2 B). However, from the RT assay, we cannot exclude a mild PKR-independent effect of PACT on viral production.

4.2.3 PACT overexpression decreases HIV-1 infectivity in TZM-bl cells

To further elucidate the PACT-mediated increase in HIV-1 expression and production, we first collected supernatants from PACT and HIV-transfected cells and tested HIV-1 production by RT

assay and observed a slight increase in HIV-1 production compared to previous experiments (Figure 4.2.3 A).

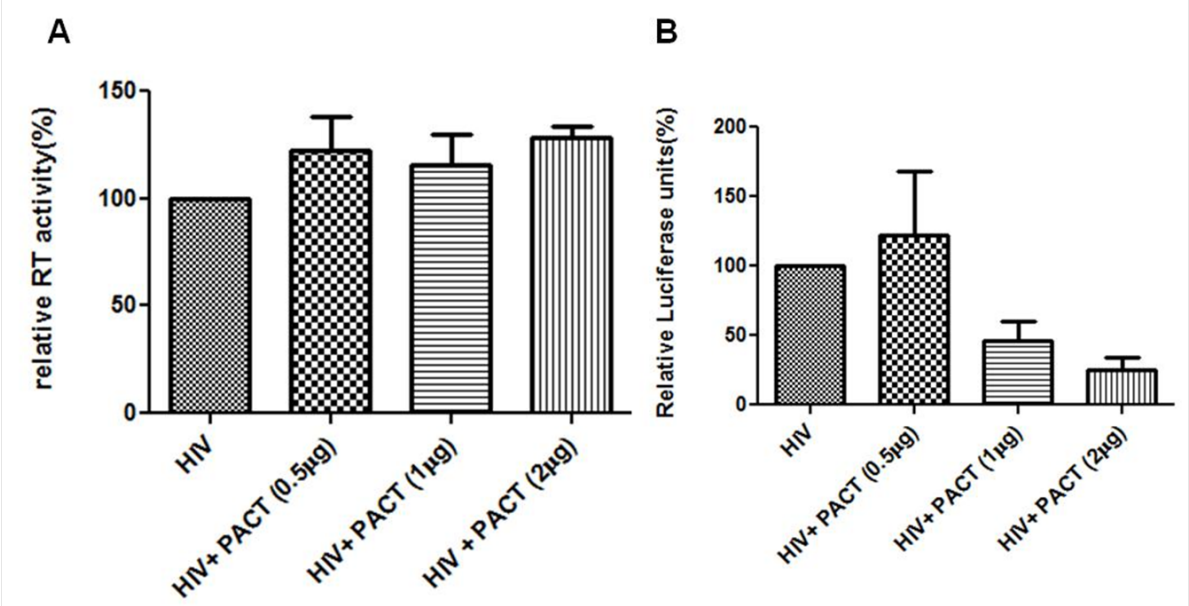

Figure 4.2.3 **PACT slightly increases HIV-1 production and decreases HIV-1 infectivity**. **A,** RT activity was measured from 5 µL supernatants from HEK293T cells transfected with 2.5µg of pNL4-3 alone or in combination with increasing amounts of pCMV2-Flag-PACT (0.5, 1 and 2 µg) and supplemented DNA amounts using pCMV2-Flag-empty plasmid. RT values were normalized to HIV-transfected alone and are indicated in percentage (%) on the Y axis. **B,** Supernatants, from conditions as in A normalized to their respective RT values, were used to infect an HIV reporter cell line TZM-bl. 10 µL of TZM-bl lysates were assayed for luciferase activity. Values were normalized to HIV alone condition and are indicated in percentage. n=3± SEM.

We next tested if PACT- mediated increase in HIV-1 production affected HIV-1 infectivity. We used supernatants, normalized to the same RT values, from PACT and HIV-transfected cells to infect TZM-bl cells (Figure 4.2.3 B).

Surprisingly, HIV-1 virions produced from PACT and HIV-transfected cells had an increased infectivity at low levels of PACT, but a decreased infectivity at high concentrations of PACT in comparison to virions produced from HIV-1 alone transfected cells (Figure 4.2.3 B).The decrease in HIV-1 infectivity was observed in a dose-dependent manner, reaching close to 70% decrease at the highest PACT expression. This suggests that overexpressed PACT alters HIV-1 infectivity.

4.2.4 Overexpressed PACT is incorporated into HIV-1 virions

Many cellular proteins have been shown to be incorporated into HIV virions and to serve, if known, various functions[42, 178, 179]. In light of the results on the effect of PACT on HIV-1 infectivity, we decided to analyze if overexpressed Flag-PACT was incorporated into the virions. We transfected HEK293T cells with pNL4-3 and increasing amounts of Flag-PACT plasmid. We isolated virions by ultracentrifugation at 48hrs post-transfection. We next analyzed protein expression, normalized by a Bradford assay, by Western blot (Figure 4.2.4).

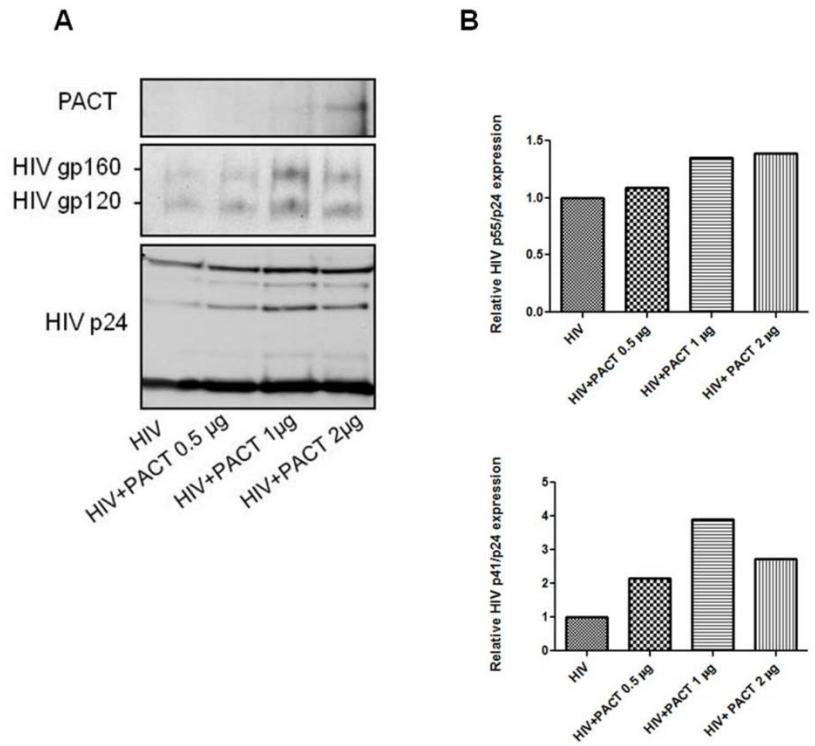

Figure 4.2.4 **Incorporation of overexpressed PACT into HIV-1 virions. A**, HIV-1 virions from HEK293T cells transfected with 2.5µg of HIV molecular clone pNL4-3 alone or in combination with increasing amounts of pCMV2-Flag-PACT (0.5, 1, 2 µg) were isolated by centrifugation and 40 µg of virions lysates were subjected to a 10% SDS-PAGE. Anti-PACT, anti-HIV gp120 and anti-HIV p24 protein antibodies were used to analyze protein expression by Western blot. **B,** Intensity of HIV p24 , p41 and p55 bands from the western blot in A were quantified using ImageJ software. The ratio of HIVp55/p24 (top) and p41/p24 (bottom) protein expression in different HIV+PACT conditions were normalized to HIV alone and plotted in a graph.

PACT protein was observed in HIV virions produced from HIV-transfected cells in combination with PACT at the highest transfection amount, 2μg, which indicates that PACT overexpression led to its incorporation into HIV virions. HIV p24 and gp120 protein expression were also analyzed (Figure 4.2.4 A). HIV p24 expression changed slightly when PACT was transfected in combination with HIV. In order to account for the possible difference in the quantity of virions collected in each conditions, we normalized the ratio of HIV p55/p24 and HIV p41/p24 protein expression to the ratio in HIV-1 virions without PACT (Figure 4.2.4 B). Using this method, we observed that PACT overexpression induced a slight increase in the ratio of HIV p55/p24 protein expression and a pronounced increase in the ratio of HIV p41/p24 protein expression (Figure 4.2.4 B). This suggests that PACT overexpression might have affected Gag processing. In addition, HIV Env expression increased when PACT was overexpressed, indicating an effect of PACT on HIV Env expression or incorporation into the virions.

4.2.5 PACT-interacting partners during HIV-1 production

PKR and ADAR1 were found to interact with PACT during HIV-1 replication[(148)] .

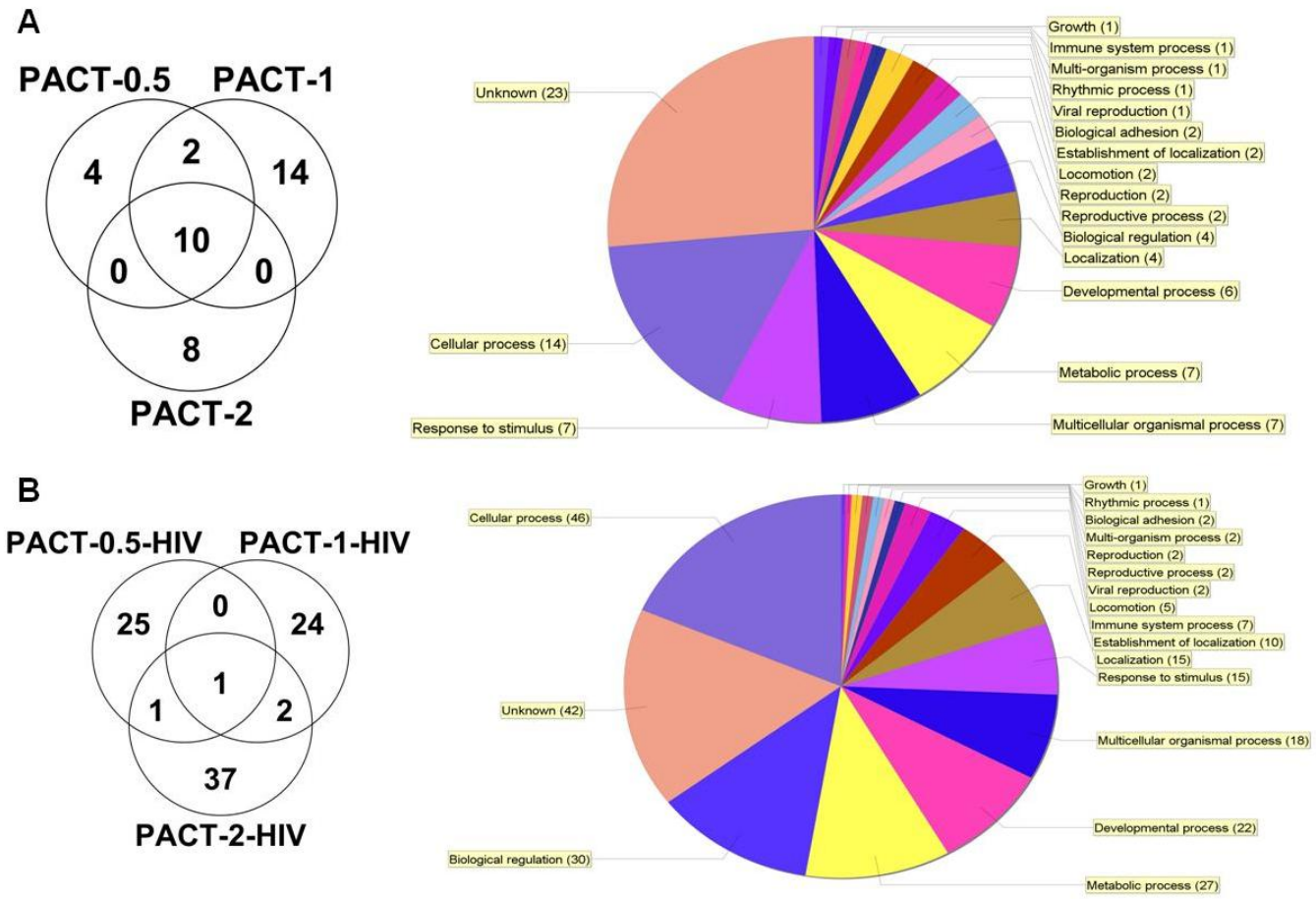

Figure 4.2.5.1 **Overview of PACT interactome during HIV-1 production. A, Left,** Venn's diagram indicating the numbers of proteins identified by MS that were pulled down in a Flag-PACT co-immunoprecipation experiment. PACT-0.5 represents proteins that were identified in a Flag pull-down from HEK293T cells transfected with 0.5 μg of pCMV2-Flag-PACT. PACT-1 and PACT-2 identified proteins were done in a similar manner as PACT-0.5. Proteins found in the control, i.e. mock-transfected HEK293T cells, were excluded from the list in the Venn's diagram. **Right,** the biological functions of total proteins (38) identified in the Flag pull-down. Due to the inability of the software to retrieve the biological ontology of 23 proteins, they are grouped in the unknown category indicated by a salmon color in the pie chart. **B, Left,** Venn's diagram indicating the numbers of proteins identified by MS that were pulled down in a Flag-PACT co-immunoprecipation. PACT-0.5-HIV represents proteins that were identified in a Flag pull-down from HEK293T cells transfected with 0.5 μg of pCMV2-Flag-PACT in combination with 2.5 μg of pNL4-3. PACT-1-HIV and PACT-2-HIV identified proteins were done in a similar manner as PACT-0.5-HIV. Proteins found in the control, i.e. HIV-transfected alone in HEK293T cells, were excluded from the list in the Venn's diagram. **Right,** the biological functions of total proteins (90) identified in the Flag pull-down. Due to the inability of the software to retrieve the biological ontology of 42 proteins, they are grouped in the unknown category indicated by a salmon color in the pie chart.

ADAR1 is a known PKR inhibitor and its interaction with PACT might play a role in the switch of PACT activity from a PKR activator to a PKR inhibitor. To identify other potential interacting partners of PACT that might also be involved in this process, we conducted a mass spectrometry

analysis of PACT-interacting partners in PACT-transfected in combination with HIV in HEK293T cells and compared them to PACT-transfected alone HEK293T cells (Figure 4.2.5). We used different amounts of PACT in order to detect proteins interacting with PACT at different levels. Proteins illustrated in Figure 4.2.5 were proteins not shared with controls which were composed of 2 antibody controls, mock-transfected cells and HIV-transfected alone cells.

In PACT-transfected alone HEK293T cells, 38 proteins were found and 10 among those proteins were common to all samples transfected with different amount of PACT (Figure 4.2.5 A). The biological functions of the 38 proteins found are shown in Figure 4.2.5 A (Right) and vary from metabolic processes to locomotion. The functions of a certain number of proteins were not included in the biological process chart provided by Scaffold due to a technical issue with the software.

In PACT+HIV-transfected cells, there were 90 proteins identified to interact with PACT (Figure 4.2.5 B). There was only 1 common protein identified in the three conditions which was a keratin related protein and was probably a contamination. The biological functions of the 90 proteins found vary also from immune processes to responses to stimuli (Figure 4.2.5 B).

Overall, there were no proteins shared between PACT-transfected alone and PACT-transfected in combination with HIV which suggests that HIV-1 expression induces a complete change in PACT interactome. HIV Env was the only viral protein found in PACT+HIV-transfected cells and was detected in HIV+PACT at 2 µg. Other expected interactions were not found, such as TRBP, ADAR1 and PKR which interactions were previously seen in IP experiments[*(148, 163, 180)*].

These interactions might still be present but below the detection threshold of the mass spectrometer.

We performed a second mass spectrometry analysis optimized to minimize background and increase the low probability of interactions observed in the previous mass spectrometry (Figure 4.2.5.2). We analyzed proteins pulled down by a Flag antibody but still bound to the beads. The beads were digested and proteins were identified by mass spectrometry. Using this methodology we were able to pull down 330 proteins interacting with PACT at more than 95% probability of these interactions being true.

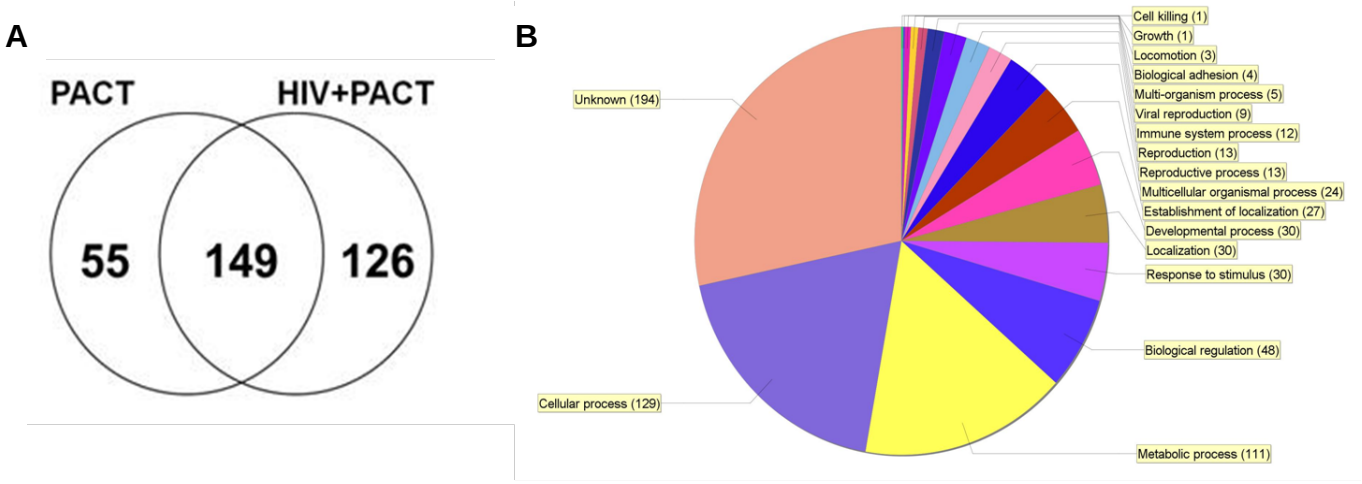

Figure 4.2.5.2 **Second mass spectrometry analysis of PACT interactome during HIV-1 production. A,**Venn's diagram indicating the numbers of proteins identified by MS that were pulled down in a Flag-PACT co-immunoprecipation experiment in HEK293T cells transfected with 2 µg of pCMV2-Flag-PACT shown as PACT or co-transfected with 2.5 µg of pNL4-3 shown as HIV+PACT. Proteins found in the controls, i.e. mock-transfected and HIV-transfected alone HEK293T cells, were excluded from the list in the Venn's diagram. **Right,** the biological functions of total proteins (330) identified in the Flag pull-down. Due to the inability of the software to retrieve the biological ontology of 194 proteins, they are grouped in the unknown category indicated by a salmon color in the pie chart.

149 proteins, such as ADAR1, interacted with PACT in the presence and absence of HIV (Figure 4.2.5.2 A). 55 PACT-interacting proteins were identified in the absence of HIV and 126 proteins

in the presence of HIV such as PKR and eIF2α (Figure 4.2.5.2 A). All the identified proteins are involved in various biological functions such as cellular processes as illustrated in Figure 4.2.5.2 B.

Chapter 5. Discussion

ISGs, which are induced by IFN following viral infection, have evolved various mechanisms to antagonize the replication of viruses. PKR is one of the most studied ISGs and is able to efficiently inhibit HIV-1 replication *in vitro* by blocking cellular and HIV-1 mRNAs translation via eIF2α phosphorylation. Our lab and others have demonstrated that PKR activity is inhibited during HIV-1 production by ADAR1, TRBP and recently, by PACT[(98, 102, 148, 151)].

5.1 PKR and ADAR1 are induced in HIV-1 infected primary cells

In this project, for the first time, we show that PKR activity is not sustained in HIV-1 infected PBMCs. This data is in concordance with what has been previously observed in HIV-1 infected Jurkat cell lines and illustrates the importance of PKR during HIV-1 infection[(102)]. Furthermore, we observed an upregulation of PKR expression which correlated with an increase in viral replication (Figure 4.1.1 B). Without stimulation, PKR expression in PBMCs has been reported to be at basal levels and almost undetectable by Western blot analysis[(181)]. This suggests that HIV-1 infection upregulates PKR expression either by inducing IFN production and subsequent expression of ISGs such as PKR or by another mechanism that would upregulate PKR expression in an IFN production-independent manner. An IFN-independent mechanism leading to increased PKR expression by p53 activation has been described previously[(117)] . Although IFN production was not measured in HIV-1 infected PBMCs, we have indirectly observed the production of IFN by detecting an increased expression of ADAR1 p150, which is another ISG (Figure 4.1.1). This could explain the increase in PKR expression by IFN production.

HIV-1 infected PBMCs responded to IFN treatment by inducing PKR expression and activation as we would have predicted. In addition, this also suggests that IFN treatment alleviates PKR activation blockade in HIV-infected PBMCs by either inducing optimal levels of PKR expression and subsequent activation not seen without IFN treatment or by mechanisms induced by the production of other ISGs. Nevertheless, IFN treatment *in vivo* for HIV infection has not been successful to date due to discrepancies between studies and to the induction of immune activation leading to an increased depletion of $CD4^+$ T cells[75, 76]. Therefore, more studies are required to assess the role and effects of IFN during HIV infection. Although the majority of cells in PBMCs are $CD4^+$ T cells, we would like to analyze, as a future direction, PKR activation in HIV-infected isolated primary $CD4^+$ T cells. This experiment would help us to better underline the effects of HIV-1 infection on PKR activation in isolated primary $CD4^+$ T cells.

In general, ISGs are categorized as only antiviral proteins but ADAR1 has also a proviral activity during HIV-1 infection. Our lab has observed an increase in HIV-1 expression and production due to the inhibition of PKR by ADAR1[102]. Furthermore, ADAR1p150 is also increased upon HIV-1 infection in PBMCs and may play an important role during viral replication in HIV-1 infected patients (Figure 4.1.1). Among all the proteins tested in HIV-1 infected patients (RIG-I, MDA-5, phospho and total eIF2α and PACT) in viremic-untreated patients and ART-treated patients, p-PKR had a positive correlation with viral replication. The limited amount of proteins in our samples did not let us to verify the concentration of TRBP and ADAR1, but they will be analyzed in future experiments. Apart from its role in innate immunity, ADAR1 is required in the maintenance of hematopoietic stem cells (HSCs)[182]. Indeed, ADAR1 protects HSCs from apoptosis induced by IFN and ADAR1-null HSCs suffer defects in

proliferation[182-185]. It is tempting to suggest that HIV has evolved a mechanism to activate ADAR1 and subsequently to maintain a pool of target cells, along with PKR inhibition. TRBP has also been shown to be a potent PKR inhibitor [147, 154, 186] and its expression in patients will be of crucial importance. More analyses of samples from a larger pool of HIV-1 infected untreated and treated patients are needed to validate our observations.

5.2 The change of PACT`s function in HIV-1 expressing cells is dependent on PKR and alters HIV-1 infectivity

In addition to PKR inhibition by ADAR1, PACT switches its PKR activating functions to become a PKR inhibitor by an HIV-1 induced mechanism (Figure 4.2.1). It was shown that HIV-1 infection does not elicit a stress and prevents formation of stress granules which are known to correlates with PKR activation[187],[188]. The lack of stress could explain the inability of PACT to activate PKR in the context of HIV but does not explain its active inhibitory effect on PKR as seen in Figure 4.2.1.

Furthermore, PACT-mediated PKR inhibition increases HIV-1 expression and production and we show here that this activity is dependent on the presence of PKR (Figure 4.2.2). The increase of HIV-1 production by addition of PACT was smaller in Figure 4.2.3A than in the Figure 4.2.2B. This difference could have been due to a possible change in the conditions of the samples in Figure 4.2.2B because they were treated with a non-targeting siRNA. PACT is known to participate in other innate immune pathways such as the RIG-I and Mda5 immune pathways during Sendai Virus and Middle Eastern Respiratory Syndrome Corona virus infection[189, 190], but this is likely not the case here as it would lead to an activation of PACT`s function rather

than a change[148]. Although we cannot exclude a partial PACT effect independent of PKR, the data presented here clearly indicate that the PACT/PKR pathway is targeted and altered during HIV infection.

When we looked at the effect of PACT on HIV-1 infectivity, we observed that PACT overexpression increased HIV-1 infectivity at low levels, but decreased it at higher levels in TZM-bl cells (Figure 4.2.3). Indeed, HIV-1 virions produced from HIV+PACT 2μg-transfected HEK293T cells decreased the infectivity by about 80% (Figure 4.2.3). The observed decrease in HIV-1 infectivity was not PKR dependent, as HIV virions from PACT+HIV-transfected HEK293T cells whose PKR expression was silenced by siRNAs did not restore HIV-1 infectivity (data not shown). Moreover, overexpressed PACT induced a modification of Gag processing as observed in collected HIV virions (Figure 4.2.4 A). Indeed, we observed a small in the ratio of HIV p55/p24 protein expression of HIV+PACT virions increased as well as a large increase in the ratio of HIV p41/p24 protein expression in comparison to their respective ratios in HIV virions with no PACT overexpression (Figure 4.2.4 B). In addition, an increase in HIV Env protein concentration was observed in HIV+PACT virions (Figure 4.2.4 A).

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Another dsRBP called Staufen1 has been reported to be incorporated into HIV virions [191]. The main difference between Staufen1 and PACT is that endogenous and overexpressed Staufen1 are incorporated into HIV virions whereas only overexpressed PACT was found in the virions. Endogenous PACT could also have been present at low levels but was not detected with our antibody. Although Staufen1 aids in viral replication through binding to Gag, Staufen1 overexpression led to a decrease in HIV-1 infectivity similar to what we found with PACT [191].

Protein overexpression might facilitate the incorporation of the overexpressed protein in HIV virions, disrupt viral assembly and maturation leading to a decrease in infectivity. This would explain that presence of PACT in HIV virions correlates with a decreased infectivity. The isolated virions were not purified from any cellular contaminants that could have been picked up during isolation and thus, constitutes a limitation to this study. More analyses from properly purified virions using a sucrose gradient for example, need to be conducted. Because we observed that low levels of overexpressed PACT molecules increase HIV infectivity, endogenous PACT is likely beneficial for the overall virion organisation and consequently its infectivity (Figure 4.2.3). Further analyses that would look at virion infectivity with a decreased PACT expression could determine if, like for Staufen1, an optimal amount of PACT molecules is required for appropriate HIV-1 virion infectivity.

5.3 A proteomic study identifies a complete change of PACT-interacting molecules in HIV-expressing cells

PACT was found in a cellular proteomic screen for cellular factors required for HIV replication. A decrease in HIVp24 ELISA was observed when the expression of PACT was silenced[(172)]. In the same study, TRBP was also identified as a cellular factor required for HIV replication. Our laboratory has shown previously that TRBP inhibits PACT's function by heterodimerization[(163, 180)]. However, in the context of HIV-1 production, a decreased TRBP expression did not fully restore PACT activating function indicating that TRBP-PACT interaction is not solely responsible for PACT switch of function [(148)]. Moreover, our lab has observed an interaction between PACT and ADAR1 in the presence and absence of HIV-1 which could indicate a possible implication of ADAR1 in the switch of PACT activity.

To further identify other proteins that might be involved in this mechanism, PACT interacting-partners in mock or in HIV-expressing HEK293T cells were identified in a mass spectrometry analysis (Figure 4.2.5). HIV-1 expression induced a complete change of PACT-interacting partners in comparison to the mock. Indeed, out of 90 proteins found in HIV-expressing HEK293T cells, none was found in the mock (Appendices 1 and 2). As shown in figure 4.2.5.1, those 90 proteins are involved in various biological processes such as metabolic processes, cellular immune responses and viral reproduction. This indicates that HIV expression does not only affect PACT-interacting partners implicated in cellular immune responses but rather proteins involve in a variety of cellular processes. In the latter, two proteins were included and are the platelet-derived growth factor-α receptor (PDGFRα) and the KIN17 protein.

PDGFRα is a cellular receptor for the platelet growth factor and is known to be involved in cellular proliferation as well as immune signalling such as the src-kinases and induction of IFN-like responses[192, 193]. In addition, PDGFRα is a mandatory cellular receptor for human cytomegalovirus and plays a role in JEV infection as well as in simian sarcoma virus infection [192-195]. PACT-PDGFRα direct or indirect interaction with PACT could suggest that PACT might be involved in PDGFRα –related signalling pathways but further validations of this interaction such as IPs need to be conducted.

KIN17 is a DNA/RNA- binding protein involved in cellular responses to genotoxic stress such as irradiation [196, 197]. Its role in viral reproduction was shown by its interaction with the Simian Virus 40 (SV40) T antigen [198]. Indeed, KIN17 expression is induced in cells expressing the SV40 T antigen and plays a role in DNA replication[198]. In this screen, we used HEK293Tcells which is a cell line that was transformed with the SV40T antigen to enhance expression of transfected plasmids containing an SV40 origin of replication. It is plausible that KIN17 was

pulled down indirectly due to its interaction with SV40. This hypothesis is further reinforced by the fact that KIN17 was only identified in cells that overexpressed the highest amount of PACT, i.e. 2 μg of plasmid.

The mass spectrometry analysis of digested peptides was also subjected to an HIV database to identify HIV-1 proteins that might be involved in PACT's switch of function. Only HIV Env was detected as a PACT-interacting partner and could play a role in the observed changes in HIV Env (gp120 and gp160) expression in virions (Figure 4.2.4).

Through optimisation of the immunoprecipitation protocol as well as using detection of proteins through beads digestion, the second mass spectrometry study gave us less background. Indeed, a total of 330 PACT interacting proteins were identified at more than 95% probability of the interaction being true. When we looked at PACT interacting proteins induced only in presence of HIV, we found 126 proteins from which PKR and eIF2α were part of. ADAR1-PACT interaction was observed in the presence and absence of HIV which was found previously in HIV-infected Jurkat Cell ADAR1 co-IP [(148)]. In this second mass spectrometry study, HIV Env was not pulled down but instead, HIV Gag-Pol polyprotein. Due to time constraints, we did not have time to further analyze the interactions found in this second mass spectrometry study.

In this study, we used transfected PACT to obtain more material. Our aim is to confirm these results in a more physiological context of HIV-infected lymphocytes (Jurkat cells or PBMCs). We will redo the experiment in those cells and immunoprecipitate with a PACT antibody prior to sending it for mass spectrometry analysis. If we cannot obtain enough material by this method, we will confirm the proteins identified by overexpressing PACT and immunoprecipitation in HIV-1 infected Jurkat cells. These assays will help to identify the cellular or viral proteins involved in the change of PACT`s function.

Chapter 6. Conclusion

The innate immune system is altered during HIV infection. PKR is part of the IFN response pathway, a component of the innate immune system. Although activation of PKR is observed at the beginning of HIV-1 infection, it is inhibited when the virus replicates actively. PKR inhibition correlates with an increased expression of ADAR1 and PACT during HIV-1 infection.

PKR inhibition during HIV-1 infection is attributable to concerted actions of dsRBPs such as TRBP, ADAR1 and PACT as shown by the data from our lab[111, 148]. TRBP blocks PKR activation in part by sequestering TAR RNA and preventing it to act as a PKR activator. In addition, TRBP and ADAR1 inhibit PKR activation through a direct inhibitory binding to PKR. PACT inhibits PKR activation and increases HIV-1 expression and production in HIV-expressing cell lines but the mechanism of this change of action remains unknown.

In the first part of the project, we reinforced the importance of PKR during HIV-1 infection by demonstrating PKR inhibition in HIV-1 infected PBMCs. Moreover, we observed a direct correlation between PKR phosphorylation and active viral replication in PBMCs from HIV-infected untreated patients which suggests the presence of newly infected cells in those patients.
In the second part of the project, we tried to elucidate PACT switch of function and have found that PACT-mediated increase in HIV-1 expression and production required the presence of PKR; overexpressed PACT affected HIV-1 infectivity and was incorporated into HIV-1 virions. Furthermore, an analysis of PACT-interacting partners by mass spectrometry shed the light on

the complete change of the PACT interactome induced by the presence of HIV-1 and establishes a pedestal for the continuation of this study.

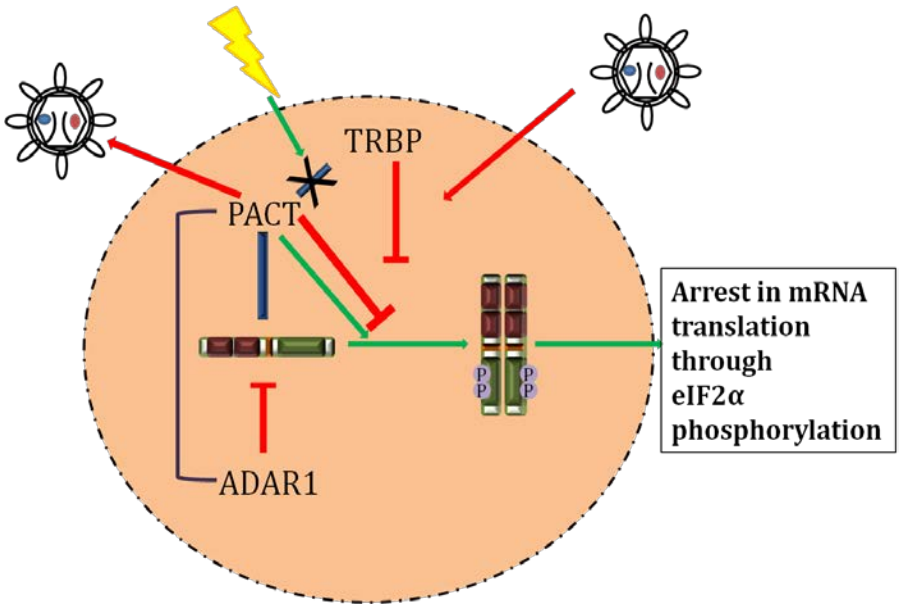

Figure 6.1 Model of PKR/PACT pathway during HIV-1 infection. Cellular stress, in yellow, disrupts PACT-TRBP interactions and allows PACT to activate PKR trans-autophosphorylation and subsequent arrest in mRNA translation in the cell through eIF2α phosphorylation. During HIV infection, PACT changes from PKR activator to PKR inhibitor and increases HIV production. PACT's switch of function might be through binding to TRBP and ADAR1.

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A-1. Complete list of PACT-interacting partners identified in a proteomic screen during HIV-1 production in HEK 293T cells

	Identified Proteins (90)	Accession Number
1	VAV2_HUMAN Guanine nucleotide exchange factor VAV2 [Homo sapiens (Human) [9606]]	P52735 (+2)
2	I3L2N8_HUMAN Zinc finger CCCH domain-containing protein 7A (Fragment) [Homo sapiens (Human)]	I3L2N8
3	Keratin, type I cytoskeletal 10 n=1 Tax=Homo sapiens RepID=K1C10_HUMAN	P13645 (+2)
4	E9PNZ4_HUMAN Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5 (Fragment) [Homo sapier	E9PNZ4 (+14)
5	Keratin, type II cytoskeletal 2 oral n=1 Tax=Homo sapiens RepID=K22O_HUMAN	Q01546
6	BI2L1_HUMAN Brain-specific angiogenesis inhibitor 1-associated protein 2-like protein 1 [Homo sap	Q9UHR4
7	RP1_HUMAN Oxygen-regulated protein 1 [Homo sapiens (Human) [9606]]	P56715
8	Keratin, type II cytoskeletal 5 n=1 Tax=Homo sapiens RepID=K2C5_HUMAN	P13647 (+4)
9	PGFRA_HUMAN Platelet-derived growth factor receptor alpha [Homo sapiens (Human) [9606]]	P16234
10	SEM4D_HUMAN Semaphorin-4D [Homo sapiens (Human) [9606]]	Q92854 (+1)
11	TITIN_HUMAN Titin [Homo sapiens (Human) [9606]]	Q8WZ42 (+17)
12	ATPG_HUMAN ATP synthase subunit gamma, mitochondrial [Homo sapiens (Human) [9606]]	P36542 (+1)
13	SHRM4_HUMAN Protein Shroom4 [Homo sapiens (Human) [9606]]	Q9ULL8
14	BHE22_HUMAN Class E basic helix-loop-helix protein 22 [Homo sapiens (Human) [9606]]	Q8NFJ8 (+3)
15	PREDICTED: peptidyl-prolyl cis-trans isomerase A-like [Homo sapiens [9606]]	UPI0000037A9D
16	E9PLB1_HUMAN Mitogen-activated protein kinase kinase kinase 11 (Fragment) [Homo sapiens (Hum	E9PLB1 (+2)
17	GRK5_HUMAN G protein-coupled receptor kinase 5 [Homo sapiens (Human) [9606]]	P34947 (+5)
18	IFM5_HUMAN Interferon-induced transmembrane protein 5 [Homo sapiens (Human) [9606]]	A6NNB3
19	B4DGW1_HUMAN Inactive serine/threonine-protein kinase VRK3 [Homo sapiens (Human) [9606]]	B4DGW1 (+2)
20	F90AC_HUMAN Putative protein FAM90A12P [Homo sapiens (Human) [9606]]	A8MX19
21	PRKDC_HUMAN DNA-dependent protein kinase catalytic subunit [Homo sapiens (Human) [9606]]	P78527 (+3)
22	NASP_HUMAN Nuclear autoantigenic sperm protein [Homo sapiens (Human) [9606]]	P49321 (+7)
23	Q6P444-2 Isoform 2 of Mitochondrial fission regulator 2 [Homo sapiens (Human) [9606]]	Q6P444-2
24	A5XEH6_HUMAN WNK lysine deficient protein kinase 1 (Fragment) [Homo sapiens (Human) [9606]]	A5XEH6 (+9)
25	B3KPE1_HUMAN Ubiquitin carboxyl-terminal hydrolase [Homo sapiens (Human) [9606]]	B3KPE1
26	F5H0F1_HUMAN Coiled-coil domain-containing protein 62 (Fragment) [Homo sapiens (Human) [9606	F5H0F1
27	H0YCK4_HUMAN Transmembrane protein 223 (Fragment) [Homo sapiens (Human) [9606]]	H0YCK4
28	TRIPB_HUMAN Thyroid receptor-interacting protein 11 [Homo sapiens (Human) [9606]]	Q15643 (+5)
29	RBM23_HUMAN Probable RNA-binding protein 23 [Homo sapiens (Human) [9606]]	Q86U06 (+6)
30	VPS11_HUMAN Vacuolar protein sorting-associated protein 11 homolog [Homo sapiens (Human) [96	Q9H270 (+6)
31	Q9NSS9_HUMAN Putative uncharacterized protein DKFZp761E10121 (Fragment) [Homo sapiens (Hun	Q9NSS9
32	B4DV59_HUMAN REST corepressor 3 [Homo sapiens (Human) [9606]]	B4DV59 (+7)
33	CBPC2_HUMAN Cytosolic carboxypeptidase 2 [Homo sapiens (Human) [9606]]	Q5U5Z8 (+3)
34	RELN_HUMAN Reelin [Homo sapiens (Human) [9606]]	P78509 (+2)
35	P3H2_HUMAN Prolyl 3-hydroxylase 2 [Homo sapiens (Human) [9606]]	Q8IVL5 (+1)
36	A0N4V7_HUMAN HCG2039797 (Fragment) [Homo sapiens (Human) [9606]]	A0N4V7
37	J3QRE9_HUMAN Keratin, type I cytoskeletal 39 [Homo sapiens (Human) [9606]]	J3QRE9 (+1)
38	PREDICTED: mucin-19-like [Homo sapiens [9606]]	UPI000387C59A
39	PREDICTED: MMS19 nucleotide excision repair protein homolog isoform X4 [Homo sapiens [9606]]	UPI000387C754 (+1)
40	B4DDX6_HUMAN cDNA FLJ57296 [Homo sapiens (Human) [9606]]	B4DDX6 (+2)
41	U5S1_HUMAN 116 kDa U5 small nuclear ribonucleoprotein component [Homo sapiens (Human) [960	Q15029 (+11)
42	FA156_HUMAN Protein FAM156A/FAM156B [Homo sapiens (Human) [9606]]	Q8NDB6 (+1)
43	ITPR1_HUMAN Inositol 1,4,5-trisphosphate receptor type 1 [Homo sapiens (Human) [9606]]	Q14643 (+13)
44	Q9H5G7_HUMAN cDNA: FLJ23452 fis, clone HSI06554 [Homo sapiens (Human) [9606]]	Q9H5G7
45	RTDR1_HUMAN Rhabdoid tumor deletion region protein 1 [Homo sapiens (Human) [9606]]	Q9UHP6 (+5)

46	H0Y459_HUMAN Nuclear receptor corepressor 1 (Fragment) [Homo sapiens (Human) [9606]]	H0Y459
47	M0QZD8_HUMAN Protein LOC388210 (Fragment) [Homo sapiens (Human) [9606]]	M0QZD8 (+1)
48	B4GT2_HUMAN Beta-1,4-galactosyltransferase 2 [Homo sapiens (Human) [9606]]	O60909 (+1)
49	GDIA_HUMAN Rab GDP dissociation inhibitor alpha [Homo sapiens (Human) [9606]]	P31150 (+1)
50	ESCO1_HUMAN N-acetyltransferase ESCO1 [Homo sapiens (Human) [9606]]	Q5FWF5 (+1)
51	U6BLW8_9PLVG Envelope glycoprotein [Simian-Human immunodeficiency virus [57667]]	U6BLW8 (+3)
52	BNIP2_HUMAN BCL2/adenovirus E1B 19 kDa protein-interacting protein 2 [Homo sapiens (Human) [9606]]	Q12982 (+1)
53	MAGBA_HUMAN Melanoma-associated antigen B10 [Homo sapiens (Human) [9606]]	Q96LZ2
54	B4DW22_HUMAN cDNA FLJ56974, moderately similar to Myeloid cell surface antigen CD33 [Homo sapiens (Human) [9606]]	B4DW22
55	O75555_HUMAN ABC transporter MOAT-B isoform (Fragment) [Homo sapiens (Human) [9606]]	O75555
56	UBP31_HUMAN Ubiquitin carboxyl-terminal hydrolase 31 [Homo sapiens (Human) [9606]]	Q70CQ4 (+2)
57	KIN17_HUMAN DNA/RNA-binding protein KIN17 [Homo sapiens (Human) [9606]]	O60870 (+3)
58	H0Y8Q5_HUMAN Probetacellulin (Fragment) [Homo sapiens (Human) [9606]]	H0Y8Q5
59	H7C234_HUMAN Dual-specificity phosphatase 28 (Fragment) [Homo sapiens (Human) [9606]]	H7C234
60	G3V3A5_HUMAN Tetratricopeptide repeat protein 6 [Homo sapiens (Human) [9606]]	G3V3A5 (+3)
61	HFM1_HUMAN Probable ATP-dependent DNA helicase HFM1 [Homo sapiens (Human) [9606]]	A2PYH4 (+3)
62	B4E3L9_HUMAN Bromodomain-containing protein 1 [Homo sapiens (Human) [9606]]	B4E3L9 (+3)
63	NSRP1_HUMAN Nuclear speckle splicing regulatory protein 1 [Homo sapiens (Human) [9606]]	Q9H0G5 (+3)
64	H0YD73_HUMAN 26S proteasome non-ATPase regulatory subunit 13 (Fragment) [Homo sapiens (Human) [9606]]	H0YD73
65	GNN_HUMAN Tetratricopeptide repeat protein GNN [Homo sapiens (Human) [9606]]	Q6P2S7 (+1)
66	LARP6_HUMAN La-related protein 6 [Homo sapiens (Human) [9606]]	Q9BRS8
67	Q502X4_HUMAN SAT2 protein (Fragment) [Homo sapiens (Human) [9606]]	Q502X4
68	STIM1_HUMAN Stromal interaction molecule 1 [Homo sapiens (Human) [9606]]	Q13586 (+5)
69	PREDICTED: ubiquitin conjugation factor E4 A isoform X1 [Homo sapiens [9606]]	UPI000387B62A
70	CO7_HUMAN Complement component C7 [Homo sapiens (Human) [9606]]	P10643 (+2)
71	D3YE67_HUMAN ABO transferase (Fragment) [Homo sapiens (Human) [9606]]	D3YE67
72	PPM1A_HUMAN Protein phosphatase 1A [Homo sapiens (Human) [9606]]	P35813 (+3)
73	H0Y8L0_HUMAN PHD finger protein 3 (Fragment) [Homo sapiens (Human) [9606]]	H0Y8L0
74	F1C628_HUMAN Platelet glycoprotein Ia (Fragment) [Homo sapiens (Human) [9606]]	F1C628 (+1)
75	K7ESB7_HUMAN Dedicator of cytokinesis protein 6 (Fragment) [Homo sapiens (Human) [9606]]	K7ESB7
76	REL_HUMAN Proto-oncogene c-Rel [Homo sapiens (Human) [9606]]	Q04864 (+2)
77	ZN790_HUMAN Zinc finger protein 790 [Homo sapiens (Human) [9606]]	Q6PG37 (+2)
78	OAS2_HUMAN 2'-5'-oligoadenylate synthase 2 [Homo sapiens (Human) [9606]]	P29728 (+11)
79	ARHGH_HUMAN Rho guanine nucleotide exchange factor 17 [Homo sapiens (Human) [9606]]	Q96PE2
80	ZMYM2_HUMAN Zinc finger MYM-type protein 2 [Homo sapiens (Human) [9606]]	Q9UBW7 (+3)
81	F5H3Y4_HUMAN Probable ATP-dependent RNA helicase DHX37 [Homo sapiens (Human) [9606]]	F5H3Y4 (+1)
82	PLIN3_HUMAN Perilipin-3 [Homo sapiens (Human) [9606]]	O60664 (+3)
83	BMP3_HUMAN Bone morphogenetic protein 3 [Homo sapiens (Human) [9606]]	P12645 (+9)
84	GOGB1_HUMAN Golgin subfamily B member 1 [Homo sapiens (Human) [9606]]	Q14789 (+10)
85	NAA25_HUMAN N-alpha-acetyltransferase 25, NatB auxiliary subunit [Homo sapiens (Human) [9606]]	Q14CX7 (+2)
86	H0YFD2_HUMAN Protein KRI1 homolog (Fragment) [Homo sapiens (Human) [9606]]	H0YFD2
87	ADAL_HUMAN Adenosine deaminase-like protein [Homo sapiens (Human) [9606]]	Q6DHV7 (+4)
88	B4DKG6_HUMAN cDNA FLJ53559 [Homo sapiens (Human) [9606]]	B4DKG6
89	F4MH30_HUMAN Ubiquitously transcribed tetratricopeptide repeat protein Y-linked transcript variant 63 [Homo sapiens (H	F4MH30
90	B3KSZ4_HUMAN cDNA FLJ37346 fis, clone BRAMY2021310, highly similar to Transcriptional repressor p66 beta [Homo sapien	B3KSZ4

A-2. Complete list of PACT-interacting partners identified in a proteomic screen in mock-

transfected HEK 293T cells

	Identified Proteins (38)	Accession Number
1	Keratin, type I cytoskeletal 14 n=1 Tax=Homo sapiens RepID=K1C14_HUMAN	P02533 (+1)
2	F8VV57_HUMAN Keratin, type II cytoskeletal 5 (Fragment) [Homo sapiens (Human) [9606]]	F8VV57 (+1)
3	Keratin, type II cytoskeletal 2 epidermal n=1 Tax=Homo sapiens RepID=K22E_HUMAN	P35908
4	K7ENW6_HUMAN Keratin, type I cytoskeletal 16 (Fragment) [Homo sapiens (Human) [9606]]	K7ENW6 (+1)
5	H7C055_HUMAN Tetratricopeptide repeat protein 7A (Fragment) [Homo sapiens (Human) [9606]]	H7C055
6	cDNA FLJ78504, highly similar to Homo sapiens keratin 6A (KRT6A), mRNA n=1 Tax=Homo sapiens RepID=A8K2I0_HUMAN	A8K2I0 (+2)
7	L0R5A1_HUMAN Alternative protein CSF2RB [Homo sapiens (Human) [9606]]	L0R5A1
8	Q5DT20_HUMAN Hornerin [Homo sapiens (Human) [9606]]	Q5DT20 (+1)
9	Keratin, type I cytoskeletal 24 n=1 Tax=Homo sapiens RepID=K1C24_HUMAN	Q2M2I5
10	J3QQT9_HUMAN 60S ribosomal protein L23 (Fragment) [Homo sapiens (Human) [9606]]	J3QQT9
11	D3DWB6_HUMAN Ubiquitin carboxyl-terminal hydrolase [Homo sapiens (Human) [9606]]	D3DWB6 (+7)
12	H0YH81_HUMAN ATP synthase subunit beta (Fragment) [Homo sapiens (Human) [9606]]	H0YH81 (+1)
13	H6VRG2_HUMAN Keratin 1 [Homo sapiens (Human) [9606]]	H6VRG2
14	B4DY56_HUMAN ATP synthase subunit alpha [Homo sapiens (Human) [9606]]	B4DY56 (+3)
15	PREDICTED: uncharacterized protein LOC101929561 [Homo sapiens [9606]]	UPI000387C49D
16	F8WCL5_HUMAN Nebulin [Homo sapiens (Human) [9606]]	F8WCL5 (+12)
17	PCNT_HUMAN Pericentrin [Homo sapiens (Human) [9606]]	O95613 (+6)
18	Keratin, type I cytoskeletal 19 n=1 Tax=Homo sapiens RepID=K1C19_HUMAN	P08727
19	E9PIJ5_HUMAN Transmembrane protease serine 13 [Homo sapiens (Human) [9606]]	E9PIJ5 (+10)
20	METK2_HUMAN S-adenosylmethionine synthase isoform type-2 [Homo sapiens (Human) [9606]]	P31153
21	MYH13_HUMAN Myosin-13 [Homo sapiens (Human) [9606]]	Q9UKX3
22	keratin, type II cytoskeletal 4 [Homo sapiens [9606]]	UPI00001AEBB1
23	PKP3_HUMAN Plakophilin-3 [Homo sapiens (Human) [9606]]	Q9Y446 (+1)
24	Q14C86-6 Isoform 6 of GTPase-activating protein and VPS9 domain-containing protein 1 [Homo sapiens (Human) [9606]]	Q14C86-6
25	B4DIG0_HUMAN M-phase inducer phosphatase 2 [Homo sapiens (Human) [9606]]	B4DIG0 (+5)
26	DSG1_HUMAN Desmoglein-1 [Homo sapiens (Human) [9606]]	Q02413 (+1)
27	SIKE1_HUMAN Suppressor of IKBKE 1 [Homo sapiens (Human) [9606]]	Q9BRV8 (+1)
28	A7XZE4_HUMAN Beta tropomyosin isoform [Homo sapiens (Human) [9606]]	A7XZE4 (+39)
29	STN1_HUMAN CST complex subunit STN1 [Homo sapiens (Human) [9606]]	Q9H668
30	J3KN16_HUMAN Proteasome-associated protein ECM29 homolog [Homo sapiens (Human) [9606]]	J3KN16
31	Q5T5C7_HUMAN Serine--tRNA ligase, cytoplasmic [Homo sapiens (Human) [9606]]	Q5T5C7
32	D6RHY1_HUMAN UDP-glucuronosyltransferase 3A2 [Homo sapiens (Human) [9606]]	D6RHY1 (+1)
33	MELT_HUMAN Ventricular zone-expressed PH domain-containing protein homolog 1 [Homo sapiens (Human) [9606]]	Q14D04 (+1)
34	E5RH12_HUMAN Steroidogenic acute regulatory protein, mitochondrial (Fragment) [Homo sapiens (Human) [9606]]	E5RH12 (+3)
35	B4DGC3_HUMAN cDNA FLJ57921, highly similar to Apolipoprotein D [Homo sapiens (Human) [9606]]	B4DGC3 (+2)
36	B2R8P1_HUMAN cDNA, FLJ93994, highly similar to Homo sapiens phosphate cytidylyltransferase 1, choline, alpha isoform (I	B2R8P1 (+3)
37	D7NTK9_HUMAN Bone morphogenetic protein 3 (Fragment) [Homo sapiens (Human) [9606]]	D7NTK9 (+1)
38	A2VCR0_HUMAN MAPRE1 protein (Fragment) [Homo sapiens (Human) [9606]]	A2VCR0 (+1)

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The PKR activator, PACT, becomes a PKR inhibitor during HIV-1 replication

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Abstract

Background: HIV-1 translation is modulated by the activation of the interferon (IFN)-inducible Protein Kinase RNA-activated (PKR). PKR phosphorylates its downstream targets, including the alpha subunit of the eukaryotic translation Initiation Factor 2 (eIF2α), which decreases viral replication. The PKR Activator (PACT) is known to activate PKR after a cellular stress. In lymphocytic cell lines, HIV-1 activates PKR only transiently and not when cells replicate the virus at high levels. The regulation of this activation is due to a combination of viral and cellular factors that have been only partially identified.

Results: PKR is transiently induced and activated in peripheral blood mononuclear cells after HIV-1 infection. The addition of IFN reduces viral replication, and induces both the production and phosphorylation of PKR. In lymphocytic Jurkat cells infected by HIV-1, a multiprotein complex around PKR contains the double-stranded RNA binding proteins (dsRBPs), adenosine deaminase acting on RNA (ADAR)1 and PACT. In HEK 293T cells transfected with an HIV-1 molecular clone, PACT unexpectedly inhibited PKR and eIF2α phosphorylation and increased HIV-1 protein expression and virion production in the presence of either endogenous PKR alone or overexpressed PKR. The comparison between different dsRBPs showed that ADAR1, TAR RNA Binding Protein (TRBP) and PACT inhibit PKR and eIF2α phosphorylation in HIV-infected cells, whereas Staufen1 did not. Individual or a combination of short hairpin RNAs against PACT or ADAR1 decreased HIV-1 protein expression. In the astrocytic cell line U251MG, which weakly expresses TRBP, PACT mediated an increased HIV-1 protein expression and a decreased PKR phosphorylation. In these cells, a truncated PACT, which constitutively activates PKR in non-infected cells showed no activity on either PKR or HIV-1 protein expression. Finally, PACT and ADAR1 interact with each other in the absence of RNAs.

Conclusion: In contrast to its previously described activity, PACT contributes to PKR dephosphorylation during HIV-1 replication. This activity is in addition to its heterodimer formation with TRBP and could be due to its binding to ADAR1. HIV-1 has evolved to replicate in cells with high levels of TRBP, to induce the expression of ADAR1 and to change the function of PACT for PKR inhibition and increased replication.

Keywords: Human immunodeficiency virus type 1 (HIV-1), Protein kinase RNA-activated (PKR), PKR activator (PACT), Eukaryotic translation initiation factor 2 (eIF2α), Lymphocytes, Astrocytes

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Background

Human immunodeficiency virus type 1 (HIV-1) mRNA expression is controlled at the transcriptional, processing and translational levels [1-3]. The main translational mechanism is a cap-mediated scanning from its 5' end but additional mechanisms occur including internal ribosome entry site in gag, programmed −1 ribosomal frameshift to produce Gag-Pol and discontinuous ribosome scanning to translate Env [4-6]. HIV-1 translation is modulated by viral components, like Trans-Activation Response element (TAR) RNA [7-10] and by cellular factors including translation factors, Protein Kinase RNA activated (PKR), TAR RNA Binding Protein (TRBP), PKR Activator (PACT), the La autoantigen, Staufen1 and the Adenosine Deaminase Acting on RNA (ADAR)1 [9,11-15]. The positive factors act by releasing the block due to the TAR structure, by inhibiting PKR or by inhibiting PACT [7-9,16-20].

The interferon (IFN)-inducible PKR is a key double-stranded RNA-binding protein (dsRBP), and a serine/threonine kinase. Its activation leads to autophosphorylation and the phosphorylation of its downstream targets, including the alpha subunit of the eukaryotic translation initiation factor 2 (eIF2α). Phosphorylated eIF2α (P-eIF2α) prevents translational initiation of viral and cellular mRNAs. PKR is central in the host innate defense strategies with strong antiviral and antigrowth activities [21-24]. In addition, its N-terminus forms a complex with proteins involved in cellular signaling pathways to mediate the activation of the NF-κB protein complex, which contributes to the induction of inflammatory cytokines [25,26]. PKR is extremely effective in restricting HIV-1 expression and replication *in vitro* [12,19,27-30]. Despite this observed activity, HIV-1 replicates efficiently in many permissive cell lines and primary cells, suggesting that the kinase activity of PKR in natural infection of lymphocytes is tightly regulated [17].

Many viruses that replicate efficiently have means to inactivate PKR and the HIV-1 Tat protein is one of these countermeasures [31-33]. Cells also avoid PKR activation using dsRNA sequestration or protein-protein interactions, likely as a normal process to allow their growth. Examples of direct interaction include the cellular protein $p58^{IPK}$, which binds to PKR and prevents its dimerization, tRNA-dihydrouridine synthetase-2, TRBP and ADAR1, which bind through their dsRNA Binding Domains (dsRBDs) and exert a strong inhibitory activity [12,19,22,32,34-40]. Besides dsRNA and heparin, PACT, the cytokine MDA-7/interleukin 24 and the transcription factor E2F-1 induce PKR activation [32,41-45]. PKR activation upon virus infection is also observed in some specialized cells. For example, cardiomyocytes are cells with high activation of PKR and PKR-like ER protein kinase (PERK) upon coxsackievirus infection due to a downregulation of $p58^{IPK}$ by the virus [46]. Similarly, astrocytic cells represent an example of naturally HIV-resistant cells with high PKR activation. In these cells, TRBP is expressed at very low amounts and cannot counteract PKR activation induced by the virus [47-49]. In contrast, in HIV-infected lymphocytes PKR activation is reduced when the virus reaches high concentrations and this is due in parts to the expression of TRBP, ADAR2 and to an increased ADAR1 expression that inhibits PKR activation [12,17,50,51].

PACT and its murine homolog RAX, are stress-inducible PKR activators [42,44,52]. They are proapoptotic proteins that induce apoptosis upon cellular stress by PKR activation [52-54]. PACT has two dsRBDs and a C-terminus domain called Medipal by homology with TRBP. All three domains in PACT homodimerize and interact with PKR and TRBP [20,55,56]. The Medipal domain mediates activation of PKR or inhibition by TRBP [18,55,57-59]. A cellular stress dissociates TRBP-PACT interactions and allows PACT activation of PKR. Therefore, PACT acts as a PKR activator in cells with low TRBP concentration or after stress induction, whereas it acts as a PKR inhibitor in cells with high TRBP content [13,18,20,55,60]. Its activity has not been tested in HIV-infected cells. Here, we observed that PKR is transiently induced and activated in HIV-1 infected peripheral blood mononuclear cells (PBMCs) with increased expression of both ADAR1 and PACT. We show that PACT binds to PKR during HIV-1 infection and that its activity is changed from an activator into an inhibitor of PKR in HIV-permissive cells and in astrocytic cells, which do not replicate HIV-1 efficiently. This change of function may be related to an interaction between ADAR1 and PACT.

Results

PKR is transiently induced and activated in PBMCs at the beginning of HIV-1 infection

We have previously shown that HIV-1 infection of the lymphocytic Jurkat T cell line induces PKR activation during the first days of infection, followed by an inactivation during high HIV-1 replication [12]. To determine if this regulation is also true in primary cells, we infected PBMCs from healthy donors with the pNL4-3 HIV-1 clone (Figure 1). To determine the importance of PKR activation during an IFN response in these cells and its impact on HIV-1 replication, half of the culture was treated with IFN at day 7 and IFN was maintained in the medium up to day 14. Following viral infection, reverse transcriptase (RT) activity was visible at day 6 and reached a peak at day 12, whereas the addition of IFN at day 7 induced a dramatic decrease in RT activity at day 8 (Figure 1A). Cell samples were gathered every two days and analyzed by Western blotting (Figure 1B). We first observed a very low basal level of PKR in uninfected cells (D0). PKR expression was induced from day 4 to 10 with a higher induction at day 6. It was activated (P-PKR) mainly at day 6 followed by deactivation. In contrast, when IFN was added at day 7,

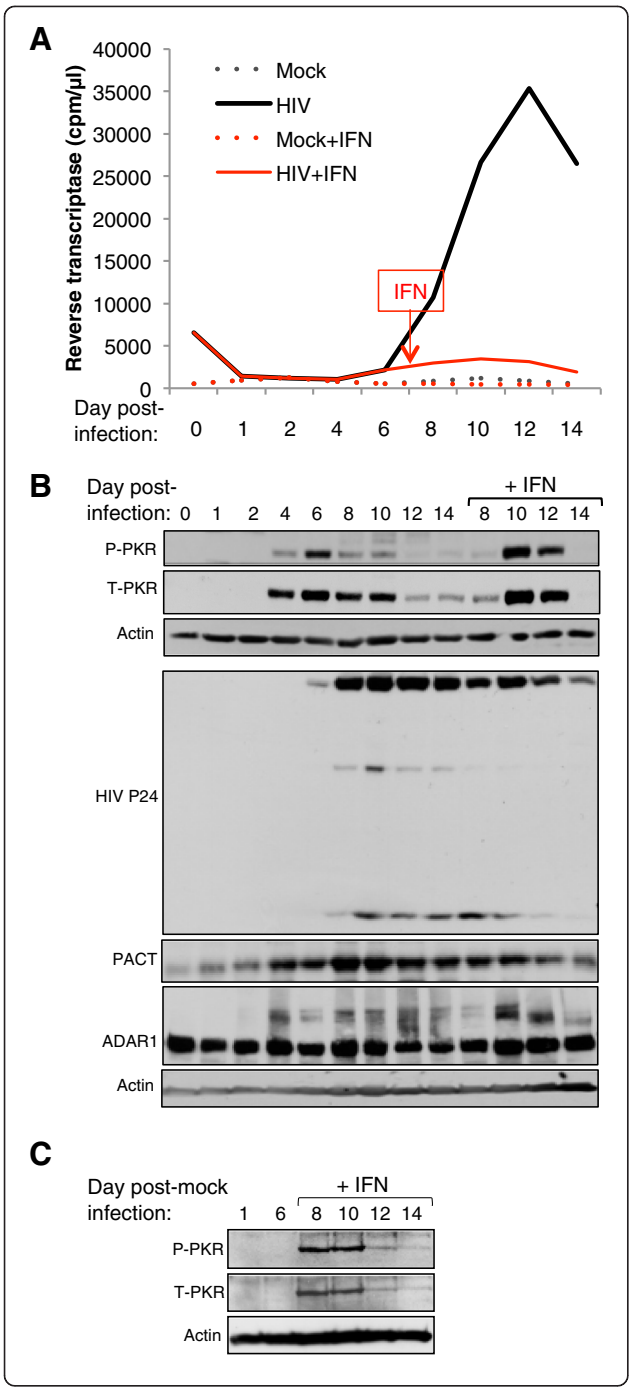

Figure 1 PKR is activated after HIV-1 infection and inhibited during active HIV-1 replication. A) HIV-1 pNL4-3 kinetics from infected PBMCs. 6.5×10^7 PBMCs from a healthy donor were infected with HIV-1 pNL4-3. At day 7, cells were separated in two flasks and IFN α/β (10000U/mL) was added to the cells in one of them up to day 14. Aliquots of cell supernatant were collected at different times and assayed for RT activity. **B)** Protein expression of pNL4-3-infected PBMCs. 50 µg of whole-cell extracts from pNL4-3 -infected PBMCs from different harvest times were subjected to a 10% SDS PAGE and blotted with anti-P-PKR, anti-PKR, anti-HIV-p24, anti-PACT, anti-ADAR1 and anti-actin antibodies as indicated. **C)** Protein expression of mock-infected PBMCs. 6.5×10^7 PBMCs from the same donor as in B were cultured and passed at the same time as in B. IFN α/β was added similarly from day 7 to 14. 50 µg of whole-cell extracts from mock-infected PBMCs from the indicated times were subjected to a 10% SDS PAGE and blotted with anti-P -PKR, anti-PKR and anti-actin antibodies as indicated.

PKR was induced and activated 3 days after (D10-12). Because ADAR1 expression is induced upon HIV-1 infection in Jurkat cells [12], we also evaluated its expression in this experiment. We found that ADAR1 p150 was induced at day 4 and was maintained up to day 14. IFN further induced its expression at day 10. To determine if PACT could likewise have a role in the regulation of PKR during HIV-1 infection of PBMCs, we also evaluated its expression. Surprisingly, we found an increase in PACT expression concomitant with ADAR1 p150 increase just before the expression of Gag protein was visible. In this case, PACT was not further induced by IFN. Interestingly, in a mock infection of the same cells, PKR was induced and activated one day after the addition of IFN (Figure 1C), suggesting that this induction is delayed by two days in HIV-1 infected cells. These results show that, PKR is transiently induced and activated in primary lymphocytes and deactivated when the virus replicates actively and that ADAR1 and PACT may play a role in this regulation.

PACT belongs to a multiprotein complex formed around PKR during HIV-1 infection

Many viral and cellular factors prevent PKR activation resulting in active viral infections and cell growth [32,33]. In the case of HIV-1 infection, the viral protein Tat, large amounts of TAR RNA, cellular proteins TRBP and ADAR1 all contribute to PKR inhibition [17]. Because cells also express PKR activators, and because we observed an increase in PACT expression during HIV-1 infection, we questioned whether PACT could contribute to PKR activation to enhance cell response and balance its inhibition by other factors. We have previously demonstrated that PACT is an activator or an inhibitor of PKR depending on TRBP expression in stressed or non-stressed cells [18,20,55]. We also observed that PACT expression is slightly increased at the peak of infection in Jurkat cells (Figure 2, input). We next determined if PACT was present in the complex formed around PKR during HIV-1

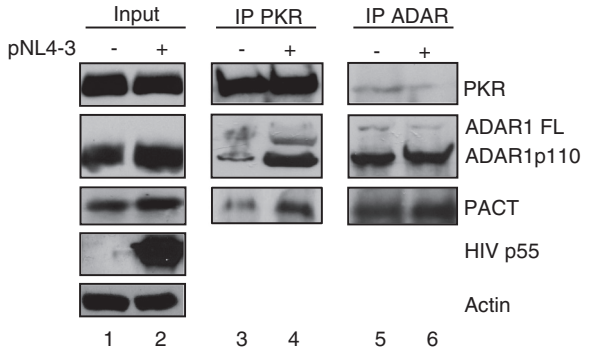

Figure 2 Increased ADAR1-PKR and PACT-PKR interactions during HIV-1 infection. Jurkat cells were mock-infected or infected with HIV-1 pNL4-3. Cell lysates were collected at day 15, which corresponds to the peak of infection for the pNL4-3-infected Jurkat cells. These were immunoprecipitated with anti-PKR or anti-ADAR1. 50 μg of proteins from each lysate (input; lanes 1–2) and the PKR (lanes 3–4) or ADAR (lanes 5–6) immunoprecipitated complexes were run on a 10% SDS-PAGE and blotted using anti-PKR, anti-ADAR1 (against full-length p150 and p110), anti-PACT, anti-HIV-p24 and anti-actin.

infection of lymphocytes. By immunoprecipitation (IP) with a PKR antibody, we observed that PACT interaction with PKR is increased at the peak of infection (Figure 2). This result resembles the previously observed increase in ADAR1 production and interaction [12]. By IP with an ADAR1 antibody, we also found that PACT is in the same complex as ADAR1, therefore suggesting that these two proteins are part of the multiprotein complex around PKR in HIV-1 infected cells.

PACT is a PKR inhibitor in HIV-1 transfected HEK 293T cells

We next questioned whether the role of PACT in a complex with PKR during HIV-1 infection would be as an activator or an inhibitor. To determine this role on viral protein expression and virion production, we transfected HEK 293T cells with pNL4-3 in the absence or presence of transfected PKR and evaluated the activity of a PACT expressing vector on viral expression and on PKR activation (Figure 3). Transfection of the HIV-1 molecular clone induced PKR and eIF2α phosphorylation (Figure 3A and B, lane 2). When cells were transfected with pNL4-3 and PACT in the absence of overexpressed PKR, PACT was able to increase HIV-1 protein expression and virion production up to 2.3 fold (Figure 3A). Surprisingly, increasing amounts of PACT clearly prevented PKR and eIF2α phosphorylation, indicating that the protein acts as an inhibitor of endogenous PKR and contributes to the enhancement of HIV-1 translation and consequently to the increased virion production.

As previously observed [12,49], transfected PKR reduced the expression of HIV-1 proteins and viral production and we show here that this is due to the concomitant increase in the ratio between P-PKR and PKR (Figure 3B, lane 3). In this case, increasing amounts of PACT restored viral protein expression and virion production up to 7 fold over the PKR-inhibited RT amount. The large amount of PKR did not allow appropriate quantification of the P-PKR/PKR ratio, but the P-eIF2α /eIF2α ratio clearly indicated that low amounts of PACT prevented the phosphorylation of eIF2α and increasing amounts restored HIV-1 protein expression and virion production (Figure 3B).

PACT, ADAR1 and TRBP inhibit PKR and eIF2α phosphorylation and increase HIV-1 protein expression

To compare the activity of the different dsRBPs that contribute to HIV-1 expression and may inhibit PKR activation in HIV-1-infected cells, we next overexpressed PACT, ADAR1, TRBP and Staufen1 with pNL4-3 in the absence or presence of transfected PKR (Figure 4). When PKR was not overexpressed, all four proteins induced a mild increase of HIV-1 protein expression and virion production reflected by HIV-1 p24 expression in cells and RT assay in the supernatant (Figure 4A). In this assay, a dramatic difference was observed in PKR and eIF2α phosphorylation between the four dsRBPs. PACT, ADAR1 and TRBP completely inhibited PKR and eIF2α phosphorylation, whereas Staufen1 only induced a modest reduction, suggesting that the first three dsRBPs increase virus expression mainly by acting on PKR, whereas Staufen1 increases virus production by a PKR-independent mechanism. When PKR was overexpressed, PACT, ADAR1 and TRBP restored PKR-inhibited HIV-1 expression and virion production, but Staufen1 did not (Figure 4B). The level of HIV-1 p24 expression reflected a complete restoration of viral protein expression with PACT, ADAR1 and TRBP, but only a low increase by Staufen1 over PKR inhibition. The P-PKR/PKR and the P-eIF2α/eIF2α ratio were difficult to evaluate due to the high expression of transfected PKR, but suggests that PACT, ADAR1 and TRBP induce an additional mechanism, which also contributes to the restoration of viral expression in the context of overexpressed PKR.

shRNAs against PACT and ADAR1 inhibit HIV-1 expression

To further determine the role of endogenous PACT on HIV-1 expression and to compare with the function of ADAR1, we generated short hairpin RNAs (shRNAs) against PACT and against ADAR1 mRNAs to decrease their protein expression (Figure 5). Cotransfection of HEK 293T cells with pNL4-3 together with the shRNA1 or 2 against PACT (P1 and P2, two variants of the same sequence), the shRNA against ADAR1 or a combination of shRNA ADAR1 and shRNA P2 against PACT all decreased HIV-1 protein expression and viral production.

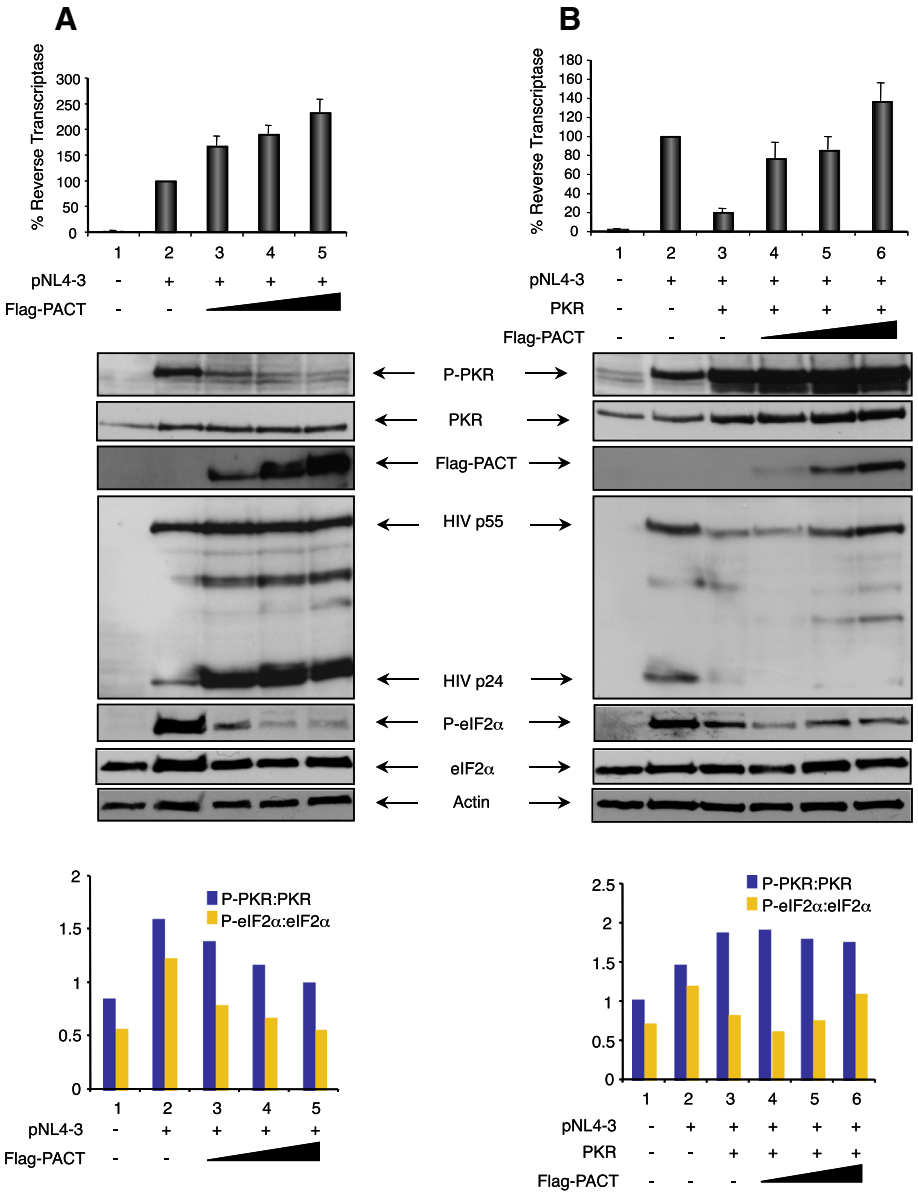

Figure 3 PACT increases HIV-1 protein expression and virion production in HEK 293T cells by PKR and eIF2α inhibition. A) PACT inhibits endogenous PKR and eIF2α phosphorylation and increases pNL4-3 expression. HEK 293 T cells were mock transfected (lane 1), transfected with 2 μg pNL4-3 (lanes 2–5) and 0.5 μg (lane 3), 1.0 μg (lane 4) or 1.5 μg (lane 5) of pCMV2-Flag-PACT. pCMV2 was added to reach the same amount of transfected DNA. (Top) % RT activity is the ratio between the RT level in the presence of PKR and PACT versus pNL4-3 alone normalized to 100%. Shown is the average of 6 independent transfections ± SEM. (Middle) Immunoblot of cell extracts of a representative experiment from the same transfected cells using antibodies against P-PKR, PKR, Flag, HIV-1 p24, P-eIF2α, eIF2α and actin. (Bottom) Ratio of phosphorylated PKR (P-PKR) versus PKR and P-eIF2α versus eIF2α. The band intensity was digitalized using Adobe Photoshop software from the bands shown above. P-PKR /PKR and P-eIF2α/eIF2α ratio was calculated by dividing the P-PKR or P-eIF2α intensity by the total PKR or eIF2α intensity of each band. **B)** PACT reverses PKR inhibition of pNL4-3. HEK 293 T cells were mock transfected (lane 1), transfected with 2 μg pNL4-3 (lanes 2–6), 0.5 μg pcDNA1-PKR (lanes 3–6), 0.5 μg (lane 4), 1.0 μg (lane 5) or 1.5 μg (lane 6) of pCMV2-Flag-PACT. Empty corresponding plasmids were added to reach the same amount of transfected DNA. (Top) % RT activity is calculated as in **A)**. Shown is the average of 6 independent transfections ± SEM. (Middle) Immunoblot of cell extracts of a representative experiment from the same transfected cells using the same antibodies as in **A)**. (Bottom) Ratio of phosphorylated PKR versus PKR and P-eIF2α versus eIF2α. The band intensities were calculated as in **A)**.

Whereas both shRNAs PACT induced a modest increase of PKR phosphorylation, the shRNA ADAR1 consistently enhanced it. A combination of shRNA2 PACT and shRNA ADAR1 for the same final amount resulted in an intermediate effect on viral production compared to the two shRNAs alone (Figure 5, lane 5 compared to lanes 3 and

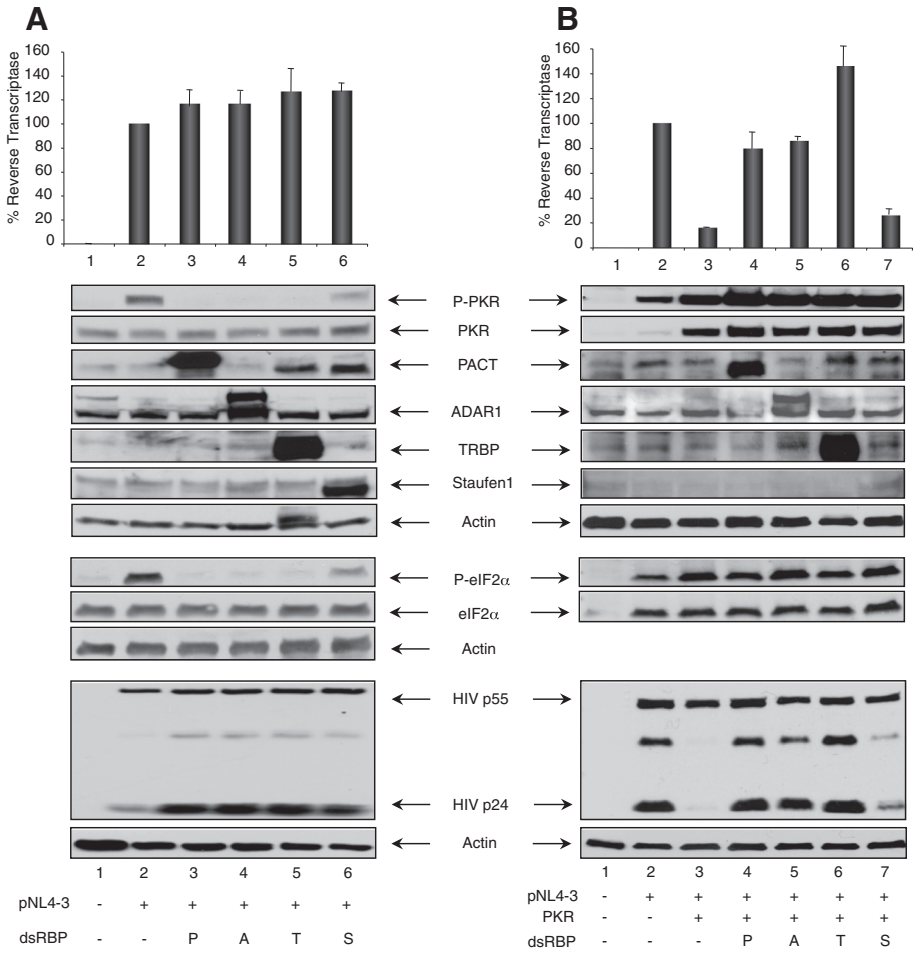

Figure 4 PACT, ADAR1 and TRBP inhibit PKR activation in HIV-1 producing cells. A) PACT, ADAR1 and TRBP, but not Staufen1, inhibit PKR and eIF2α phosphorylation. HEK 293T cells were mock transfected (lane 1), transfected with 2 μg pNL4-3 (lanes 2–6), and 1.5 μg of pCMV2-Flag-PACT (lane 3), pcDNA3.1-ADARp150-V5 (lane 4), pcDNA3-TRBP2 (lane 5) or pCMV-RSV-Staufen1-HA (lane 6). pcDNA3 was added to reach the same amount of transfected DNA. (Top) RT activity from cell supernatants normalized to 100% in the absence of PKR or dsRBPs. Shown is the average of 6 independent transfections ± SEM. (Bottom) Immunoblot of 150 μg of cell extract of a representative experiment from the same transfected cells using antibodies against P-PKR, PKR, P-eIF2α, eIF2α, PACT, ADAR1, TRBP, Staufen1, HIV-1 p24 and actin as indicated. Probing for P-eIF2α and eIF2α were performed on a separate membrane and the corresponding actin is shown. **B)** PACT, ADAR1 and TRBP, but not Staufen1, restore PKR inhibition of HIV-1 protein expression and virion production. HEK 293T cells were mock transfected (lane 1), transfected with 2 μg pNL4-3 (lane 2–7), 0.5 μg pcDNA1-PKR (lanes 3–7) and 1.5 μg of pCMV2-Flag-PACT (lane 4), pcDNA3.1-ADARp150-V5 (lane 5), pcDNA3-TRBP2 (lane 6) or pCMV-RSV-Staufen1-HA (lane 7). The empty pCMV2 vector was used to supplement transfections such that the same amount of DNA was transfected into each well. (Top) % RT activity is calculated as in **A)**. Shown is the average of 6 independent transfections ± SEM. (Bottom) 150 μg of each cell extract was analyzed by immunoblot against P-PKR, PKR, P-eIF2α, eIF2α, PACT, ADAR1, TRBP, Staufen1, HIV-1 p24 and actin as indicated. Shown is a representative experiment from the same transfected cells. Probing for P-eIF2α and eIF2α were performed on the same membrane as HIV-1 p24 and have the same actin.

4), suggesting an additive effect. In agreement with the data with PACT overexpression, these results suggest that in these cells, PACT contributes to the enhanced HIV-1 protein production in combination with other proteins.

PACT is a PKR inhibitor in HIV-1 transfected U251MG astrocytic cells

We next wanted to determine if the function of PACT as a PKR inhibitor during HIV-1 replication in lymphocytes as well as in HIV-1 production in HEK 293T cells could be due to TRBP heterodimers formation [18]. To do this, we evaluated PACT's activity in U251MG astrocytic cells which naturally express low levels of TRBP. We first confirmed that Flag-PACT activates PKR in astrocytes and induces eIF2α phosphorylation as previously shown [18]. We also verified the activity of PACTΔ13 (also called PACT305 or PACTΔ1), a truncated PACT lacking 13 amino acids in its C-terminus that constitutively activates PKR and is poorly sensitive to TRBP inhibition. Similar to previous results [18], PACT and PACTΔ13 induced PKR

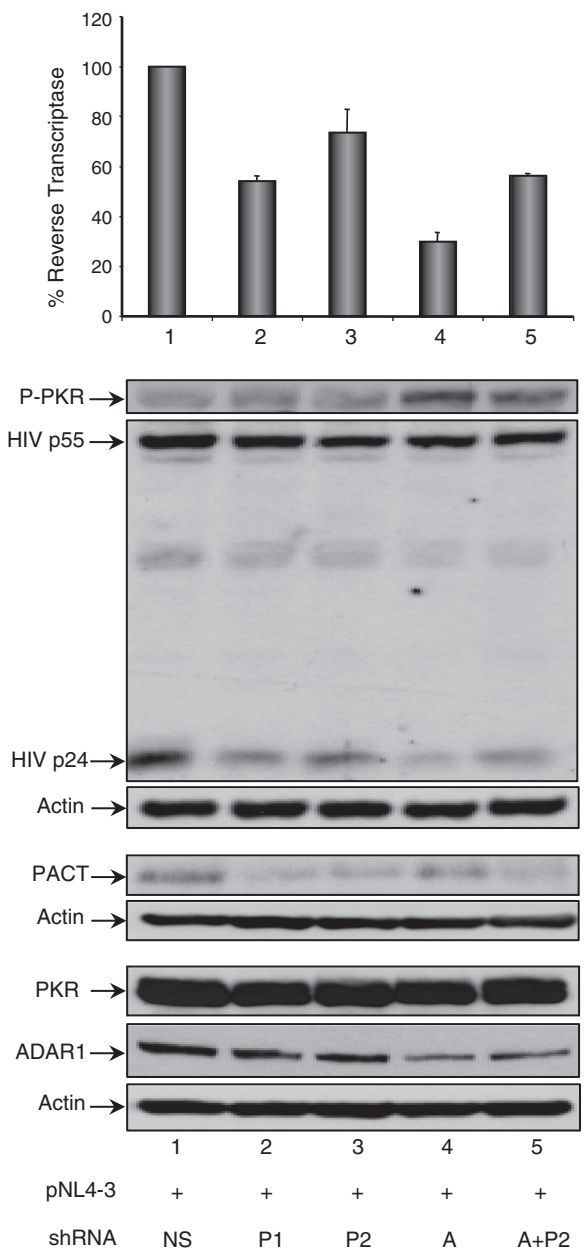

Figure 5 shRNAs against PACT and ADAR1 decrease HIV-1 protein expression and virion production. HEK 293T cells were co-transfected with 0.2 μg pNL4-3 (lane 1–5), and 2 μg of shRNAs NS (lane 1), 2 μg of shRNA1 PACT (lane 2), 2 μg of shRNA2 PACT (lane 3), 2 μg of shRNA ADAR1 (lane 4) and a combination of shRNA2 PACT and shRNA ADAR1 at 1 μg each (lane 5). 100 μg of cell lysates were resolved by SDS-PAGE and analyzed by immunoblotting using antibodies against P-PKR, PKR, HIV-1 p24, PACT, ADAR1, and actin. NS stands for non-specific, P1 and P2 stand for two different shRNAs against PACT and A stands for shRNA against ADAR1.

and eIF2α phosphorylation with PACTΔ13 being highly active at low doses (Figure 6A). We then repeated the experiment in U251MG cells expressing HIV-1 proteins. Indeed, if PACT is a PKR activator in astrocytes expressing HIV-1 like in Figure 6A, it could be ascribed to the lack of TRBP-PACT heterodimer formation [18,20,55]. In contrast, if PACT is a PKR inhibitor in these cells, the effect would not solely be due to TRBP. We observed that overexpression of PACT in HIV-transfected U251MG astrocytoma cells induced an increased expression of HIV-1 protein production and inhibited PKR and eIF2α phosphorylation (Figure 6B). This result shows that PACT becomes a PKR inhibitor in astrocytes when these cells express HIV-1 proteins. The low level of HIV-1 virion production was increased by up to 4-fold, which is similar to what was previously observed with TRBP [12,49]. PACTΔ13 is a potent PKR activator in HIV-non-infected astrocytes ([18] and Figure 6A) and mediates apoptosis through PKR activation in HT1080 cells [61]. In U251MG cells transfected with HIV-1, we observed that PACTΔ13 lost its activating property on PKR and eIF2α phosphorylation (compare Figure 6C to 6A). Compared to wild-type PACT, it lost the enhancement in HIV-1 protein expression and virion production (compare Figure 6C to 6B).

PACT and ADAR1 directly interact in cells

The experiments in Figure 6 demonstrate that TRBP-PACT interaction cannot solely explain the change in PACT function in HIV-1 expressing cells. We therefore wanted to determine if this change could be due to a virally-induced mechanism. Because we showed that ADAR1 expression is induced during HIV-1 replication both in a lymphocytic cell line [12] and in PBMCs (Figure 1), we tested if ADAR1 and PACT could interact directly in cells. We transfected HEK 293T cells with either Flag-PACT or ADAR1 p150-V5, immunoprecipitated respectively with anti-Flag or anti-V5 antibody and blotted for either the endogenous ADAR1 or PACT (Figure 7). We recovered both proteins in the immunoprecipitate with either antibody, showing an interaction (Figure 7, lanes 4). When we treated the extracts with Benzonase, which contains RNAses against ss and dsRNAs, we clearly recovered ADAR1 with an anti-Flag showing an interaction between ADAR1 and PACT in the absence of RNA. The reverse IP with Benzonase was not as distinct, but also showed a likely direct interaction. Taken together, these results show that ADAR1 binds to PACT with or without RNAs, which could explain the reversal of PACT's function in HIV-1 producing cells.

Discussion

During HIV-1 infection, IFNα/β is mainly produced by plasmacytoid dendritic cells and acts on infected cells, but this cell response is not sufficient to clear the virus in patients [62]. Our results show that PBMCs do respond to IFN by producing IFN-stimulated genes (ISGs) and inhibiting HIV-1 replication (Figure 1). Therefore,

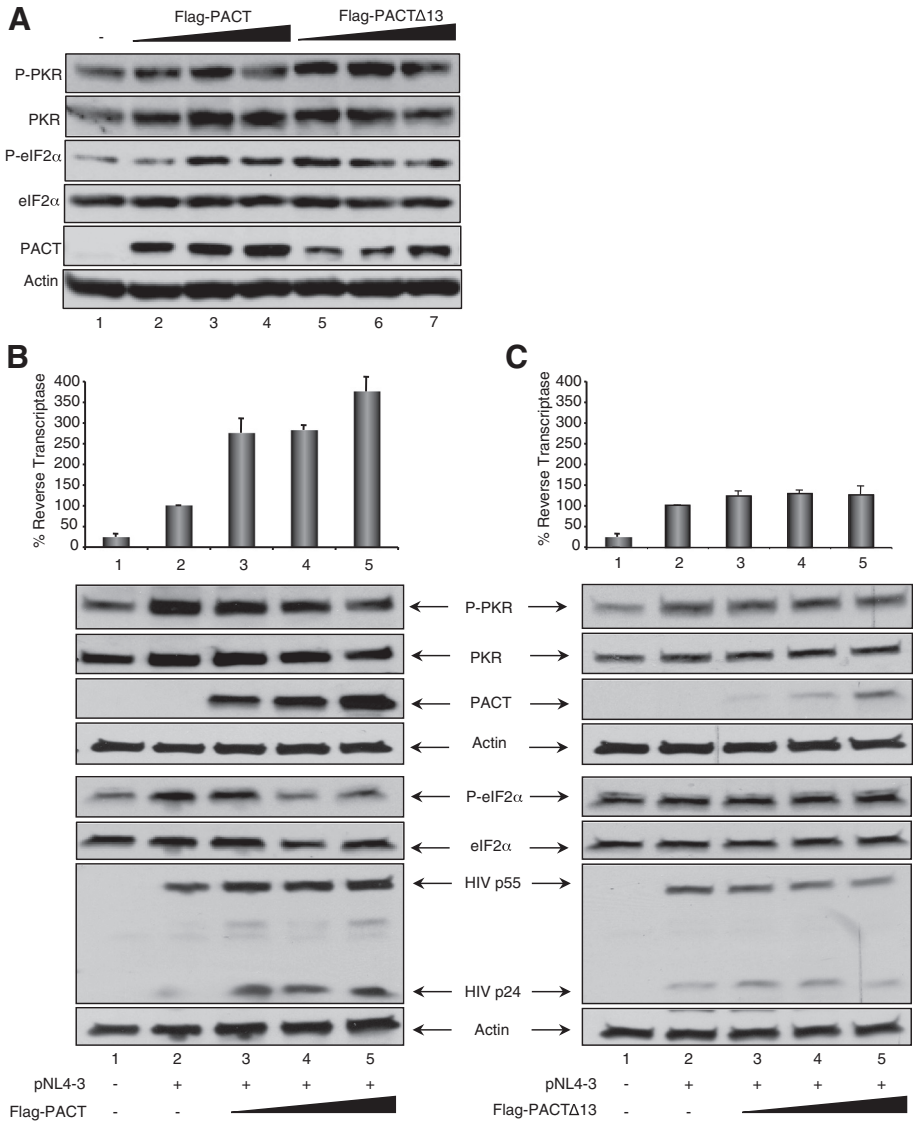

Figure 6 PACT and PACTΔ13 are not PKR activators in HIV-producing U251MG astrocytes. A) PACT and PACTΔ13 activate PKR and eIF2α in U251MG astrocytes. U251MG astrocytes were mock transfected (lane 1), transfected with 0.5 μg (lane 2 and 5), 1 μg (lane 3 and 6), 2 μg (lane 4 and 7) of pCMV2-Flag-PACT or pCMV2-Flag-PACTΔ13 as indicated. 100 μg of cell lysates were resolved by SDS-PAGE and analyzed by immunoblotting using antibodies against P-PKR, PKR, HIV-1 p24, Flag and actin. **B)** PACT moderately inhibits PKR activation and causes an increase of HIV-1 production in U251MG astrocytes. U251MG astrocytes were mock transfected (lane 1), transfected with 2 μg pNL4-3 (lanes 2–5) and 0.5, 1 and 2 μg of pCMV2-Flag-PACT (lanes 3–5). The corresponding empty plasmid, pCMV2, was supplemented to each transfection to have the same amount of DNA transfected in each well. (Top) The RT activity is displayed as a percentage of activation compared to pNL4-3 alone. Results shown are an average of 3 independent transfections ± SEM. (Bottom) Cell extracts were immunoblotted and probed using antibodies against P-PKR, PKR, Flag, HIV-1 p24, P-eIF2α, eIF2α and actin. Shown is a representative experiment. **C)** PACTΔ13 affects neither PKR activation nor HIV-1 production in U251MG astrocytes. U251MG astrocytes were mock transfected (lane 1), transfected with 2 μg pNL4-3 (lanes 2–5) and 0.5, 1 and 2 μg of pCMV2-Flag-PACT13 (lanes 3–5). The corresponding empty plasmid, pCMV2, was supplemented to each transfection to have the same amount of DNA transfected in each well. (Top) The RT activity was displayed as a percentage of activation compared to pNL4-3 alone. Results shown are an average of 3 independent transfections ± SEM. (Bottom) Cell extracts were immunoblotted and probed using antibodies against P-PKR, PKR, Flag, HIV-1 p24, P-eIF2α, eIF2α and actin. Shown is a representative experiment.

the lack of *in vivo* efficacy cannot be ascribed to a lack of cell response to IFN. It could be due to either an insufficient amount of IFN production or to a block in the downstream effects of IFN or both. IFNα/β also has adverse effects, which limits its therapeutic use [63-65], emphasizing the need to better understand the downstream effects of ISGs and their regulation in HIV-1 -infected cells. Among the ISGs, PKR and its activator PACT can either contribute to translational inhibition, proliferation arrest and apoptosis through eIF2α, I-κB

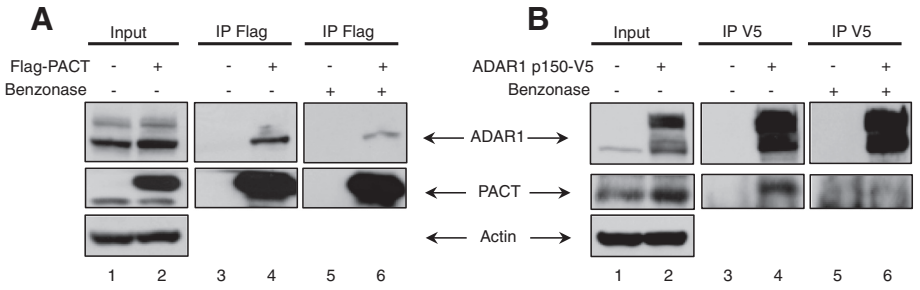

Figure 7 PACT interacts with ADAR1. A) Transfected Flag-PACT interacts with endogenous ADAR1. HEK 293T cells were not transfected (lanes 1, 3, 5), or transfected with 2 μg of pCMV2-Flag-PACT (lanes 2, 4, 6). IP was performed with 2 mg of protein and anti-Flag antibody and treated with Benzonase (lane 5, 6). 150 μg of cell lysate (input or immunoprecipitates) were resolved by 10% SDS-PAGE and analyzed by immunoblotting using antibodies against ADAR1, PACT and actin. **B)** Transfected ADAR1-V5 interacts with endogenous PACT. HEK 293T cells were not transfected (lanes 1, 3, 5), or transfected with 2 μg of pcDNA3.1-ADARp150-V5 (lanes 2, 4, 6). IP was performed with 2 mg of protein and anti-V5 antibody and treated with Benzonase (lane 5, 6). 150 μg of cell lysate (input or immunoprecipitates) were resolved by 10% SDS-PAGE and analyzed by immunoblotting using antibodies against ADAR1, PACT and actin.

phosphorylation or IFNβ induction when PKR is activated [52-54,61,66,67], or to increased viral replication and NF-κB signaling when it is not activated [12,17,25,26,68]. Because the PKR/PACT axis is part of the innate immune response to viruses, the elucidation of its activity is important to understand the inefficient response during HIV-1 replication. We and others have shown that PKR is extremely effective in restricting HIV-1 replication *in vitro* [12,27-30,49]. Furthermore, knocking down PKR by small interfering RNAs (siRNAs) or expressing a transdominant mutant of PKR increases HIV-1 production [49]. Despite this activity, HIV-1 replicates efficiently in many cells, suggesting that the activity of PKR in natural infection is highly regulated [17]. We therefore investigated the activation or deactivation of PKR during HIV-1 infection and the activity of exogenous IFN on PKR induction and activation. The transient activation of PKR followed by an absence of activation during HIV-1 infection of PBMCs (Figure 1) resembles the one observed with lymphocytic cell lines infected with X4 or R5 HIV-1 strains [12]. The transient activation of PKR in PBMCs suggests that this part of the innate immune response is active but is also tightly regulated during the infection of primary lymphocytes and monocytes in patients. Interestingly, the addition of IFN inhibited virus growth and induced PKR induction and activation. PKR induction was delayed by two days compared to the mock infection emphasizing that the presence of the virus postpones its expression. Furthermore, ADAR1 and PACT were induced at day 4 suggesting that an early protein from the virus may contribute to their expression.

The regulation of PKR activation is the result of the action of activators and inhibitors. The equilibrium reached after a viral infection contributes to a high or a weak cell response that will either activate innate immunity and block viral replication or let the virus replicate [32]. In the case of HIV-1 infection, the TAR RNA is likely one of the main activators of PKR at the beginning of the infection, but may become an inhibitor if produced in large amounts in the cell [69]. The HIV-1 Tat protein is also an inhibitor of PKR acting by substrate competition [31]. Besides direct viral countermeasures, viruses also evolved to replicate in cells that have the appropriate cellular components to allow their replication [70]. Viruses can also induce the production of cellular proteins that will counteract an antiviral cell response. A cell that expresses high amounts of PKR inhibitors certainly favors HIV-1 replication. HIV-1 replicates in cells that express a large amount of TRBP that inhibits PKR [39,49]. HIV-1 also induces ADAR1 production, which contributes to PKR inhibition and RNA editing and favors viral replication [12,50,71]. We show here that ADAR1 is also induced in PBMCs (Figure 1B), which corroborates this effect in primary cells. Because TRBP not only acts on PKR, but also prevents PACT activity on PKR [18,55], we originally thought that PACT may activate PKR and that the end-up result of the PKR status would be a balance between PKR activators and PKR inhibitors. The identification of PACT in a protein complex with PKR, TRBP and ADAR1 during HIV-1 infection suggested a role for PACT but raised the question of its function within this complex (Figure 2).

When overexpressed in HIV-1-expressing cells, PACT inhibited PKR and eIF2α phosphorylation and consequently increased HIV-1 expression (Figures 3 and 4). PACT inhibition of PKR activation and consequently on eIF2α phosphorylation was very dramatic on endogenous PKR (Figures 3A and 4A), indicating that PACT reverses its function in HIV-1-producing cells. When PKR was overexpressed, the effect of PACT on PKR activation was only visible on the P-PKR/PKR and P- eIF2α/eIF2α ratio (Figure 3B), but clearly reversed PKR inhibition of HIV-1 production, suggesting that PACT may also act through another kinase like PERK, or directly on eIF2α, or it

may have an additional activity to increase viral expression. The mechanism of this increased viral expression despite high PKR phosphorylation may be related to the phosphorylation of HIV-1 Tat by PKR [72] or to a transcriptional activity of PACT similar to the recently observed recruitment of PACT, TRBP and Dicer to the promoter of nuclear receptors [73]. Furthermore, PACT inhibition by shRNAs decreased HIV-1 protein expression similarly to shRNAs against ADAR1 (Figure 5). Together, the increased expression of PACT during HIV-1 replication in PBMCs (Figure 1), the increased PKR-PACT interaction at the peak of infection (Figure 2), PACT activity upon over-expression (Figures 3 and 4) and results with shRNAs (Figure 5) contribute to reach the same conclusion that PACT is a PKR inhibitor during HIV-1 replication. There are several explanations that could explain this activity: i) TRBP is present in high amounts in HEK 293 T cells and forms heterodimers with all PACT molecules, which reverses PKR activation; ii) the large amount of ADAR1 induced by HIV-1 binds to PACT and reverses its function; or iii) an HIV-1 component or an HIV-induced component will change PACT from an activator into an inhibitor of PKR.

Our results in the astrocytic cells U251MG show that the first hypothesis by the formation of TRBP-PACT heterodimers cannot be the sole explanation, strongly suggesting that an HIV-1 component or an HIV-induced component mediates the change in PACT function in HIV-1-expressing cells (Figure 6). This component prevents PACT from being a PKR activator and changes it into a PKR inhibitor with a similar activity as TRBP on PKR. Furthermore, PACT Δ13, although not naturally produced in cells, has been shown to be a strong activator of PKR, because it is constitutively active and not regulated by TRBP [18,20,61]. Its loss of activity in HIV-1-expressing astrocytes reinforces the idea that an HIV-1 or HIV-induced component reverses PACT activating function on PKR independently of TRBP (Figure 6C). The second possibility would be that the HIV-mediated increase in ADAR1 expression mediates a change in PACT function by direct binding. Our IP assays show that it may be the case because the two proteins are in the same complex during HIV-1 infection (Figure 2) and that they interact in the absence of RNA (Figure 7). We cannot exclude that another mechanism may be involved as well. PACT-Dicer interaction [74] or PACT induction of RIG-I upon Sendai virus infection [75] seems unlikely here because it would lead to viral restriction or enhanced innate immune response respectively, which we do not observe during HIV-1 infection or after PACT overexpression of HIV-1-expressing cells (Figures 1, 3, 4, 6). Therefore, ADAR1-PACT interaction is currently the most likely mechanism, which may contribute, at least in part, to the change in PACT activity during HIV-1 infection.

Our results show that three cellular proteins, TRBP, ADAR1 and PACT contribute to the inhibition of PKR and eIF2α phosphorylation observed in HIV-1-infected cells (Figure 4). All of them are dsRBPs, therefore raising the question if all proteins of this family act similarly. Staufen1 was used as another dsRBP that has a positive activity on the virus by binding to Gag and by increasing translation from TAR-containing RNAs [9,76]. In agreement with its PKR-independent mechanism on translation [9], we found that Staufen1 did not inhibit PKR activation supporting a combined inhibition of PKR by TRBP, ADAR1 and PACT and a different mechanism for Staufen1 via Gag multimerization, HIV-1 assembly and encapsidation of genomic RNA [77-79], all contributing to viral replication. Further studies will determine if PKR forms a different protein complex at the beginning of HIV-1 infection when PKR and eIF2α are activated, if PACT has a different activity in this context and how it may contribute to the pathogenicity induced by the virus.

Conclusions

Previous results have characterized PACT as a stress-inducible PKR activator. In contrast, we show here that PACT becomes a PKR inhibitor during HIV-1 replication. PACT belongs to a multiprotein complex including the PKR inhibitors TRBP and ADAR1 formed around PKR during high viral replication. Results strongly suggest that PACT reversion of PKR activation comes in addition to its control by TRBP and could be due to its interaction with ADAR1 and other HIV-1 or an HIV-induced component. These data show that HIV-1 has evolved using several mechanisms to overcome the innate cell response.

Methods

Cells and transfections

HEK 293T (ATCC CRL-11268) and U251MG [49] cells were maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle's Medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (HyClone), 2 mM L-glutamine, and 1% penicillin-streptomycin (Invitrogen). Jurkat T cells (ATCC TIB-152) were maintained in RPMI-1640 (Invitrogen) supplemented similarly.

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors previously selected to be negative for HIV, HTLV-I and II, HCV, CMV and syphilis. Blood sample collection was approved by the ethics review board of McGill University.

For transfection of HEK 293T and U251MG cells with plasmids, cells were plated in six-well plates at 50% confluence 24 h prior to transfection using polyethylenimine (PEI) following manufacturer's protocol (Polysciences). Transfection of HEK 293T cells with shRNA vectors (2 μg/well in a 6-well plate) was performed 24 h after plating using TransIT-LT (Mirus) as described [80]. pNL4-3

(0.2 μg/well in a 6-well plate) was then transfected using TransIT-LT 24 h after transfection of the shRNAs. Supernatants and lysates were then collected 48 h after transfection of pNL4-3 as described [81].

Plasmids and shRNA synthesis

HIV-1 clone pNL4-3, pcDNA1-PKR, pCMV2-Flag-PACT, pCMV2-Flag-PACTΔ13, pcDNA3-TRBP2, pcDNA3.1-ADARp150-V5, and pcDNA3-RSV-Staufen1-HA were previously described [12,55,82].

shRNAs targeting PACT and ADAR1 were cloned into the psiRNA vector (InvivoGen) using sequences obtained from the Sigma-Aldrich website. The sense (S) and antisense (AS) oligonucleotide sequences of the shRNAs are:

PACT 1: (S) 5′ -
ACCTCGCGCCAATGGACAATATCAATCTCGA
GATTGATATTGTCCATT GGCGCTT - 3′ and (AS) 5′ -
CAAAAAGCGCCAATGGACAATATCAATCTC
GAGATTGATATTGTCC ATTGGCGCG - 3′.
PACT 2: (S) 5′ -
ACCTCGCGCCAATGGACAATATCAATACTCGAG
AATTGATATTGTCCATT
GGCGCTT- 3′ and (AS) 5′ -
CAAAAAGCGCCAATGGACAATATCAATTCTC
GAGTATTGATATTGTCCATTGGCGCG - 3′.
ADAR1:(S) 5′ -
ACCTCGCTGTTAGAATATGCCCAGTTACTCGA
GAAACTGGGCATATTCTA
ACAGCTT- 3′ and (AS) 5′ -
CAAAAAGCTGTTAGAATATGCCCAGTTTC
TCGAGTAACTGGGCATATTCTAACAGCG - 3′.

After annealing at 80°C for 2 minutes, the shRNAs were ligated into BbsI-digested psiRNA.

Transfection of HIV-1 molecular clones and RT assay

HEK 293T were transfected as above with pNL4-3 proviral DNA. Cell supernatants were collected 48 h post transfection and viral production assayed for standard RT assay. RT assay was as previously described [81] with modifications described in [83]. Supernatants from transfected HEK 293T cells were used for infection of Jurkat or PBMCs.

The RT assay from the supernatant of transfected U251MG cells was carried out in a similar manner, with the exception of a longer incubation period at 37°C (3 h) and 10 μl being spotted onto the DEAE paper as previously [84]. This was to account for the low amount of virus production in U251MG astrocytes.

HIV-1 viral infection of Jurkat cells and PBMCs

HIV-1 Jurkat cells infection was previously described [12]. For PBMCs HIV-1 infection, cells were stimulated with 0.6 μg/ml phytohaemagglutinin (Sigma cat. # 12646) for three days in supplemented RPMI (Invitrogen). 24 h prior to infection, recombinant human interleukin 2 (IL-2) (R&D Systems, cat. # 202-IL) was added to the cells for a final concentration of 10 ng/ml. 6.5×10^7 cells were infected with HIV-1 cell supernatant corresponding to 1.3×10^7 cpm measured by standard RT assay in a final volume of 2.5 ml supplemented RPMI in polypropylene round-bottom tube, and incubated for 2 h at 37°C. RPMI supplemented with IL-2 for a final concentration of 10 ng/ml was then added to the cell-virus mixture, transferred to a T25 flask and incubated at 37°C. The cells were fed on average every two days with fresh medium supplemented with IL-2 (10 ng/ml). Supernatant and cell samples were collected at different times and assayed for RT activity, immunoblotting and IP when indicated.

Immunoprecipitation and immunoblotting

IP with infected Jurkat cells was previously described [12]. For IP from HEK 293T cells, 48 h post-transfection, cells were washed twice with PBS and lysed in the cold lysis buffer with protease inhibitors. For each IP, 50 μl of protein G agarose fast flow compact beads (Sigma) were washed with TNEN (50 mM Tris–HCl [pH 7.4], 100 mM NaCl, 1 mM EDTA [pH 8], 0.5% NP40 (Sigma)) and left rotating at 4°C for overnight incubation at 4°C with 5 μg of anti-V5 antibody (Invitrogen). 500 μg to 2 mg of cell extract was added to the beads for overnight incubation at 4°C. The beads were washed 3 times with 1 ml of cold lysis buffer, 5 times with 1 ml cold PBS and resuspended in SDS loading dye. When indicated, the beads were treated with 250 U/ml of Benzonase® (Sigma) in 50 mM Tris–HCl, 1 mM MgCl2, pH 8.0 for 30 min at 37°C. Bound proteins were eluted by boiling the beads for 5 min and separated by 10% SDS-PAGE. The immunoprecipitates were analyzed by a Western blot analysis using the anti-ADAR1 (from Dr. BL Bass) or anti-PACT (Medimabs) antibodies.

For immunoblotting, HEK 293T, or Jurkat T cells extracts were prepared, separated and transferred on a Hybond ECL nitrocellulose membrane (GE Healthcare) as previously described [55]. Membranes were blocked for 1 h in 5% nonfat dry milk and Tris-buffered saline-0.1% Tween 20 (TBST). Membranes were incubated overnight at 4°C with the primary antibody. After five washes in TBST, membranes were incubated with Horseradish Peroxidase-conjugated secondary goat anti-rabbit or goat anti-mouse antibody (GE Healthcare). Anti-P-PKR (Abcam) and anti-P-eIF2α (Invitrogen) was blotted in 3% BSA/TBST overnight. After immunoblotting with an antibody, the membranes were washed in TBST overnight or stripped

and reused to detect other proteins. The bands were visualized using ECL (GE Healthcare). Primary antibodies used for immunoblotting were: monoclonals anti-PKR 71–10 [85] obtained from Dr. A. Hovanessian, anti-Actin (Chemicon) at a 1/5000 dilution, anti-HIVp24 183-H12-5C [86], at a 1/1000 dilution, anti-Flag (Sigma) at a 1/5000 dilution, polyclonal anti-TRBPjbx [18] at a 1/500 dilution, anti-P-PKR (Abcam), anti-PACT (Medimabs), anti-Staufen1, anti-ADAR1, anti-P-eIF2α (Invitrogen) and anti-eIF2α (Cell Signaling), at a 1/1000 dilution. The anti-Staufen1 antibody was generated at the Cell Imaging and Analysis Network (McGill University, Montréal, Canada) using purified full-length His-tagged Staufen1 protein. For multiple blotting, when the same membrane cannot be blotted again, extracts from the same experiment were separated by SDS PAGE and blotted on a new membrane. In this case, actin is shown for each membrane. Where indicated, the bands where quantified by densitometry analysis as described [80].

Abbreviations

HIV-1: Human immunodeficiency virus type 1; PKR: Protein kinase RNA-activated; eIF2α: Alpha subunit of the eukaryotic translation initiation factor 2; IFN: Interferon; dsRBP: Double-stranded RNA binding protein; dsRBD: Double-stranded RNA binding domain; ADAR1: Adenosine deaminase acting on RNA 1; TRBP: TAR RNA binding protein; PACT: PKR activator; PBMCs: Peripheral blood mononuclear cells; siRNA: Small interfering RNA; shRNA: Short hairpin RNA; RT: Reverse transcriptase; IP: Immunoprecipitation.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

The work was originally designed by GC, JFG and AG, followed by ES, AD, SB, TE, LS and AG. GC, ES, AD, SB, JFG, TE and LS performed the experiments and analyzed the corresponding data. JPR, AJM, RCP provided reagents and analyzed data. AG has supervised all the work and wrote the final version. All authors have participated to the writing and have approved the final version.

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HIV-1 translation and its regulation by cellular factors PKR and PACT

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ABSTRACT

The synthesis of proteins from viral mRNA is the first step towards viral assembly. Viruses are dependent upon the cellular translation machinery to synthesize their own proteins. The synthesis of proteins from the human immunodeficiency virus (HIV) type 1 and 2 RNAs utilize several alternative mechanisms. The regulation of viral protein production requires a constant interplay between viral requirements and the cell response to viral infection. Among the antiviral cell responses, the interferon-induced RNA activated protein kinase, PKR, regulates the cellular and viral translation. During HIV-1 infection, PKR activation is highly regulated by viral and cellular factors. The cellular TAR RNA Binding Protein, TRBP, the Adenosine Deaminase acting on RNA, ADAR1, and the PKR Activator, PACT, play important roles. Recent data show that PACT changes its function from activator to inhibitor in HIV-1 infected cells. Therefore, HIV-1 has evolved to replicate in cells in which TRBP, ADAR1 and PACT prevent PKR activation to allow efficient viral protein synthesis. This proper translation will initiate the assembly of viral particles.

1. Introduction

Eukaryotic translation is regulated from initiation to elongation and termination steps (Dever and Green, 2012; Fraser, 2009; Hinnebusch and Lorsch, 2012). The co-evolution between viruses and their hosts results in a mutual interplay between their regulatory mechanisms. Viruses are entirely dependent on cellular translation factors for the production of their proteins. Viruses interfere with host translation mechanisms by subverting cellular factors for their own use, but have also evolved mechanisms to either shut-off or modify cellular translation, which may contribute to their pathogenicity (Li et al., 2013; Mohr and Sonenberg, 2012; Walsh et al., 2013). In addition, the development of specific mechanisms for the production of their own proteins ensures their replication within the cell (Firth and Brierley, 2012; Komarova et al., 2009; Reineke and Lloyd, 2011; Roberts et al., 2009).

In response to virus invasion, cells have evolved mechanisms to counteract the negative impact of viruses and ensure their survival. The immediate innate immune response is mediated by external and internal sensors (Kumar et al., 2011), which recognize the viral components and trigger mechanisms leading to the production of cytokines and interferon (IFN)α/β proteins (Borden et al., 2007; Randall and Goodbourn, 2008). IFNs are secreted, bind to receptors in a paracrine and autocrine manner on different cell types to trigger signalling cascades leading to the production of IFN-stimulated

Abbreviations: ADAR, adenosine deaminase acting on RNA; ds, double-stranded; dsRBD, dsRNA binding domain; dsRBP, dsRNA binding protein; eIF, eukaryotic translation initiation factor; HCV, Hepatitis C virus; HDV, Hepatitis D virus; HIV, human immunodeficiency virus; HTLV, Human T-cell leukaemia virus; IFN, Interferon; IRES, internal ribosome entry site; IKK, inhibitor of nuclear factor kappa-B kinase; IP, immunoprecipitation; ISRE, IFN Stimulated Response element; IRF, IFN regulatory factor; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; MDA-7, melanoma-associated gene 7; MEK, MAPK kinase; miRNA, micro RNA; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; OAS, 2′-5′oligoadenylate synthetase; PABP, PolyA binding Protein; PAMPs, pathogen-associated molecular patterns; PACT, PKR Activator; PDCs, plasmacytoid dendritic cells; PKR, Protein Kinase RNA activated; RAX, PKR-associated protein X; RHA, RNA Helicase A; RT, reverse transcription; RLR, RIG-I like receptor; RNAi, RNA interference; RRE, Rev Response Element; S-HDag, small delta antigen; slfn, Schlafen; ss, single-stranded; SIV, simian immunodeficiency virus; siRNA, small interfering RNA; STAT, signal transducers and activators of transcription; TAR, trans-activation responsive element; TLRs, Toll-like receptors; TRAF, TNFR-associated factor; TRBP, TAR RNA Binding Protein; UTR, untranslated region; VHS, virus host shutoff; VSV, Vesicular stomatitis virus; ZBD, Z-DNA binding domain.

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genes (ISGs) (Randall and Goodbourn, 2008; Sadler and Williams, 2008). Most ISGs have antiviral activities, but some of them, like the adenosine deaminase acting on RNA (ADAR1) have both antiviral and proviral functions (Gélinas et al., 2011; Samuel, 2011). Among the ISGs, the protein kinase RNA-activated (PKR) and the 2′-5′ oligoadenylate synthetases (OAS) are activated by double-stranded (ds)RNA. They block the initiation of translation or activate a latent endoribonuclease, RNAse L, respectively, both resulting in inhibition of cellular and viral protein synthesis (Hovanessian, 2007). To ensure viral replication and cell survival, the activity of these enzymes is regulated by viral and cellular factors (Dabo and Meurs, 2012; Garcia et al., 2006, 2007).

In this review, we will analyze the mechanisms involved in the translation of *Human immunodeficiency viruses* (HIV)-1 and -2 and their regulation by viral and cellular factors by comparison with other viruses. The innate immunity pathway leading to control of viral translation will also be described. Recent data on the role and regulation of PKR and the unexpected change in the function of its regulator, the PKR activator (PACT) during HIV-1 replication will be described in more detail.

2. HIV-1 and HIV-2 translation

HIV replication requires constant interactions between the virus and its host and cellular mechanisms of gene expression are diverted to produce viral RNA and proteins. Once produced, HIV RNA undergoes multiple splicing to produce mRNAs for the synthesis of its proteins. In the early phase, HIV mRNA is multiply spliced into 2 kb mRNAs which are exported to the cytoplasm and translated to produce Rev, the trans-activator of transcription (Tat) and Nef. Tat and Rev go to the nucleus, where Tat increases the rate of transcription (Gatignol, 2007). In the late phase, the viral protein Rev, its RNA target Rev Response Element (RRE), and cellular factors are essential to bring the singly spliced and the genomic RNA out of the nucleus for translation (McLaren et al., 2008; Pollard and Malim, 1998; Suhasini and Reddy, 2009). HIV translation initiation occurs mainly through the cap-dependent scanning mechanism, but other mechanisms occur, including the use of an internal ribosome entry site (IRES) in the 5′UTR and in gag RNA as well as various specialized mechanisms to translate the large number of singly and doubly spliced RNAs (Bolinger and Boris-Lawrie, 2009; de Breyne et al., 2013; Purcell and Martin, 1993). Furthermore, the regulation of translational elongation involves a programmed −1 ribosomal frameshift to produce Gag–Pol from the full-length mRNA (Bolinger and Boris-Lawrie, 2009; Brakier-Gingras et al., 2012). In addition, as part of the virus-induced detrimental effects, HIV-1 reduces cellular translation by impairing eIF4E with Vpr and by cleaving eIF4G and PABP with its Protease (Castello et al., 2009; Ohlmann et al., 2002; Perales et al., 2003; Sharma et al., 2012).

2.1. cap-dependent translation

Like other viruses, *Retroviruses* use the cellular translational machinery for the synthesis of their proteins and have evolved diverse strategies to produce the appropriate amount of each protein for efficient replication (Bolinger and Boris-Lawrie, 2009; Chamond et al., 2010; de Breyne et al., 2013). HIV translation occurs mainly through a cap-mediated scanning mechanism from its 5′ end. It starts with the recognition of the 7-methyl-guanosine cap by eukaryotic translation initiation factor eIF4E cap-binding protein, which forms a complex with eIF4G and eIF4A (de Breyne et al., 2012). The 40S ribosomal subunit associated with eIF3, eIF2, GTP and Met-tRNA forms the 43S ribosome complex, binds the capped mRNA and scans it until an initiator AUG codon is found (Bolinger and Boris-Lawrie, 2009; de Breyne et al., 2013; Kozak, 1989). HIV translation initiation is slowed down by the highly structured sequence in the 5′ untranslated region (UTR) (Dorin et al., 2003; Dugré-Brisson et al., 2005; Soto-Rifo et al., 2012a; Svitkin et al., 1994). All HIV transcripts start with the formation of the trans-activation responsive element (TAR) RNA at their 5′ end. In HIV-1, the TAR RNA is composed of a stable stem-bulge-loop structure, whereas in HIV-2 RNA, this structure is duplicated (Bannwarth and Gatignol, 2005; Berkhout et al., 1990; Jeang and Gatignol, 1994; Soto-Rifo et al., 2012a). The TAR structure constitutes a block to translation, which is stronger for HIV-2 (Soto-Rifo et al., 2012a). For HIV-1, this block can be alleviated by the TAR RNA Binding protein (TRBP), by the autoantigen La, by Staufen or by DEAD box polypeptide 3 (DDX3) (Chang et al., 1994; Dorin et al., 2003; Dugré-Brisson et al., 2005; Soto-Rifo et al., 2012b; Svitkin et al., 1994), but these factors have not been studied in the context of HIV-2 for which the IRES may play an important function (Soto-Rifo et al., 2012a). Furthermore, the cellular proteins, PKR, 5′OAS, Schlafen and GCN2 negatively impact HIV-1 translation in response to IFN, stress and dsRNA (Clerzius et al., 2011; Cosnefroy et al., 2013; del Pino et al., 2012; Jakobsen et al., 2013; Li et al., 2012; Silverman, 2007). In contrast, cellular factors such as the RNA helicase A (RHA), upframeshift protein 1 and the Rev co-factors Sam68, eIF5A, human Rev-interacting protein (hRIP) and DDX3 increase cap-dependent translation efficiency of HIV-1, although eIF5A, hRIP and DDX3 also act on IRES-mediated translation (Ajamian et al., 2008; Bolinger et al., 2010; Liu et al., 2011; Soto-Rifo et al., 2012b).

2.2. IRES

IRES elements, which directly recruit the 40S ribosomal unit, have been observed in several retroviruses (Berlioz and Darlix, 1995; Camerini et al., 2008; Deffaud and Darlix, 2000; Nicholson et al., 2006; Ohlmann et al., 2000; Vallejos et al., 2012). In the *simian immunodeficiency virus* (SIV), HIV-1 and HIV-2, IRESes have been identified before or within Gag and promote the translation of transcripts expressing Gag and Gag-Pol proteins (de Breyne et al., 2013). In HIV-1, an IRES element, active during the G2/M phase has been identified in the 5′UTR, upstream of the HIV-1 Gag AUG (Brasey et al., 2003; Vallejos et al., 2011). This IRES was confirmed in various HIV-1 strains, primary HIV-1 isolates and its strength is cell-type specific (Gendron et al., 2011; Plank et al., 2013; Vallejos et al., 2012). Furthermore, it is stimulated by oxidative stress and is most functional when the cap-dependent translation is shut down by the co-expression of the *Picornavirus* proteases (Amorim et al., 2014; Gendron et al., 2011; Monette et al., 2013). Another IRES has been identified within the Gag coding region of HIV-1, driving the synthesis of a low-abundance 40-kDa Gag isoform (Buck et al., 2001). In HIV-2 and SIV, an IRES element has been identified only downstream of the Gag AUG in the genomic RNA for efficient translation of Gag and GagPol proteins. This IRES promotes the synthesis of shorter, N-truncated isoforms of Gag (Herbreteau et al., 2005; Locker et al., 2011; Nicholson et al., 2006). These IRESes within Gag of HIV-1, HIV-2 and SIV initially bind the 40S subunit and eIF3, require the function of the DEAD-box helicase eIF4A and have the specificity to recruit three initiation complexes on a single molecule to mediate the synthesis of full-length and N-truncated Gag molecules (Locker et al., 2011; Weill et al., 2010). The requirements of each IRES for specific eIFs and associated factors will likely identify conserved and different features of their translation initiation and role during the viral replication cycle.

2.3. Frameshifting

The synthesis of the Pol protein in HIV-1 and -2 is dependent on a ribosomal −1 frameshifting to produce the Gag–Pol fusion protein (Jacks et al., 1988; Wilson et al., 1988). The frameshifting

mechanism uses a heptanucleotide slippery sequence upstream of a structured frameshift stimulatory signal forming an RNA pseudoknot. The heptanucleotide interacts with the ribosome, which unwinds the lower part of the structured stimulatory signal. The very stable upper part of this structure becomes an effective frameshift stimulatory signal triggering an incomplete translocation of two nucleotides instead of three (Brakier-Gingras et al., 2012; Marcheschi et al., 2007; Mazauric et al., 2009; Namy et al., 2006; Reil et al., 1993). This programmed change in the reading frame is modulated or enhanced by viral factors such as the TAR-polyA RNA and the Tat protein as well as cellular factors such as the eukaryotic release factor 1 and the DEAD-box RNA helicase 17 (Charbonneau et al., 2012; Gendron et al., 2008; Kobayashi et al., 2010; Lorgeoux et al., 2013). Decreasing the efficiency of the frameshift decreases virus infectivity due to the presence of a genomic RNA packaging enhancer overlapping the Gag–Pol ribosomal frameshift signal. The consequent alteration of genomic RNA packaging and viral replication makes the frameshifting structure a target for antiviral strategies (Brakier-Gingras et al., 2012; Chamanian et al., 2013; Dulude et al., 2006).

2.4. Translation of spliced HIV-1 RNAs

Whereas the HIV-1 and -2 genomic RNAs are translated by a cap-scanning mechanism, IRES and frameshifting, spliced RNAs can use different mechanisms. The singly spliced HIV-1 RNA coding for the vpu and env gene is translated by both a linear and a discontinuous ribosome scanning mechanism (Anderson et al., 2007). Indeed, while Vpu translation occurs through a continuous leaky scanning mechanism, Env is also translated independently from upstream AUG signals indicating a discontinuous ribosome scanning mechanism by the use of either an IRES or a shunt mechanism. In addition, a minimal open reading frame located upstream of the vpu gene increases the translation efficiency of the downstream env gene (Krummheuer et al., 2007). Because the IRES in the 5′UTR requires only 270 nucleotides present in all spliced HIV-1 RNAs, the question arose of whether this IRES could be functional to mediate the translation of other proteins besides Gag. The doubly spliced HIV-1 RNA coding for Tat was the first to be tested and is likely translated by both a cap-dependent and an IRES mechanism in addition to being positively regulated by Tat itself (Charnay et al., 2009). The RNAs encoding Vpu, Vpr, Nef and Vif were also translated partly from the common IRES structure with the lowest activity for Vpr and the highest for Vif in Jurkat cells (Plank et al., 2013, 2014).

These different mechanisms allow an efficient translation of the HIV-1 and -2 RNAs. In every step, cellular factors are involved and contribute to the appropriate regulation of viral protein synthesis. Recent data have shown the important contribution of cellular dsRNA binding proteins (dsRBPs) acting on viral translation as part of the IFN pathway (Bolinger and Boris-Lawrie, 2009; Clerzius et al., 2011; de Breyne et al., 2013).

3. Translational control as a cell response to virus infection

3.1. Innate immune responses to viruses

Antiviral mechanisms have evolved in complexity in concordance with pathogens. Upon virus entry, the innate immune response is triggered by interactions between pathogen-associated molecular patterns (PAMPs) and pathogen recognition receptors (PRRs). PAMPs can be viral RNAs or DNAs and are recognized by PRRs such as Toll-like receptors (TLR) and RIG-I like receptors (RLR) (Kawai and Akira, 2011; Lepelley et al., 2011; Rustagi and Gale, 2013). This recognition is followed by a series of signalling cascades leading to the production of cytokines and IFNs. The antiviral IFN response is mediated by IFNα/β (Borden et al., 2007; Randall and Goodbourn, 2008). IFNs are secreted and act by binding to their receptors on the cell surface. They then induce the Janus kinases/signal transducers and activators of transcription (JAK/STAT) signalling pathway leading to the formation of a trimer composed of the IFN regulatory factor (IRF)-9 and phosphorylated STAT1-2. This complex translocates to the nucleus and binds to the IFN Stimulated Response element (ISRE) DNA on responsive promoters, inducing the production of more than 300 ISGs (Randall and Goodbourn, 2008; Sadler and Williams, 2008) (Fig. 1).

3.2. IFN production and activity during HIV-1 infection

Following HIV-1 infection, IFNα/β is produced mainly in plasmacytoid dendritic cells through innate immune detection of viral RNA and DNA (Hosmalin and Lebon, 2006; Hughes et al., 2012; Rustagi and Gale, 2013; Schmidt et al., 2005). High levels of IFNs are observed in the serum of HIV-infected individuals, but the correlation between IFN production and pathogenicity remains unclear (Hosmalin and Lebon, 2006; Lehmann et al., 2008; von Sydow et al., 1991). IFN inhibits HIV-1 replication in primary macrophages and T cells and induces the production of ISGs, blocking several steps, including entry, reverse transcription (RT), protein synthesis, viral assembly, and virion release (Sadler and Williams, 2008; Schoggins and Rice, 2011). Among these ISGs, PKR, 2′-5′ OAS and Schlafen (slfn) 11 contribute to viral translation inhibition, whereas ADAR1 mostly contributes to increased translation.

3.3. ISGs acting on viral translation

3.3.1. PKR

PKR was initially discovered in virus infected cells as an antiviral protein induced by IFN treatment (Hovanessian, 2007; Meurs et al., 1990). PKR is activated by phosphorylation after binding to dsRNA through its two dsRNA binding domains (dsRBDs) and by dimerization (Heinicke et al., 2009). Once active, PKR phosphorylates several substrates including the alpha subunit of the translation initiation factor 2 (eIF2α). P-eIF2α blocks the ability of eIF2B to renew the eIF2•GTP•Met-tRNA$_i$ ternary complex required for cellular and viral protein synthesis initiation and is a critical component of antiviral and cell growth pathways (Gale et al., 2000). In addition to dsRNA, growth factors, cytokines, proinflammatory stimuli and oxidative stress also activate PKR, transmitting signals to the translational machinery, as well as to other factors (Dabo and Meurs, 2012; Garcia et al., 2006; Sadler and Williams, 2007). PKR is involved in the regulation of the translation of many viruses and is regulated by viral and cellular factors (Clerzius et al., 2011; Dabo and Meurs, 2012; Gale et al., 2000; Garcia et al., 2007; Munir and Berg, 2013).

3.3.2. 2′-5′ OAS and RNAse L

2′-5′ OAS is activated by dsRNA and produces 2′-5′ oligo adenylates (2-5A) from cellular ATP. 2-5A activate RNase L, which degrades cellular and viral RNAs and serves as an efficient antiviral tool by preventing viral protein expression (Hovanessian, 2007; Silverman, 2007). 2′-5′ OAS and RNAse L are active against many RNA and DNA viruses (Silverman, 2007). In several cases, the different forms of the OAS protein exert various antiviral effects (Brehin et al., 2009; Kajaste-Rudnitski et al., 2006; Kwon et al., 2013; Lin et al., 2009; Maitra et al., 1994; Simon-Chazottes et al., 2011). Viruses have also evolved mechanisms to regulate the activity of either 2′-5′ OAS or RNAse L activity through the expression of viral components or by the induction of cellular inhibitors (Martinand et al., 1999; Mertens et al., 2010; Tan et al., 2011; Tian et al., 2012; Zhao et al., 2012). In addition, the RNase L inhibitor (also called HP68 or ABCE1) induced during HIV-1 replication also has a

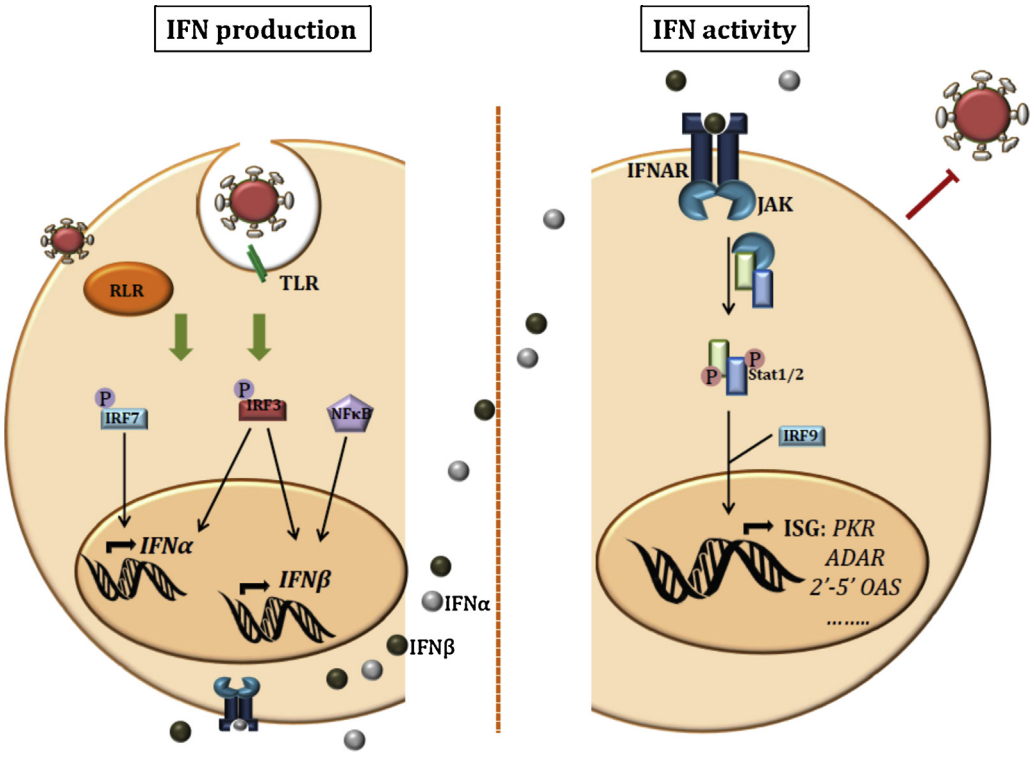

Fig. 1. IFN production and activity during a viral infection. (Left) IFN production in a cell infected by a virus. The virus is recognized by one or more PRR, such as RLR and TLRs. Activation of PRRs by the virus leads to a signalling cascade that activates the transcription of type I IFN genes (IFNα, IFNβ) by three activated transcription factors (IRF3, IRF7 and NF-κB). IFNα/β is secreted from the infected cell. (Right) IFN activity. IFNα/β binds to the IFN alpha-receptor (IFNAR) on the surface of non-infected cells and infected cells. IFNAR-IFNα/β interaction leads to activation of JAK-STAT signalling pathway and formation of a trimer with the transcription factor IRF9. This trimer translocates in the nucleus and binds to the ISRE DNA on responsive promoters, inducing the production of more than 300 ISGs. For clarity purposes, the pathways are simplified and each arrow represents several steps.

post-translational role by regulating HIV-1 capsid assembly (Lingappa et al., 2006; Silverman, 2007; Tian et al., 2012).

3.3.3. *Schlafen 11 (slfn11)*

The Schlafen (slfn) family of proteins are involved in the control of cell proliferation, T cell development as well as the regulation of bacterial and viral infections (Mavrommatis et al., 2013). Several members of the murine and human Slfn proteins are ISGs. Slfn11 has been recently shown to restrict HIV-1 replication. Slfn11 has an RNA helicase domain and inhibits HIV-1 protein synthesis *via* a viral codon bias usage. Indeed, HIV-1 uses a relatively different synonymous codon usage (also known as the codon bias) than the one used by the host (Coleman et al., 2008; Meintjes and Rodrigo, 2005). It was shown that Slfn11 selectively inhibited the expression of an HIV-1 Gag vector using the viral codon bias usage while it did not affect a similar HIV-1 Gag vector that was codon-optimized for human cell expression (Li et al., 2012). How HIV-1 counteracts this effect remains unknown (Jakobsen et al., 2013; Li et al., 2012).

3.3.4. *ADAR1*

ADAR1 mediates Adenosine to Inosine (A-to-I) editing on dsRNAs, including mRNAs, small RNAs and viral RNAs. Inosines are recognized as Guanosines by the translational machinery and are considered as A-to-G mutations leading to alteration of translation. ADAR1 contains two N-terminal Z-DNA binding domains (ZBD), three dsRBDs and a catalytic domain mediating the editing function (Galeano et al., 2012; Hogg et al., 2011; Hundley and Bass, 2010; Nishikura, 2010). Viral edited transcripts have been observed in infected cells but surprisingly, the function of this editing is mostly an enhancement of viral replication (Gallo and Locatelli, 2012; Gélinas et al., 2011; Samuel, 2011). In HIV-infected cells, ADAR1 editing was characterized in the 5′UTR, in the Tat, Rev and Env genes, stimulated virion release and increased virion infectivity. Overall these modified sequences increased viral replication, but the exact mechanism is not understood (Doria et al., 2009; Phuphuakrat et al., 2008). Similarly, ADAR2 also edits HIV-1 RNA, increases viral production and virion release, but does not affects viral infectivity (Doria et al., 2011). In contrast, in one study, A-to-G mutations by ADAR1 editing affected Rev-RRE binding and RNA transport resulting in decreased viral replication (Biswas et al., 2012). The difference between these studies is not currently understood but may be due to different experimental settings. Furthermore, ADAR1 has an additional function by binding to PKR and inhibiting its activation, which results in enhanced HIV-1 translation (Clerzius et al., 2009; Doria et al., 2009).

4. Involvement of PKR in the translation of HIV and other viruses

4.1. *PKR pathway: a central target by many viruses*

PKR is one of the most studied ISGs and is involved in antiviral responses to many viruses. In addition, PKR is implicated in cellular responses to stress such as regulation of certain components of the mitogen-activated protein kinases (MAPK) pathway and cellular responses to cytokines (Silva et al., 2004; Williams, 1999). PKR is also involved in other pathways such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation through the association with TNFR-associated factor (TRAF) in the inhibitor of nuclear factor kappa-B kinase (IKK) complex (Dabo and Meurs, 2012), in the activation of the inflammasome (Lu et al., 2012) and in the regulation of the insulin pathway (Nakamura et al., 2010).

4.1.1. PKR structure

PKR is a 551 amino acid (aa) long serine/threonine kinase and is composed of two dsRBDs in its N-terminal end and one catalytic domain in its C-terminal end (Sadler and Williams, 2007) (Fig. 2). PKR activation can be RNA-dependent by binding to viral RNA species through its dsRBD or RNA-independent through interactions with cellular factors (Clerzius et al., 2011). To activate PKR in a RNA-dependent manner, the two dsRBDs bind to RNA species of a minimum of 15 bp length and subsequent activation requires at least 30 bp long RNAs (Heinicke et al., 2009). PKR activation is not dependent on the nucleotide sequence but on the RNA structure of the dsRNA. As a result, PKR cannot be activated by single-stranded (ss)RNA or dsDNA. A large number of viruses, including DNA viruses, can produce dsRNA structures and therefore have the potential to activate PKR. However, this can vary greatly, depending on the intracellular localization or concentration of these RNA structures or competition for binding to viral or cellular effectors (Heinicke et al., 2009). As a result, certain classes or categories of viruses can potently activate PKR and others do not elicit a PKR response (Heinicke et al., 2009). For instance, PKR can remain inactive in *Sendai virus*-infected cells through the action of the viral C-protein, which restricts the generation of large amounts of viral dsRNA (Takeuchi et al., 2008). In contrast, PKR is activated in *Human T-cell leukaemia virus* (HTLV)-1–2 infected cells (Cachat et al., 2013).

4.1.2. PKR activators

A limited number of cellular factors can activate PKR. The mRNA encoding IFNγ presents a particular pseudoknot contained in its 5′UTR and first 26aa coding sequence, with sufficient flexibility to be used as a translation template and PKR activator, thus controlling its own expression (Cohen-Chalamish et al., 2009). PACT and its murine homolog the PKR-associated protein X (RAX) were the first proteins identified as PKR activators in the absence of RNA (Ito et al., 1999; Patel and Sen, 1998). PACT and RAX are activated by stress and their mechanism of action is further discussed in Section 5. Additional cellular proteins that activate PKR in a RNA-independent manner include ISG15 (Okumura et al., 2013) and the tumour suppressor melanoma-associated gene 7(MDA-7) (Pataer et al., 2005). Whether triggered by RNA-dependent or independent stimuli, PKR kinase activity is switched on by dimerization of PKR molecules in their 3rd basic region and subsequent phosphorylation on threonine 446 and threonine 551 on the C-terminal end, due to the reciprocal action between PKR dimers (Dabo and Meurs, 2012; Dey et al., 2005) (Fig. 2).

Once activated as a kinase, PKR phosphorylates the serine 51 of the translation initiation factor, eIF2α. This phosphorylation blocks the GDP–GTP exchange necessary for the recruitment of the ternary initiation complex Met tRNA-GTP-eIF2 to the 40S ribosome (Langland et al., 2006). The resulting inhibition of the synthesis of eIF2α-dependent proteins affects a large number of cellular and viral proteins and thus participates to the antigrowth and antiviral activity linked to PKR. Indeed, a number of viruses are able to escape the control mediated by eIF2α phosphorylation, for instance by using other combinations of initiation factors. Among those are the *Picorna-like cricket paralysis virus*, the *Alphavirus Sindbis* and *Semliki forest virus* or *Hepatitis C virus* (HCV) (reviewed in (Dabo and Meurs, 2012)). Of note, translation of HCV mRNA is using eIF2A, an alternative initiator tRNA-binding protein, instead of eIF2α (Kim et al., 2011). Interestingly, this gives an advantage for the synthesis of the HCV proteins while that of the eIF2α-dependent cellular proteins is affected from the HCV-mediated PKR activation, in particular the effectors of the antiviral response such as IFN (Arnaud et al., 2010) and IFN-stimulated genes (Garaigorta and Chisari, 2009). Some cellular proteins can also escape the eIF2α-mediated control of translation. This may sustain the cellular defence in case of viral infection, through activation of apoptosis (Teng et al., 2014) or autophagy (Lussignol et al., 2013; Talloczy et al., 2002). However, it can also be detrimental as the translation of some eIF2α-independent enzymes can be linked to neurodegenerative disorders, such as Alzheimer disease (Mouton-Liger et al., 2012).

4.1.3. PKR targets

The initiation factor eIF2α is considered as the major substrate of PKR. In addition, PKR can trigger the phosphorylation of a number of other proteins involved in different stress or signalling pathways but those may not be direct or specific PKR substrates (Fig. 2). B56α, a regulatory subunit of the phosphatase protein PP2A, can be phosphorylated *in vitro* by PKR but its phosphorylation *in vivo* requires eIF2α activation and serine/threonine kinase AKT (Ruvolo et al., 2008). The dsRBP ILF-2/NF90 forms a complex with PKR and can be phosphorylated by PKR *in vitro* on its dsRBD (Parker et al., 2001). NF90, is present in stress granules, where it functions as a regulator of PKR activation, probably through competition for dsRNA structures (Wen et al., 2014). The tumour suppressor p53 can be phosphorylated by PKR on Ser37 both *in vitro* and *in vivo*. PKR and PACT are also involved in signalling pathways leading to p53 activation. A sumoylation-dependent mechanism with promotion of p53 phosphorylation and translational activation leads to G1 arrest (Bennett et al., 2012). Viral proteins can also be phosphorylated by PKR. The HIV-1 Tat protein may interfere with the correct positioning of eIF2α at the kinase domain of PKR and becomes phosphorylated instead (Brand et al., 1997). Tat phosphorylation increases its interaction with TAR and enhances transcription (Endo-Munoz et al., 2005). PKR also phosphorylates the 24 kDa small delta antigen (S-HDag) of *Hepatitis delta virus* (HDV), which has a negative effect on HDV replication (Chen et al., 2002).

4.2. PKR regulation by viral and cellular factors

Given that PKR activation is implicated in diverse cellular pathways, its kinase activity is regulated by various mechanisms. PKR is a target for many viral and cellular proteins, the latter being induced or not by viruses. These inhibitors are categorized here by their mode of inhibition on PKR (Fig. 3).

4.2.1. Degradation of PKR or the dsRNA activator

Virus-induced PKR protein degradation is a radical measure to counteract inhibition of protein synthesis. *Poliovirus* infection elicits a strong PKR activation and eIF2α phosphorylation, which is followed by PKR degradation. Although PKR–RNA interaction is required, PKR is degraded by cellular proteases and not by viral proteases but the mechanism remains unclear (Black et al., 1993; Langland et al., 2006). Another example is the *Rift valley fever virus* (RVFV), from the *Bunyaviridae* family, which codes for the Non Structural protein NSs. This protein plays a crucial role in RVFV virulence such as suppression of host transcription including that of IFN (Billecocq et al., 2004; Ikegami et al., 2009). In addition, the RVFV NSs protein triggers PKR degradation through a proteasome-dependent pathway (Habjan et al., 2009; Ikegami et al., 2009; Kalveram et al., 2013). The ability of NSs protein to induce PKR degradation was confirmed during *Toscana virus* infection, another *Phlebovirus* of the *Bunyaviridae* family (Kalveram and Ikegami, 2013). *Herpes simplex virus* (HSV) codes for a virus host shutoff (VHS) RNase protein. VHS degrades viral and cellular RNA structures that could potentially activate PKR (Sciortino et al., 2013). VHS activity acts in cooperation with MAPK kinase (MEK) expression. Indeed, PKR activation was restored only in ΔVHS virus-infected MEK deficient HT1080 cells, which is in concordance with a previous study showing PKR inhibition by MEK during HSV replication (Farassati et al., 2001; Taddeo et al., 2013).

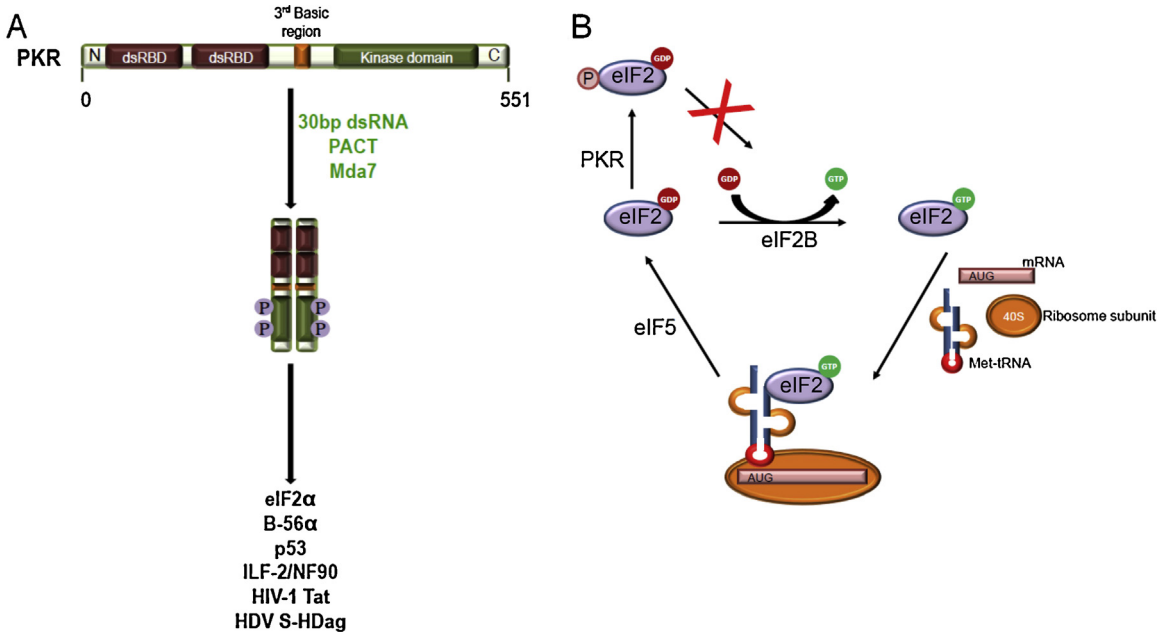

Fig. 2. PKR activation and response pathway. (A) PKR protein domains are composed of two dsRBDs, a third basic region and a kinase domain on the C-terminus. Upon activation by 30 bp dsRNA, PACT or MDA-7, PKR molecules dimerize through their 3rd basic region and induce a conformational change that activates the kinase domain. PKR molecules trans-autophosphorylate at threonine 446 and 551. Activated PKR phosphorylates its substrates, eIF2α, B56α, the tumour suppressor p53, ILF2-NF90, the HIV-1 Tat and the S-HDag of HDV. (B) The initiation of mRNA translation is mediated by eIF2, a heterotrimeric complex composed of α, β and γ subunits. Activation of the initiation of mRNA translation is accomplished by the exchange of GDP bound to eIF2 to GTP by the guanine nucleotide exchange factor eIF2B. This enables the formation of a quaternary complex composed of eIF2–GTP, the mRNA to be translated, the methionine transfer RNA (Met-tRNA) and the 40S ribosomal subunit that initiates mRNA translation. eIF2 GTP to GDP state is mediated by eIF5, a GTPase-activating protein. In stress and viral conditions, eIF2α subunit is phosphorylated by PKR which blocks the GDP–GTP exchange required for the initiation of mRNA translation.

4.2.2. Limiting the availability of the dsRNA activator

The E3L protein of *Vaccinia virus* (VV), the prototypic member of the *Orthopoxviruses*, contains a C-terminal dsRBD and suppresses the activation of PKR by sequestering dsRNA. E3L also contains an N-terminal ZBD similar to the first one from ADAR1 and this domain also inhibits PKR by direct binding (Romano et al., 1998). Other members of the *Poxviridae* family, such as *Myxoma virus* and *Swinepox virus* can also suppress PKR activation (Myskiw et al., 2011). Recent data show that the nucleic acid-binding ability of the E3L ZBD from the *variola virus*, is not required to inhibit PKR

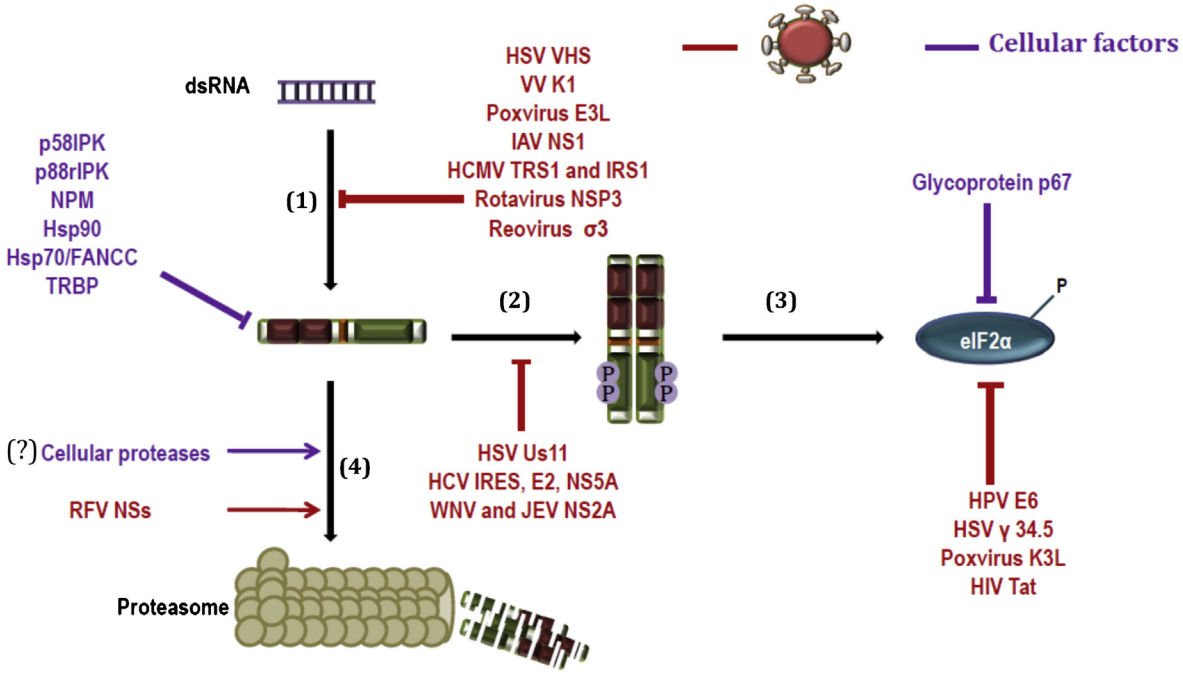

Fig. 3. PKR pathway is targeted by viral and cellular factors. PKR inhibitory viral factors are represented in red. Cellular inhibitory factors are in purple. Inhibitory mechanisms include: (1) limiting the availability of the dsRNA activator; (2) prevention of PKR activation by PKR sequestration; (3) inhibiting the phosphorylation of the substrate eIF2α; (4) targeting PKR for degradation in the proteasome.

and that E3L might also mediate other anti-viral responses (Thakur et al., 2014). A number of other viral dsRBPs are able to sequester dsRNA and thus prevent PKR activation, such as the *Reovirus* σ3, the *influenza virus* NS1, the *Rotavirus* NSP3 (Langland et al., 2006) and *Human cytomegalovirus* TRS1 and IRS1 proteins (Bierle et al., 2013). Another strategy to prevent PKR activation is to act on the production of dsRNA molecules. Indeed, the K1 protein from VV inhibits PKR activation during infection in HeLa cells by diminishing the production of intermediate gene transcripts that have the potential to activate PKR (Willis et al., 2011). The mechanism of action is unclear but does not involve dsRNA-K1 interactions as shown by co-immunoprecipitation (IP) experiments (Shisler and Jin, 2004; Willis et al., 2011). During *West Nile virus* infection, the association of replicating RNA within endoplasmic reticulum membrane vesicles prevents their ability to enter the cytoplasm and to trigger early activation of PKR, thus ensuring efficient viral replication. This membrane protection is overcome by actively replicating viruses at later times of infection and the viral RNA enters the cytoplasm through small pores opening from those vesicles, where it can be recognized by cellular sensors, including PKR. This results in PKR activation, eIF2α phosphorylation and subsequent formation of stress granules (Courtney et al., 2012).

4.2.3. Sequestration of PKR

Viruses have evolved with proteins that directly bind to PKR and inhibit its activation (Garcia et al., 2007; Langland et al., 2006). The nonstructural protein 2A (NS2A) of *Japanese encephalitis virus*, a *Flavivirus*, can prevent PKR dimerization. Interestingly, this leads to a suppression of eIF2α phosphorylation and arrest of protein synthesis that is specifically mediated by PKR and not by the other eIF2α kinases (Tu et al., 2012). The nonstructural protein NS5A and the structural protein E2 of HCV have also been reported to inhibit PKR by sequestration, either by interacting with the PKR dimerization domain (NS5A) or *via* a pseudosubstrate mechanism by mimicking the PKR autophosphorylation site located between the two dsRBDs of PKR (E2) (reviewed in (Dabo and Meurs, 2012)). Although these mechanisms of action remain possible, there is at present no definitive experimental evidence that they operate in real conditions of HCV infection. These examples point out that many uncertainties still exist regarding the mechanisms by which viruses counteract the host response. Viruses have also evolved with strategies to manipulate cellular factors that counteract the innate immune response. Several mechanisms involve a series of stress-related chaperone proteins such as Heat Shock proteins (Garcia et al., 2006; Geller et al., 2012; Padwad et al., 2010). One example is the NP protein of the *Influenza A virus*, which counteracts PKR activation using p58(IPK), a tetratricopeptide repeat-containing co-chaperone. The mechanism of action involves the dissociation of p58(IPK), from the heat shock protein 40 (hsp40) and binding of p58(IPK) to PKR (Melville et al., 1999). Indeed, infection with *Influenza A virus* triggers a kinase cascade, starting with activation of p38 MAPK and leading to activation of MK2 and MK3 which can then form a tetrameric protein complex with p88(rIPK)/p52(rIPK) and p58(IPK) to bind to and inhibit PKR (Luig et al., 2010).

4.2.4. Acting on eIF2α and translation

Viruses also bypass PKR activation by targeting its major downstream effector, eIF2α. The K3L protein of VV acts as a pseudosubstrate inhibitor of PKR through a motif homologous to residues 79–83 in eIF2α, which mediates eIF2α-PKR binding (Kawagishi-Kobayashi et al., 1997; Rice et al., 2011). Furthermore, K3L anti-PKR activity is enhanced when the VV is challenged by serial propagation in human cells (Elde et al., 2012). Another example is the HSV protein γ_1 34.5 involved in viral DNA synthesis and viral pathogenesis. γ_1 34.5 triggers eIF2α dephosphorylation by binding to its phosphatase PP1α, therefore limiting PKR-mediated inhibition of translation (Langland et al., 2006). Independently of its inhibition of PKR, γ_1 34.5 negatively regulates the IRF3 kinase, TBK1, which thus contributes to inhibit IFN induction and favours viral replication and spread *in vivo* (Ma et al., 2012).

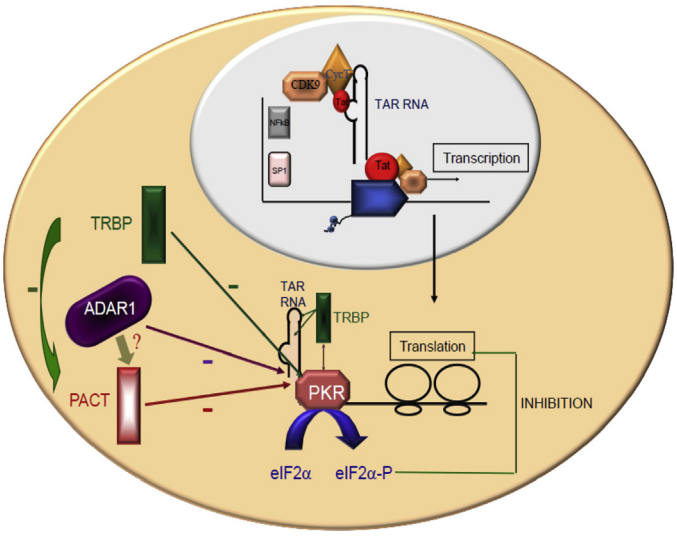

Fig. 4. PKR activation and inhibition in an HIV-1 infected cell. HIV-1 transcription occurs in the nucleus. Tat combined with the cellular Cyclin T1 and CDK9 bind to the TAR RNA present on all HIV-mRNA transcripts to activate transcription. In the cytoplasm, TAR-containing mRNAs activate PKR response leading to phosphorylation of eIF2α and subsequent translational inhibition of all mRNAs. Cellular factors such as TRBP and ADAR1 inhibit PKR activation. PACT activates PKR in stress conditions but its activity is inhibited when it forms heterodimers with TRBP. Furthermore, PACT becomes a PKR inhibitor in HIV-1 infected cell, which may be mediated *via* an interaction with ADAR1.

4.3. Translational regulation of HIV-1 by PKR

As part of the innate immune system, PKR regulates HIV-1 mRNA translation. HIV-1 infection elicits a PKR response in two phases: an activation phase and an inhibition phase. In each phase, PKR interacts with viral and cellular factors, which regulate its activity (Fig. 4).

4.3.1. PKR activation

Following HIV-1 genome integration into the host's chromosome, HIV RNA transcripts are synthesized, all starting with the TAR RNA stem-loop structure. The TAR RNA is recognized by PKR and plays a dual role in its regulation. At low levels, TAR RNA is a potent PKR activator, whereas high concentrations of TAR inhibit PKR activation (Cole, 2007; Lemaire et al., 2008; Maitra et al., 1994). Furthermore, TAR dimerization promotes the binding of two PKR molecules, therefore inducing its activation (Heinicke et al., 2009). It is possible that other dsRNAs structures from the HIV-1 RNA activate PKR, but those have not been studied. PKR is extremely effective in restricting HIV-1 translation and replication in cell culture suggesting that it is effectively activated upon viral expression (Adelson et al., 1999; Benkirane et al., 1997; Clerzius et al., 2009, 2013; Dimitrova et al., 2005; Muto et al., 1999; Ong et al., 2005). Furthermore, knocking down PKR by small interfering (si) RNAs or expressing a transdominant mutant of PKR increases HIV-1 production (Ong et al., 2005). Infection of the lymphocytic cell line Jurkat by HIV-1 shows an activation of the already expressed PKR at the beginning of the infection (Clerzius et al., 2009), whereas during the infection of peripheral blood mononuclear cells (PBMCs) by HIV-1, PKR is induced and activated within the first days of the infection (Clerzius et al., 2013). In each case, this activation is not sustained throughout HIV replication and PKR is deactivated when

the virus replicates at high levels (Clerzius et al., 2009, 2013). Cumulative data show that viral and cellular factors contribute to PKR deactivation in HIV-1 expressing cells (Clerzius et al., 2011).

4.3.2. PKR inhibition

4.3.2.1. Tat. The HIV-1 Tat protein is essential for viral transcription by increasing the level of basal transcription when bound to TAR RNA (Bannwarth and Gatignol, 2005; Gatignol, 2007). Tat was shown to prevent PKR phosphorylation and activation by sequestering dsRNA, by directly binding to PKR and by being a pseudo substrate mimicking eIF2α (Brand et al., 1997; Cai et al., 2000; McMillan et al., 1995). In addition, PKR phosphorylates Tat, which results in an increase in Tat–TAR interaction and increased transcription (Endo-Munoz et al., 2005). Because of the low level of Tat being produced and its high transcriptional activity during HIV-1 replication, it is difficult to evaluate the contribution of Tat to PKR inhibition during viral replication. Studies integrating transcriptional control by Tat during viral latency or viral persistence may help to elucidate the contribution of Tat–PKR interactions to the regulation of HIV-1 replication (Mbonye and Karn, 2014; Van Lint et al., 2013; Wilhelm and Bell, 2013).

4.3.2.2. TRBP. TRBP is a cellular protein discovered by its ability to bind to HIV-1 TAR RNA (Daniels and Gatignol, 2012; Gatignol et al., 1991). It has two dsRBDs and a KR-helix motif that mediates TAR binding (Daviet et al., 2000; Erard et al., 1998; Gatignol et al., 1993). In human cells, TRBP acts as a Dicer partner for binding siRNAs and has a key function in the RNA interference (RNAi) mechanism (Chendrimada et al., 2005; Daniels and Gatignol, 2012; Daniels et al., 2009; Haase et al., 2005). TRBP is involved in the generation of micro (mi)RNAs as it is required for Dicer to cleave precursor miRNAs in the cytoplasm (Chakravarthy et al., 2010; Ma et al., 2008). TRBP–Dicer complex then forms of a ternary complex with an Argonaute protein, which represents the initial complex necessary for RNAi function (MacRae et al., 2008; Suk et al., 2011). TRBP is required for the retention of si/miRNAs in the complex and contributes to the selection of the guide RNA strand for appropriate mRNA silencing (Gredell et al., 2010; Takahashi et al., 2014). In the context of HIV, TRBP sequesters TAR RNA and decreases the amount of RNA available for PKR activation (Park et al., 1994). Moreover, TRBP directly binds to PKR and strongly inhibits PKR activation (Benkirane et al., 1997; Daher et al., 2001). A model for the role of TRBP–PKR interactions in HIV-1 replication comes from astrocytes, which are brain cells that do not efficiently replicate the virus. Astrocytes have a major block in the translation of HIV-1 structural proteins due to a heightened PKR activation (Gorry et al., 1999; Ong et al., 2005). This PKR activation is due to a weak TRBP expression in astrocytes as compared to HeLa or lymphocytes and the consequent absence of a PKR squelching mechanism. The low TRBP expression in astrocytes is due to the weak induction of its promoter in part caused by the reduced amount of NF-Y transcription factors in astrocytes compared to lymphocytes (Bannwarth et al., 2001, 2006; Ong et al., 2005). In addition, TRBP inhibits PACT-mediated PKR activation through TRBP–PACT interactions (Daher et al., 2009; Laraki et al., 2008; Singh et al., 2011). Silencing or overexpressing TRBP shows that the protein favours HIV-1 replication every time it has been tested, which is likely due to its role as a PKR inhibitor, but may also be related to its function in RNAi (Christensen et al., 2007; Daniels and Gatignol, 2012; Eekels et al., 2011; Sanghvi and Steel, 2011).

4.3.2.3. ADAR1. ADAR1 and PKR interact with each other and ADAR1 inhibits PKR activation during viral infections, therefore inhibiting PKR-mediated eIF2α phosphorylation and subsequent arrest in global protein synthesis (Gélinas et al., 2011; Samuel, 2011; Wang and Samuel, 2009). This activity enhances viral replication of *Vesicular stomatitis virus* (VSV) (Li et al., 2010; Nie et al., 2007). ADAR1–PKR interaction also inhibits PKR-induced stress granule formation and promotes the replication of *Measles virus* (Okonski and Samuel, 2013; Toth et al., 2009). The expression of ADAR1 is induced upon HIV-1 infection in Jurkat cells and in PBMCs (Clerzius et al., 2009, 2013). In addition, ADAR1 interaction with PKR is enhanced when HIV-1 is actively replicating. ADAR1 increases HIV-1 protein production and alleviates PKR-mediated inhibition of HIV-1 production. Knockdown of ADAR1 expression in HIV-transfected HEK 293T cells partially restores PKR activation (Clerzius et al., 2009, 2013; Doria et al., 2009). This suggests that ADAR1 may be part of a multiprotein complex, which inhibits PKR during HIV-1 replication, but also in other viral infections (Clerzius et al., 2011; Gélinas et al., 2011; Samuel, 2011).

5. Involvement of PACT in the translation of HIV-1 and other viruses

5.1. Identification of PACT and RAX as PKR activators

PACT cDNA has been cloned in a yeast two-hybrid screen of a human cDNA library using an inactive PKR mutant as bait (Patel and Sen, 1998). Mammalian cells transfected with PACT showed an enhanced phosphorylation of PKR and eIF2α and translation inhibition. Overall, these assays demonstrated that PACT is a cellular PKR activator in the absence of dsRNA or viral infection (Patel and Sen, 1998). RAX cDNA was cloned from a mouse Interleukin-3 (IL-3)-dependent cDNA library for its interaction with a kinase-deficient PKR (Ito et al., 1999). PACT and RAX expressed in bacteria, purified and devoid of any contaminating dsRNA, activated PKR *in vitro* (Ito et al., 1999; Patel and Sen, 1998). Endogenous PACT and RAX induce apoptosis upon various oxidative stresses through PKR activation (Ito et al., 1999; Lee et al., 2007; Patel et al., 2000; Singh et al., 2009). Although the two proteins have the same length and differ by only 6 aa, in the original experiments, transfected PACT was able to directly activate PKR, whereas transfected RAX required IL-3 deprivation or diverse stress such as arsenite, thapsigargin or H_2O_2 to activate PKR (Ito et al., 1999; Patel and Sen, 1998). This discrepancy was solved after sequencing the cloned PACT cDNA, which had an accidental mutation inducing a frameshift in its C-terminal end, leading to a truncated PACT deleted for the last 13 aa called PACT305 (Peters et al., 2001) or PACTΔ13 (Daher et al., 2009). This truncated PACT activated PKR with no stress when used in a translation inhibition assay after IFN induction (Daher et al., 2009; Gupta et al., 2003; Huang et al., 2002; Patel and Sen, 1998; Peters et al., 2001). In contrast, wild-type transfected PACT or RAX did not activate PKR, increased gene expression and did not induce apoptosis, suggesting that both the human and the murine protein require a stress to activate PKR (Daher et al., 2009; Ito et al., 1999; Laraki et al., 2008; Li and Sen, 2003; Yang et al., 2003). Data also showed that the two dsRBDs of PACT bind PKR, whereas the C-terminal end is responsible for PKR activation (Gupta et al., 2003; Huang et al., 2002; Peters et al., 2001). Interaction assays showed that PACT homodimerizes in the two dsRBDs and in the C-terminal domain called Medipal and this PACT–PACT interaction is essential for PKR activation (Laraki et al., 2008; Singh and Patel, 2012). Activation of PACT and RAX by stress induces their phosphorylation on Serines 18, 246 and 287, required for PKR activation (Bennett et al., 2004; Peters et al., 2006; Ruvolo et al., 2001; Singh and Patel, 2012). Overall, data on human and murine proteins were reconciled and indicate that PACT and RAX are both proapoptotic proteins and stress-inducible activators of PKR. Their activation by stress is mediated by the C-terminal end, but a truncation of the last 13 aa, not found in the natural context, generates a constitutive PKR activator (Daher et al., 2009; Singh and Patel, 2012).

5.2. Regulation of PACT by viral factors

During HSV infection, the viral protein Us11 binds to PKR and blocks its activation by PACT, showing an indirect activity of the virus (Peters et al., 2002). PACT can also trigger a cell response independently of its activity on PKR. It binds and activates the cytoplasmic RNA sensor DExD/H box helicase RIG-I during *Sendai virus* infection (Kok et al., 2011). This activity is counteracted by HSV Us11 and contributes to an additional level of perturbation of type I IFN production in HSV-infected cells (Kew et al., 2013). As more studies are performed with different viruses, it is likely that PACT will be found to be a direct or indirect target of viruses to avoid RIG-I or PKR activation.

5.3. Regulation of PACT by TRBP

PACT is also regulated by cellular components. While the exact mechanism leading to its phosphorylation and activation by cellular stresses remains to be elucidated, its activity in cells is regulated by the formation of heterodimers with the cellular protein TRBP. TRBP and PACT are homologous proteins, which have diverged from a common ancestor between invertebrates and vertebrates (Daniels and Gatignol, 2012). Interaction assays using the yeast two-hybrid method, co-IPs, *in vitro* interactions and colocalization have shown that TRBP and PACT interact through the two dsRBDs in an interchangeable manner (Kok et al., 2007; Laraki et al., 2008). In addition, TRBP and PACT also interact through the C-terminal Medipal domain and this interaction provides the clue to TRBP regulation of PACT activity on PKR (Laraki et al., 2008). In HeLa cells, only PACTΔ13 and not PACT activated PKR, whereas both proteins increased PKR phosphorylation in astrocytes, which express low amounts of TRBP (Daher et al., 2009; Laraki et al., 2008). PKR activation by wild-type PACT was restored in the absence of TRBP either in astrocytes, after treating cells by siRNAs against TRBP or in *tarbp2*$^{-/-}$ cells. Furthermore, TRBP–PACTΔ13 heterodimers are unstable providing an explanation for the constitutive PKR activation by the truncated mutant. PACT activity was also restored by a stress that separates the TRBP–PACT heterodimers, indicating that TRBP controls PACT activation of PKR by a stress-reversible process (Daher et al., 2009; Singh et al., 2011).

5.4. Reversal of PACT function during HIV-1 replication

HIV-1 provides another example of an additional regulation of PACT during viral infection. PKR and PACT interact during HIV-1 infection of lymphocytes as shown by co-IP and this interaction is increased at high level of virus replication (Clerzius et al., 2013). As PKR is deactivated during HIV-1 replication, the question of the role of this interaction was raised. In HEK293T cells transfected with an HIV molecular clone, PACT reversed PKR and eIF2α phosphorylation induced by the virus. Furthermore, PACT rescued PKR-mediated inhibition of HIV-1 protein synthesis. This effect was not solely due to its inactivation by TRBP because a similar, but not as strong inhibition was observed in HIV-1-expressing astrocytes. Furthermore, knockdown of PACT expression partially rescues PKR activation and inhibits viral expression in HIV-1-expressing HEK293T cells. Therefore, PACT becomes a PKR inhibitor in HIV-1 expressing cells. Although the mechanism remains to be elucidated, the increased ADAR1 expression and the direct ADAR1–PACT interaction may be part of the mechanism (Clerzius et al., 2013).

6. Conclusion

Synthesis of viral proteins through regulation of viral mRNA translation is a prerequisite for the assembly of virions. HIV-1 and -2 translation uses several mechanisms from cap-scanning mechanism to IRES, frameshifting and ribosomal shunting. The cell response to HIV-1 entry induces various pathways leading to translational alterations by cellular proteins PKR, OAS, Slfn11 and ADAR1 when they are not counteracted. PKR has the potential to inhibit HIV-1 replication, but the virus has evolved several mechanisms to bypass its activation using viral and cellular proteins. Several cellular proteins have a role in PKR inactivation. HIV-1 has evolved to replicate in cells expressing large amounts of TRBP, which directly counteract PKR and PACT activation. The virus also induces an overexpression of ADAR1, which also inhibits PKR. Finally, HIV-1 changes the function of PACT, which becomes a PKR inhibitor in HIV-1 expressing cells. These proteins may form a large complex or binary complexes to inhibit PKR activation or act differently to target various steps of the PKR pathway. We can speculate that such elaborated mechanisms have evolved to counteract a strong viral inhibition by PKR. Targeted inhibitors of specific PKR interactions may be useful in future therapeutic regimens against HIV-1.

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