Virology 393 (2009) 210–220

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Insulin-like growth factor II mRNA binding protein 1 modulates Rev-dependent human immunodeficiency virus type 1 RNA expression

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ARTICLE INFO

Article history:
Received 12 March 2009
Returned to author for revision 2 June 2009
Accepted 4 August 2009
Available online 1 September 2009

Keywords:
HIV-1
IMP1
Rev

ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) needs to overcome cellular counter mechanisms such as to successfully propagate itself. Results of our recent studies show that overexpression of insulin-like growth factor II mRNA binding protein 1 (IMP1) inhibits production of infectious HIV-1 particles through adversely affecting virus maturation. Here, we report that IMP1 interacts with HIV-1 Rev protein and its ectopic expression causes relocation of Rev from the nucleus to the cytoplasm. In accordance with this observation, ectopic expression of IMP1 severely diminishes Rev-dependent expression of CAT enzyme and disturbs HIV-1 RNA expression by causing accumulation of the multiple spliced viral RNA. Results of mutagenesis analysis further reveal that the KH4 domain represents the key element of IMP1 in modulating HIV-1 RNA expression. Taken together, these data suggest, in addition to hampering virus assembly, that IMP1 also has an effect on Rev-dependent viral RNA expression.

Introduction

Human immunodeficiency virus type 1 (HIV-1) encodes nine genes including three structural genes (*gag*, *pol* and *env*), two regulatory genes (*tat* and *rev*) and four accessory genes (*vpr*, *vif*, *vpu* and *nef*; Frankel and Young, 1998). HIV-1 life cycle falls into two phases. The early phase starts from virus entry and finishes till integration of viral cDNA into the host chromosomal DNA to form a provirus. The late phase begins with transcription of HIV-1 RNA and ends with generation of infectious virus particles. HIV-1 replicates strictly within cells by exploiting various cellular pathways and consuming cellular resources (Goff, 2007). This is well reflected by the fact that HIV-1 relies on the cellular RNA polymerase II to produce and amplify viral RNA genome as well as on the cellular translation machinery to make viral proteins. Furthermore, although HIV-1 Gag protein is capable of generating virus-like particles, in order to successfully check out of the cells, these Gag particles need to hijack the ESCRT (endosomal sorting complex required for transport) for budding and release (Fujii et al., 2007). To defend itself from HIV-1 infection, cells have evolved various means to target and block different steps of HIV-1 replication (Goff, 2007), which is well manifested by several recently discovered HIV-1 restriction factors.

IMP1 is one of the zip-code binding proteins (ZBPs) that recognize *cis*-acting RNA elements and regulate the activity of mRNA target (Jambhekar and Derisi, 2007). IMP1 and its homologues from different species form a small protein family named the VICKZ family, including IMP1, IMP2 and IMP3 in human, coding region determinant-binding protein (CRD-BP) in mouse, ZBP1 in chicken, and Vg1-RBP/Vera in *Xenopus* (Yisraeli, 2005). The VICKZ family members all possess two RNA recognition motifs (RRMs) followed by four tandem hnRNP K homology (KH) domains. IMP1 was originally discovered through its association with the leader 3 of the insulin-like growth factor II (IGF II) mRNA and is known for its ability to regulate the expression of IGF II at the early stage of mouse embryonic development (Nielsen et al., 1999). A few more RNA targets of IMP1 were subsequently identified such as H19 RNA (Runge et al., 2000), tau mRNA (Atlas et al., 2004), c-myc mRNA (Doyle et al., 1998) and PABP mRNA (Patel and Bag, 2006). A comprehensive microarray analysis of RNA composition of IMP1-containing RNP granules suggests that IMP1 potentially controls the activity of 2–3% of the cellular transcriptome (Jonson et al., 2007). The importance of IMP1 in cell life is further highlighted by the findings that the KH4-deleted IMP1 mutant acts in a dominant-negative fashion to inhibit cell migration (Natkunam et al., 2007) and that elevated expression of IMP1 is associated with several types of cancers such as colorectal carcinoma (Ross et al., 2001), brain tumors (Ioannidis et al., 2004), non-small cell lung carcinoma (Ioannidis et al., 2004), and mesenchymal tumors (Ioannidis et al., 2001).

Given that IMP1 is a typical RNA-binding protein and that its intrinsic function is to regulate the activity of its RNA target (Yisraeli, 2005), we speculated that IMP1 may also modulate HIV-1 RNA expression. Indeed, results of this study revealed that ectopic expression of IMP1 caused significant accumulation of the multiple

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doi:10.1016/j.virol.2009.08.004

spliced HIV-1 RNA, which is attributed to the ability of IMP1 to interact with Rev and to relocate Rev from the nucleus into the cytoplasm.

Results

IMP1 interacts with Rev protein and its ectopic expression causes relocation of Rev from the nucleus to the cytoplasm

IMP1 shuttles between the nucleus and the cytoplasm as a component of RNP complex and may thus have a potential role in regulating nuclear export of RNA (Yisraeli, 2005). HIV-1 Rev is also a shuttling protein and controls nuclear export of HIV-1 full-length and singly spliced RNA (Pollard and Malim, 1998). We therefore asked whether IMP1 and Rev happen to interact with each other when they traffic between the nucleus and the cytoplasm. To address this, HEK293T cells were co-transfected with the FLAG-IMP1 and Rev DNA constructs. The FLAG-IMP1 protein was then immunoprecipitated from cell lysates using the anti-FLAG antibody. The results of Western blotting showed that Rev protein was readily detected in precipitated materials (Fig. 1A, B). Deletion of the four KH domains, but not the two RRM domains or the KH3/KH4 domains, eliminated this interaction (Fig. 1A, B). Association of IMP1 and Rev was next investigated by imaging analysis to determine whether these two proteins co-localize in cells. To this end, we transfected HeLa cells with Rev-RFP DNA alone or together with the FLAG-IMP1 DNA. Rev-RFP was detected in the nucleoli in cells that were transfected only by Rev-RFP DNA (Fig. 1C). It is known that Rev binds to Crm1 and exports HIV-1 full-length RNA into the cytoplasm via the Crm1 pathway (Pollard and Malim, 1998). In the absence of Rev-RFP, Crm1 was evenly distributed within the nucleoplasm with a modest accumulation along the nuclear rim (Fig. 1C). Expression of Rev-RFP led to accumulation of Crm1 in the nucleoli, suggesting that Rev sequesters a substantial amount of Crm1 protein within the nucleoli for its own use. When both Rev-RFP and FLAG-IMP1 were expressed, a significant amount of Rev-RFP was detected within the cytoplasm and co-localized with FLAG-IMP1 (Fig. 1C). Expression of FLAG-ΔRRM(1–2), but not FLAG-ΔKH(1–4), resulted in substantial relocation of Rev-RFP from the nucleus into the cytoplasm (Fig. 1C). Interestingly, the FLAG-ΔKH(3–4) protein was unable to change the subcellular location of Rev-RFP, although Rev was efficiently co-immunoprecipitated with FLAG-ΔKH(3–4) (Fig. 1B, C), indicating that interaction of FLAG-IMP1 with Rev is necessary but insufficient to divert Rev from the nucleus into the cytoplasm. Taken together, these results suggest that IMP1 interacts with Rev and has the ability of altering Rev subcellular distribution.

Ectopic expression of IMP1 suppresses Rev-dependent expression of the CAT enzyme

Given the adverse effects of IMP1 on Rev subcellular localization, we speculated that ectopic IMP1 expression impairs the function of Rev. To test this, we utilized the pDM128 plasmid DNA that expresses the CAT enzyme in a Rev-dependent manner (Hope et al., 1990). Results of Fig. 2A show that cotransfection of FLAG-IMP1 DNA led to a 6-fold reduction of Rev-dependent CAT production. Further testing a panel of IMP1 mutants revealed that the FLAG-ΔRRM(1–2) mutant inhibited CAT expression by 2-fold as opposed to modestly stimulated CAT expression by the FLAG-ΔKH(1–4) or FLAG-ΔKH(3–4) mutants (Fig. 2B), which is in accordance with the effect of these mutants on the subcellular location of Rev (Fig. 1C). Therefore, ectopic IMP1 expression inhibits the function of Rev and the KH domains play an important role in this inhibition activity.

Since Rev-dependent gene expression requires specific access to Crm1-mediated nuclear export pathway, we next tested whether IMP1 adversely affects Rev-dependent CAT expression through specifically impairing the function of Crm1 but not TAP. Toward this end, a reporter construct named DM128-ms2×4 was employed in the following experiments. This DNA construct contains four copies of RNA binding sites for the MS2 protein (Yi, Bogerd, and Cullen, 2002). Through co-transfection with MS2-Crm1 or MS2-TAP DNA, Crm1 or TAP protein is recruited to the DM128-ms2×4 mRNA as a result of specific binding of MS2 to its RNA ligand ms2, followed by the nuclear export of DM128-ms2×4 mRNA and expression of CAT enzyme (Fig. 3A). When FLAG-IMP1 was expressed, the MS2-Crm1-dependent, but not MS2-TAP-dependent, expression of CAT enzyme was significantly reduced (Fig. 3A). Results of further studies showed that the KH domains were essential for this inhibitory activity of IMP1 (Fig. 3B). This finding was confirmed by the results of experiments performed with the GPV-ms2×6 DNA construct that encode HIV-1 Gag and Gag-Pol proteins and carries six copies of RNA binding sites for the MS2 protein. The results in Fig. 3C show that IMP1 expression specifically inhibited Crm1-dependent, but not TAP-dependent, production of Gag and Gag-Pol proteins. Taken together, the data suggest that, in addition

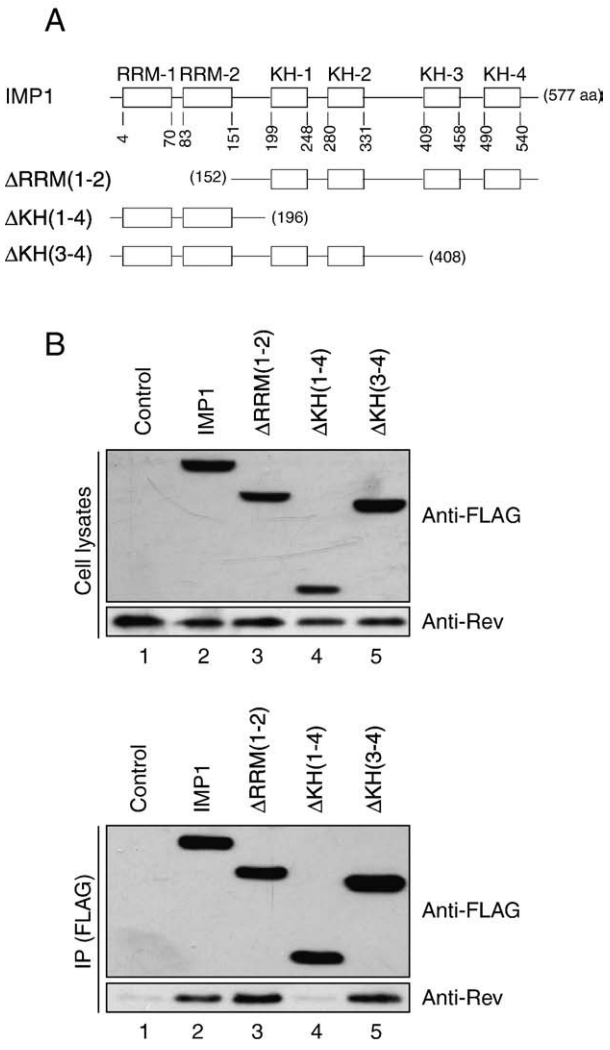


Fig. 1. Ectopic expression of FLAG-IMP1 alters subcellular localization of Rev protein. (A) Domain structure of IMP1 and its mutants. Positions of the deleted amino acids are denoted. (B) IMP1 interacts with Rev. HEK293T cells were co-transfected with the Rev DNA and the wild-type FLAG-IMP1 or the mutated FLAG-ΔRRM(1–2), FLAG-ΔKH(1–4) and FLAG-ΔKH(3–4) DNA. Cell lysates were incubated with agarose beads coated with anti-FLAG antibody to immunoprecipitate FLAG-IMP1 protein and its mutants. The precipitated materials were assessed by Western blotting using either anti-FLAG or anti-Rev antibodies. (C) Subcellular localization of FLAG-IMP1 and Rev proteins. The Rev-RFP DNA was transfected into HeLa cells together with the wild-type or mutated FLAG-IMP1 DNA. Subcellular distribution of FLAG-IMP1 and the endogenous Crm1 proteins was detected by immunofluorescence staining using anti-FLAG or anti-Crm1 antibodies. Fluorescent signals for FLAG-IMP1, Rev-RFP and Crm1 are pseudo-colored as blue, red and green, respectively.

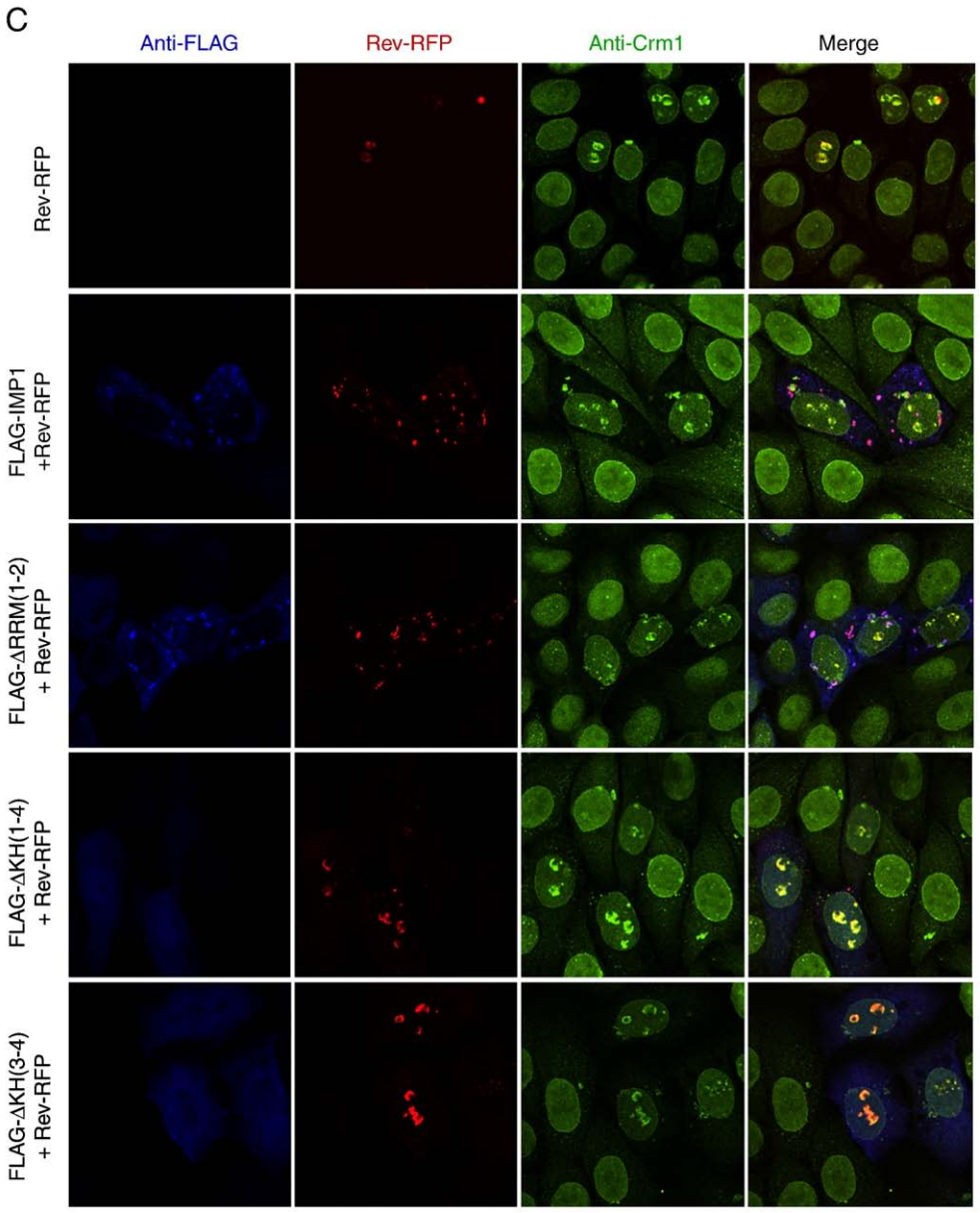


Fig. 1 (*continued*).

to altering the subcellular distribution of Rev protein, IMP1 impedes Rev-dependent gene expression by targeting the Crm1-mediated nuclear export pathway.

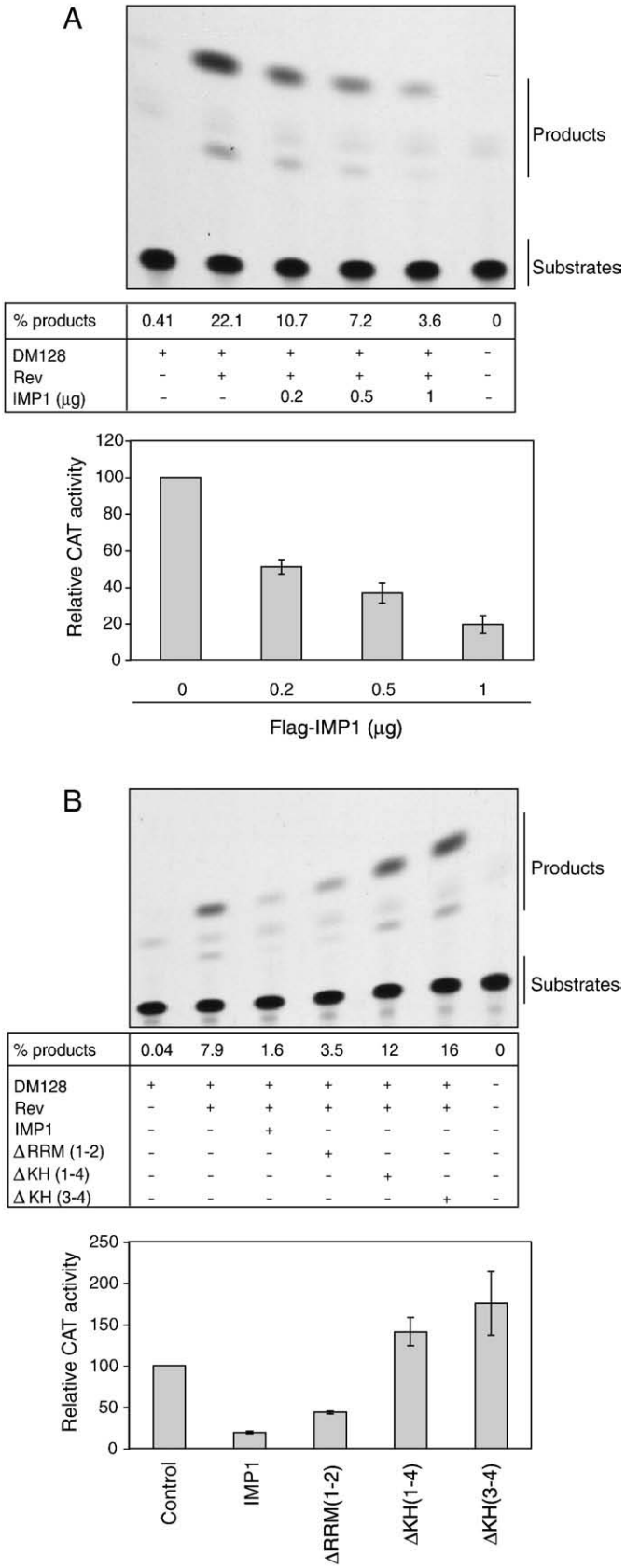
Ectopic expression of IMP1 leads to significant accumulation of multiple spliced HIV-1 RNA

Rev protein promotes the nuclear export of the unspliced and singly spliced HIV-1 RNA and thus represents a key viral factor in maintaining the balance between the unspliced and spliced viral RNA species (Pollard and Malim, 1998). The adverse effect of ectopic IMP1 on Rev function is expected to result in abnormal profiles of HIV-1 RNA expression. To test this, we transfected HEK293T cells with HIV-1 proviral DNA clone BH10 together with different amounts of FLAG-IMP1 DNA and assessed expression of viral RNA by Northern blotting. HIV-1 RNA of three different sizes were detected, and they represent the 9-kb unspliced RNA, the 4-kb singly spliced RNA and the 2-kb multiple spliced RNA (Fig. 4A). When 0.2 μg of FLAG-IMP1 DNA was used for co-transfection, levels of both the 9-kb and 2-kb viral RNA were increased with a much greater augmentation for the 2-kb species (Fig. 4A). Increasing FLAG-IMP1 expression further elevated the amount of 2 kb viral RNA as opposed to a gradual decline of 9 kb viral RNA (Fig. 4A). These results indicate that ectopically expressed FLAG-IMP1 causes accumulation of multiple spliced viral RNA.

We next tested three IMP1 mutants, including FLAG-ΔRRM(1–2), FLAG-ΔKH(1–4), FLAG-ΔKH(3–4), to determine which region of IMP1 plays the critical role in altering HIV-1 RNA expression. Each of these DNA constructs was co-transfected with BH10 DNA into HEK293T cells. The results of Northern blotting showed that the FLAG-ΔRRM(1–2) protein, but not the FLAG-ΔKH(1–4) and FLAG-ΔKH(3–4) proteins, caused significant accumulation of the 2-kb viral RNA (Fig. 4B), suggesting the indispensable role of the KH3 and KH4 domains for IMP1 to change HIV-1 RNA expression.

The KH3 and KH4 domains represent the key RNA-binding motifs of IMP1 (Nielsen et al., 2004). Results of a recent study further show that the KH4 domain directly contacts with the RNA target and that expression of a KH4-deleted mutant named EGFP-IMP1-ΔKH4, but not the wild-type EGFP-IMP1, affects cell migration (Oberman et al.,

2007). We therefore wished to further test the importance of the KH4 sequence in IMP1 modulating HIV-1 RNA expression. To this end, the EGFP-IMP1 and EGFP-IMP1-ΔKH4 DNA constructs were used to transfect HEK293T cells together with BH10 DNA. Similar to the results obtained with FLAG-IMP1, expression of EGFP-IMP1 also significantly enhanced expression of the 2-kb HIV-1 RNA albeit modestly inhibited production of the 9-kb RNA (Fig. 4C). In contrast, expression of EGFP-IMP1-ΔKH4 did not affect levels of the unspliced and spliced HIV-1 RNA (Fig. 4D). These results indicate the key role of the KH4 domain of IMP1 in modulating HIV-1 RNA expression.



The EGFP-IMP1 fusion protein potently suppresses production of infectious HIV-1 particles

While measuring the effect of EGFP-IMP1 or EGFP-IMP1-ΔKH4 on HIV-1 RNA expression, we also monitored their effects on production of Gag protein and HIV-1 particles by Western blotting and ELISA. In contrast with the modest inhibition of Gag production in HEK293T cells transfected by FLAG-IMP1 (Zhou et al., 2008), EGFP-IMP1 suppressed Gag expression by as much as 30-fold when 1 μg of EGFP-IMP1 DNA was used in transfection (Fig. 5A, B). This profound decrease of Gag expression accounts, to a large extent, for the 100-fold inhibition of virus production (Fig. 5C). Furthermore, when viruses equivalent to the same amount of HIV-1 p24 antigen were tested for their infectivity by infecting TZM-bl indicator cells, the results showed that EGFP-IMP1 diminished the infectivity of HIV-1 virions by as much as 30-fold (Fig. 5D). In contrast, the EGFP-IMP1-ΔKH4 mutant diminished Gag expression by 3-fold (Fig. 6A, B), reduced virus production by 10-fold (Fig. 6C), and decreased infectivity of HIV-1 virions by 7-fold (Fig. 6D). Further increasing the amount of EGFP-IMP1-ΔKH4 DNA to 2 μg in transfection did not inhibit HIV-1 production to a more severe extent (Fig. 6). It was noted that expression of FLAG-IMP1 or EGFP-IMP1 protein did not affect the viability of transfected cells within the period of experiments. These results suggest that the EGFP moiety greatly enhances the potency of IMP1 in suppressing production of infectious HIV-1 particles and that the KH4 domain plays an important role in this event.

Ectopic expression of IMP1 and IMP2 inhibit replication of HIV-1 in SupT1 cells

We next assessed the effect of IMP1 on HIV-1 replication in a CD4+ T-cell line named SupT1. To this end, a SupT1 cell line was generated to express FLAG-IMP1 with induction by a tetracycline derivative doxycycline (Fig. 7A). As a control, a second SupT1 cell line was engineered to express the FLAG-ΔKH(1–4) mutant whose expression does not affect HIV-1 production (Zhou et al., 2008). Wild-type HIV-1 was used to infect the FLAG-IMP1 and FLAG-ΔKH(1–4) cell lines in the absence or presence of doxycycline. The results showed that expression of FLAG-IMP1 reduced production of HIV-1, as opposed to a modest increase of virus production with expression FLAG-ΔKH(1–4) (Fig. 7B). We further tested the two homologues of IMP1, namely IMP2 and IMP3, in these experiments. The results showed that IMP2 inhibited HIV-1 replication in SupT1 cells to a more significant extent than IMP1, whereas IMP3 did not exert any effect on HIV-1 infection (Fig. 7). Together, these data support the inhibitory role of IMP1 in HIV-1 replication in CD4+ T cells and further indicate that IMP2 possesses a stronger inhibitory activity toward restricting HIV-1 infection. This explains why knockdown of IMP1 alone does not significantly affect HIV-1 production (Zhou et al., 2008).

Fig. 2. Ectopic expression of FLAG-IMP1 inhibits Rev-dependent expression of CAT enzyme. (A) Different amounts of FLAG-IMP1 DNA were transfected into HEK293T cells together with the pDM128 reporter DNA and Rev DNA. Levels of the CAT activity were measured as being described in Materials and Methods. Result from one representative experiment is shown. The average of three independent transfection experiments is summarized in the bar graph with level of CAT activity in the control experiment (0 μg of FLAG-IMP1 DNA) set as 100. (B) The pDM128 DNA, Rev DNA and the wild-type or mutated FLAG-IMP1 DNA were co-transfected into HEK293T cells. Levels of CAT activity were measured as being described in Materials and Methods. Results from three independent transfection experiments were summarized in the bar graph.

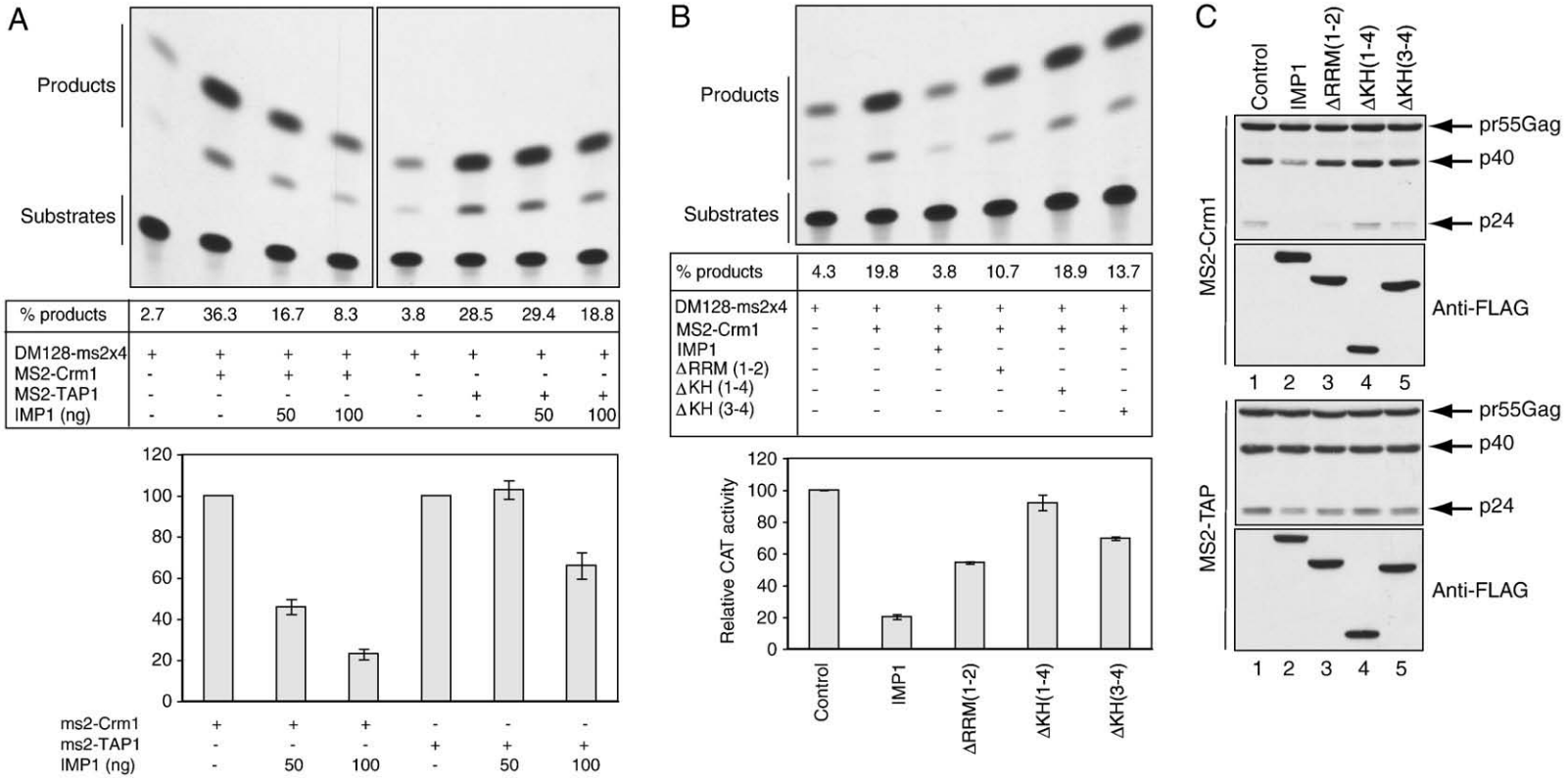


Fig. 3. FLAG-IMP1 inhibits Crm1-mediated but not TAP-mediated gene expression. (A) HEK293T cells were transfected by the DM128-ms2×4 DNA together with MS2-Crm1 or MS2-TAP in the presence of different amounts of FLAG-IMP1 DNA. (B) The DM128-ms2×4 and the MS2-Crm1 DNA was co-transfected with the wild-type or the mutated IMP1 DNA. Levels of CAT expression were measured as described in Materials and Methods. (C) The GPV-ms2×6 DNA was co-transfected with either MS2-Crm1 or MS2-TAP in the presence of the wild-type IMP1 or its mutants. Levels of Gag expression in cells were assessed in Western blots using anti-HIV-1 p24 antibody. Expression of FLAG-IMP1 and its mutants was detected using anti-FLAG antibody.

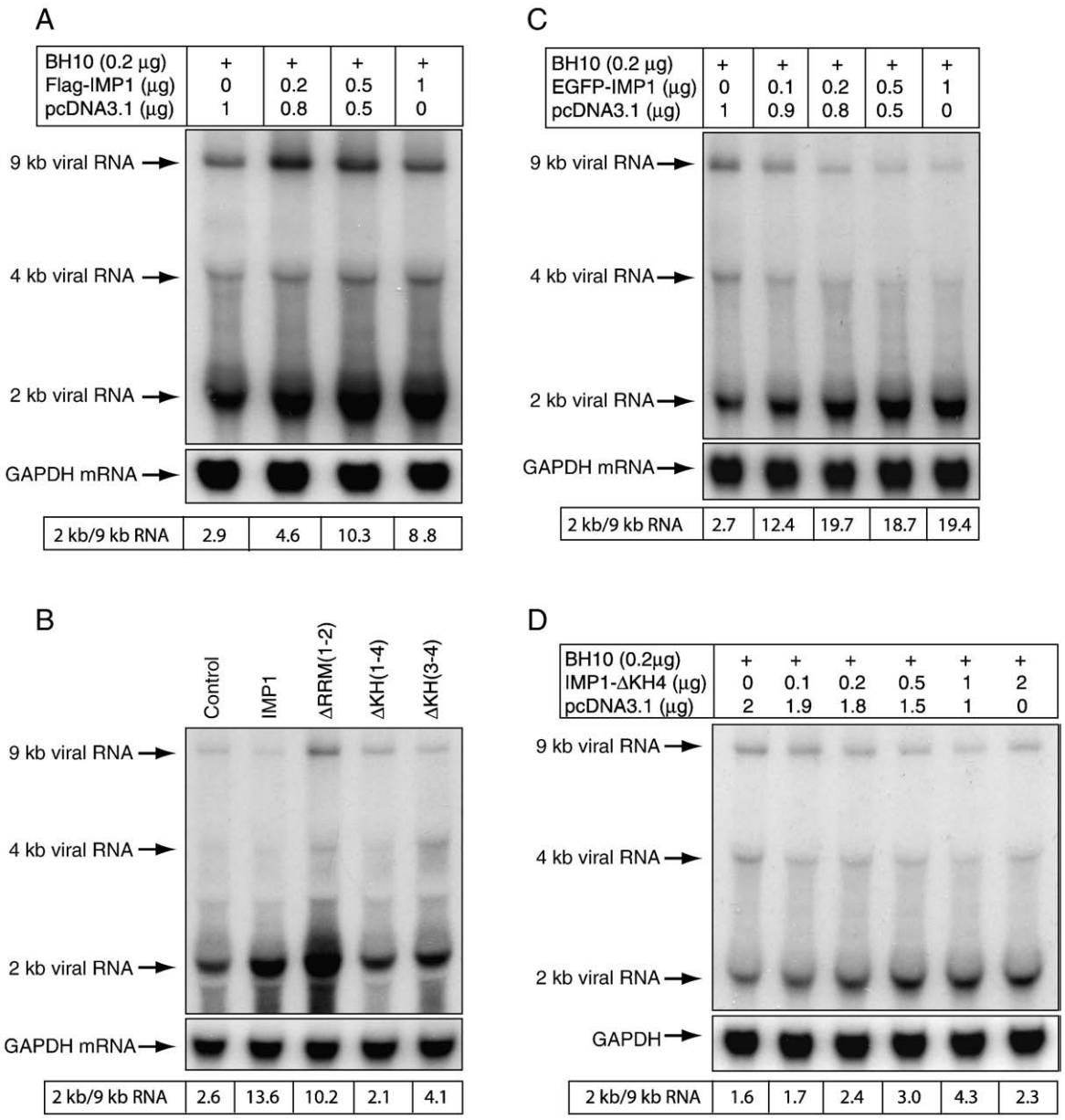


Fig. 4. Ectopic expression of FLAG-IMP1 leads to accumulation of multiple spliced HIV-1 RNA. (A) Northern blots to measure levels of the unspliced and spliced HIV-1 RNA in the presence of ectopic expression of IMP1. HEK293T cells were transfected with BH10 DNA and different amounts of FLAG-IMP1 DNA. Total cellular RNA was extracted and subjected to Northern blotting using [α-^{32}P] labeled HIV-1 DNA probes. The GAPDH mRNA was also detected as the internal control. RNA signals were measured using PhosphoImager. Ratios of the multiple spliced vs. full-length viral RNA were calculated. Results shown are the average of two independent experiments. (B) The BH10 DNA was co-transfected with the wild-type or the mutated IMP1 DNA into HEK293T cells. Viral RNA expression was assessed by Northern blotting. The ratios of the multiple spliced and full-length viral RNA are presented. Results shown are the average of two independent experiments. (C) Ectopic expression of EGFP-IMP1 causes accumulation of multiple spliced HIV-1 RNA. (D) Deletion of the KH4 domain alone abrogates the activity of IMP1 in affecting HIV-1 RNA expression.

Discussion

The full-length HIV-1 RNA undergoes complex alternative splicing, which generates more than 30 different kinds of viral RNA (Frankel and Young, 1998). On the basis of their splicing patterns and their sizes, HIV-1 RNA can be divided into three major groups, the 9-kb unspliced (full length) viral RNA, the 4-kb singly spliced (incompletely spliced) viral RNA and the 2-kb multiple spliced (completely spliced) viral RNA. At the early stage of HIV-1 gene expression, the full-length viral RNA is spliced to produce the messenger RNA to translate Tat, Rev and Nef proteins. Rev then shuttles back into the nucleus and exports the unspliced and singly spliced viral RNA into the cytoplasm to produce viral structural proteins such as Gag, Gag-Pol and Env, which marks the late stage of viral gene expression and the beginning of virus assembly. Rev plays a key role in regulating the relative levels of different viral RNA species such that optimal amounts of different viral proteins can be produced to ensure efficient virus production (Pollard and Malim, 1998). By exporting the unspliced and singly spliced HIV-1 RNA into the cytoplasm, Rev actually diminishes production of multiple spliced RNA including its own messenger RNA. This feedback mechanism creates a balance between unspliced and spliced viral RNA that relies on the level of Rev and Rev's activity. Results of this study show that overexpression of IMP1 changes the subcellular location of Rev and disturbs Rev's function. A direct consequence of this interference is less efficient export of unspliced and singly spliced viral RNA that are therefore further spliced to generate more multiple spliced viral RNA. A new balance between different HIV-1 RNA species is reached when the

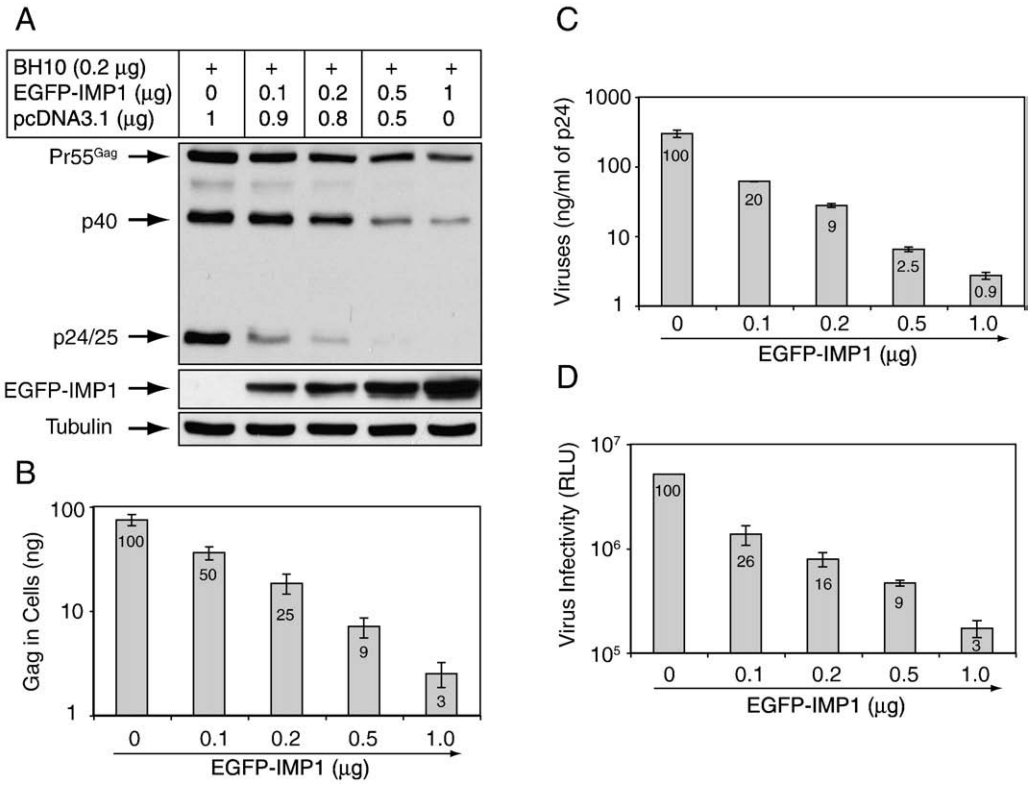


Fig. 5. EGFP-IMP1 impairs production of infectious HIV-1 particles. (A) Various amounts of EGFP-IMP1 DNA was transfected into HEK293T cells together with BH10 DNA (0.2 μg). Expression of HIV-1 Gag protein and EGFP-IMP1 was examined by Western blotting using anti-p24(CA) and anti-EGFP antibodies, respectively. Levels of tubulin were also assessed as the internal control. (B) Amounts of Gag and its derivatives (p40 and p24) in cells were determined by HIV-1 p24 ELISA. Results shown represent the average from three independent transfection experiments. (C) Levels of HIV-1 particles in the culture supernatants were determined by HIV-1 p24 ELISA. Results shown represent the average from three independent transfection experiments. (D) Infectivity of HIV-1 virions was measured by infecting the TZM-bl indicator cells using viruses equivalent to 10 ng of HIV-1 p24 antigen. Results shown represent three infection assays using viruses from three independent transfection experiments. RLU, relative luciferase unit. Values of the control experiments performed with BH10 alone are arbitrarily set at 100. Percentages of Gag expression, virus yield and virus infectivity in the presence of EGFP-IMP1 expression are calculated and indicated in the bar graphs.

reduced export activity Rev is compensated by a higher level of Rev protein produced from the accumulated multiple spliced viral RNA. This observation exemplifies how interfering with Rev function affects the balance between the unspliced and spliced viral RNA and also how the virus survives this disturbance by adjusting the levels of multiple spliced viral RNA to produce more Rev and hereby restore efficient export of the unspliced viral RNA. As a result, expression of viral Gag protein is not profoundly affected by overexpression of IMP1 (Zhou et al., 2008). We also observed that expression of FLAG-IMP1 led to modest increase in the levels of the full-length (9 kb) and singly spliced (4 kb) viral RNA (Fig. 4A). One possible mechanism behind this result is that IMP1 associates with HIV-1 RNA and increases viral RNA stability. In support of this possibility, HIV-1 RNA is co-immunoprecipitated with FLAG-IMP1 protein (unpublished data).

IMP1 is not the only cellular protein that has been reported to affect subcellular distribution of Rev. Hax-1 has been shown to cause relocation of Rev and inhibits Rev function (Modem and Reddy, 2008). Rev's distribution in cells also changes under oxidative and osmotic shocks but not with ultraviolet radiation and heat shock (Soros and Cochrane, 2001). Inhibition of RNA transcription using actinomycin D or dichlorobenzimidizole also leads to redistribution of Rev into the cytoplasm (Meyer and Malim, 1994). These results further demonstrate the shuttling nature of Rev in cells. In any case, the nucleolar location is a prerequisite for Rev to function. For instance, mutation of arginine residues in the arginine-rich domain leads to release of Rev from the nucleolus and concomitant inhibition of Rev's RNA export activity (Cochrane et al., 1990). Furthermore, randomly inserted mutations that change the subcellular localization of Rev to the cytoplasm severely impede Rev function (Wolff et al., 2006). Given that nucleoli undergo dynamic changes in response to extracellular stimuli and nutrition conditions, the nucleolar localization may allow Rev to sense these cues and regulate the rate of virus production accordingly.

As opposed to the inhibitory role of IMP1 in HIV-1 Rev function, a number of cellular factors have been shown to enhance Rev-mediated expression of HIV-1 genomic RNA. One example is DDX3 that binds to Crm1 and is essential for Rev/Crm1-mediated nuclear export of HIV-1 full-length RNA (Yedavalli et al., 2004). hRIP (human Rev-interacting protein) is required for the release of HIV-1 RNA from the perinuclear region (Sanchez-Velar et al., 2004). Sam68 (src-associated protein in mitosis) is able to either promote nuclear export of HIV-1 RNA in a Rev-independent manner or assist Rev to express RRE-containing RNA (Li et al., 2002; Modem et al., 2005; Reddy et al., 1999; Soros et al., 2001). It appears that the function of Rev is subject to a complex regulation by a number of cellular factors.

The increased levels of multiple spliced HIV-1RNA may also be a result of changed splicing efficiency at certain splice sites in HIV-1 RNA. There are four splice donor sites and eight splice acceptor sites in HIV-1 RNA (Neumann et al., 1994; Purcell and Martin, 1993). The splicing efficiency at each of these sites is modulated by the activities of exonic splicing silencers (ESS) and intronic splicing silencers (ISS) that are often bound by hnRNP A/B family or hnRNP H, as well as exonic splicing enhancers (ESE) that are selectively bound by SR proteins (Stoltzfus and Madsen, 2006). We currently cannot rule out the possibility that IMP1 may recognize one of these ESS, ISS or ESE elements and thus modulates splicing efficiency.

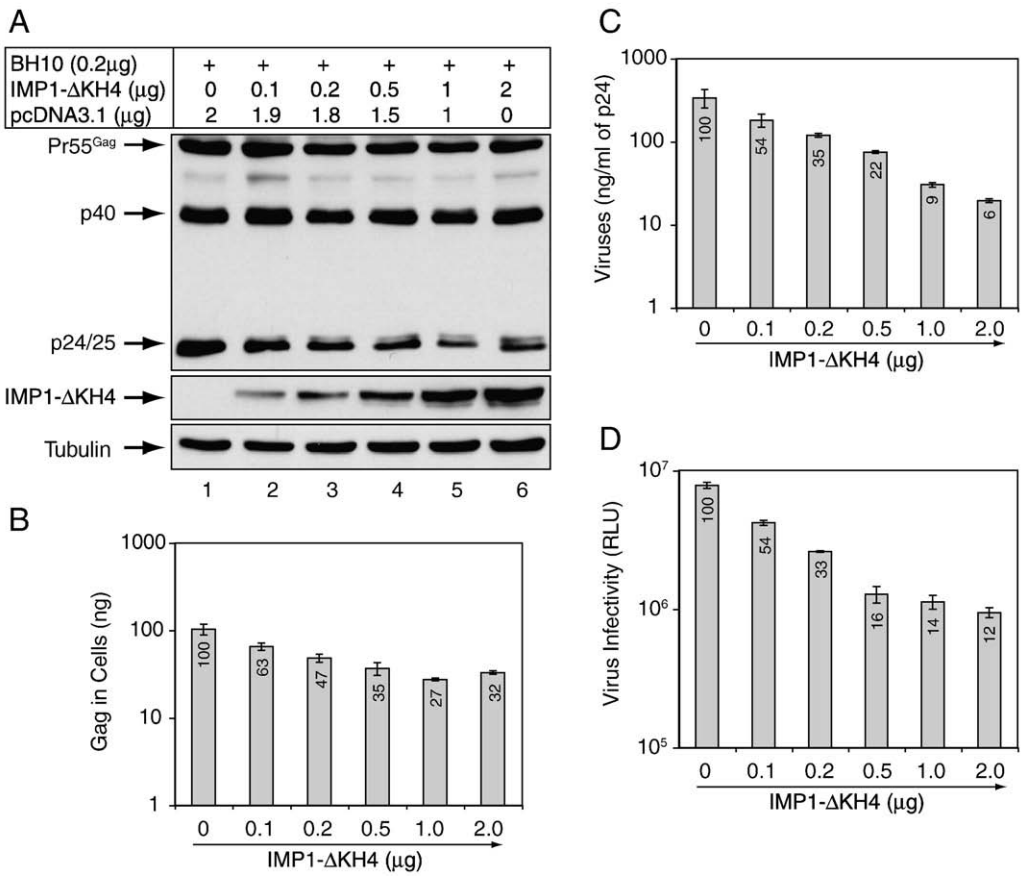


Fig. 6. Deletion of the KH4 domain alleviates the inhibitory effect of EGFP-IMP1 on production of infectious HIV-1 particles. (A) Western blots to measure expression of HIV-1 Gag protein, EGFP-IMP1-ΔKH4 and tubulin. (B) Levels of Gag protein in cells were determined by HIV-1 p24 ELISA. (C) Levels of HIV-1 particles in culture supernatants were measured by HIV-1 p24 ELISA. (D) HIV-1 infectivity was measured by infecting the TZM-bl indicator cells using viruses equivalent to 10 ng of HIV-1 p24 antigen. Data shown represent the results from three independent experiments. RLU, relative luciferase unit. Percentages of Gag expression, virus yield and virus infectivity in the presence of EGFP-IMP1-ΔKH4 expression are calculated and indicated in the bar graphs.

One interesting observation in this study is that EGFP-IMP1 is able to reduce the production of infectious HIV-1 particles by as much as 3000-fold in comparison to the 25-fold inhibition by FLAG-IMP1 (Zhou et al., 2008). This potent inhibition by EGFP-IMP1 reflects the sum of defects in several steps of HIV-1 replication; these include a 30-fold decrease of Gag expression in cells, a 100-fold diminution in virus production and a 30-fold reduction in the infectivity of HIV-1 virions. It is noted that expression of FLAG-IMP1 protein only modestly reduced Gag expression in contrast with the potent inhibition effect from EGFP-IMP1 (Zhou et al., 2008). This may have resulted from the possibility that the FLAG tag interferes with RNA-binding ability of IMP1, which is supported by the results showing that the N-terminal glutathione S-transferase (GST)-tagged or the N- and C-terminal His-tagged IMP1 binds to RNA with low affinity (Nielsen et al., 1999). The EGFP tag differs from the FLAG, GST or His tags in that EGFP is able to multimerize and may thus help IMP1 to form multimers (Jain et al., 2001). Since several IMP1 molecules bind to its RNA target in a cooperative manner (Nielsen et al., 2004), the intrinsic multimerization property of EGFP may have enhanced EGFP-IMP1 binding to RNA and consequently increases the ability of IMP1 to modulate translation.

The KH4 domain is directly involved in IMP1 binding to RNA and is essential for IMP1 oligomerization (Nielsen et al., 2004; Oberman et al., 2007). It is thus not surprising that deletion of KH4 sequence in the context of EGFP-IMP1 largely alleviates suppression of Gag expression. Interestingly, the EGFP-ΔKH4, EGFP-IMP1 and FLAG-IMP1 proteins all reduced the efficiency of virus production by approximately 3-fold following adjustment by the intracellular Gag levels. Therefore, as opposed to its important role in modulating HIV-1 RNA expression, the KH4 domain is dispensable for IMP1 to interfere with the assembly of HIV-1 particles.

In summary, results of this study reveal a new activity of IMP1 in promoting expression of multiple spliced HIV-1 RNA by interfering with the function of Rev protein. Importantly, attaching EGFP to IMP1 greatly enhances the activity of IMP1 to suppress production of infectious HIV-1 particles, which raises the possibility of using EGFP-IMP1 as a tool to develop new strategies to block HIV-1 infection.

Materials and Methods

Plasmid DNA and antibodies

The infectious HIV-1 proviral DNA clone BH10 was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. The pEGFP-IMP1 and pEGFP-IMP1-ΔKH4 DNA constructs were kindly provided by Joel K .Yisraeli (Natkunam et al., 2007). They express the EGFP-IMP1 fusion protein and the EGFP-IMP1-ΔKH4 mutant lacking the KH4 domain, respectively. The pFLAG-IMP1, pFLAG-ΔRRM(1–2), pFLAG-ΔKH(1–4) and pFLAG-ΔKH(3–4) DNA constructs were described previously (Zhou et al., 2008), they express FLAG-tagged wild-type IMP1 and IMP1 mutants. The GPV-ms2×6 DNA construct was created by replacing the four copies of CTE element within the GPV-CTEx4 DNA (Wodrich et al., 2000) with six copies of the ms2-binding sites. The anti-GFP antibody was purchased from Invitrogen, anti-FLAG and anti-tubulin antibodies from Sigma, anti-HIV-1 p24 antibody from ID Labs, Inc., anti-Crm1 antibody from Bethyl Laboratories.

Cell culture and transfection

HEK293T, HeLa and TZM-bl cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen). The TZM-bl cells were obtained from the National Institutes of Health AIDS Research and Reference Reagent program (Wei et al., 2002). One day prior to transfection, HEK293T cells were seeded in six-well plate at 5×10^5 per well. Transfection was performed with Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instruction.

Creating doxycycline-inducible SupT1 cell lines that express IMP1, IMP2 or IMP3 protein

The cDNA sequences of IMP1, IMP2 and IMP3, with the FLAG sequence attached to the 5′ end, were cloned into a doxycycline-inducible retroviral vector PUR-GOI (Clontech). The FLAG-ΔKH(1–4) cDNA was also inserted into PUR-GOI. The created DNA clones were co-transfected together with the VSV (vesicular stomatitis virus)-G DNA into the GP2-293 packaging cell line to produce retrovirus particles, which were used to infect SupT1 cells together with viruses that express the rtTA activitor. One day after infection, G418 (1 mg/ml) and puromycin (2 μg/ml) were added into the culture to select for stably infected cells. Expression of FLAG-IMP1, FLAG-IMP2, FLAG-IMP2 and FLAG-ΔKH(1–4) in the presence of doxycycline was assessed by Western blot using anti-FLAG antibody. Wild-type HIV-1 equivalent to 10 ng p24 antigen was used to infect 1×10^6 cells. Growth of viruses was monitored by measuring the level of viral reverse transcriptase activity in the culture supernatants.

HIV-1 protein analysis

HIV-1 proteins were detected either by Western blotting or by HIV-1 p24 ELISA. HEK293T cells that have been transfected with the BH10 DNA were harvested and lysed in the protein extraction buffer (Novus Biologicals). Cell lysates of 20 μg proteins were fractionated on SDS-10% PAGE followed by transfer of proteins onto polyvinylidene difluoride membranes (GE Healthcare). After blocking with 5% milk (in 1× phosphate buffered saline), the membranes were incubated with anti-HIV-1 p24 antibodies (1:1000) for 2 h at room temperature, followed by washing for three times with 1× phosphate buffered saline containing 0.05% Tween 20. The membranes were then incubated with the horseradish peroxidase conjugated secondary antibodies for 1 h at room temperature. After washing with 1× phosphate buffered saline (0.05% Tween 20), the membranes were treated with the ECL reagents (Perkin-Elmer) and exposed to the X-ray films to visualize the protein signals. The amounts of HIV-1 p24 antigen in the cell lysates or the culture supernatants were also measured using the HIV-1 p24 microELISA kit (bioMerieux) following instruction from the manufacturer.

HIV-1 RNA analysis

Levels of HIV-1 RNA in HEK293T cells that had been transfected by the BH10 DNA were measured by denaturing Northern blotting (Zhou et al., 2008). Briefly, total cellular RNA was extracted using Trizol reagent (Invitrogen) followed by electrophoresis in 1% denaturing agarose gels in a 1× MOPS (3-(*N*-morpholino) propanesulfornic acid) buffer at 100 V for 4 h. RNA was then transferred to hybond N membranes and hybridized to [α-^{32}P]-labeled HIV-1 DNA probes (HIV-1 nucleotide positions 1–2000 in BH10). RNA signals were visualized by exposure to X-ray films. Intensity of the signals was measured using a PhosphoImager (Storm 840, Amersham).

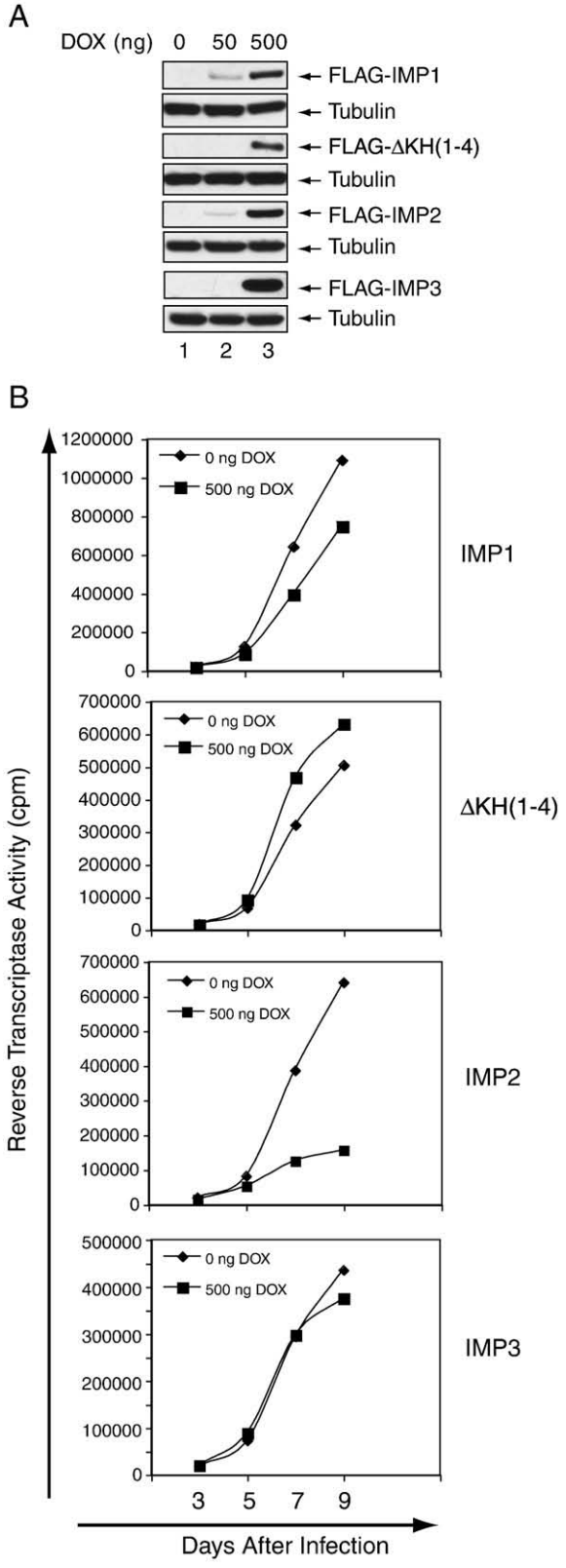


Fig. 7. Effects of expression of IMP1, IMP2 and IMP3 on HIV-1 replication in SupT1 cells. (A) Expression of FLAG-IMP1, FLAG-IMP2, FLAG-IMP3 and FLAG-ΔKH(1–4) in the presence of doxycline. SupT1 cell lines were exposed to different amounts of doxycline for 20 h before cells were harvested and lysed for Western blot analysis using antibodies against FLAG or tubulin. (B) Growth of HIV-1 in SupT1 cells with the expression of FLAG-IMP1, FLAG-IMP2, FLAG-IMP3 and FLAG-ΔKH(1–4). Each SupT1 cell lines was infected with wild-type HIV-1 in the absence or presence of doxycycline (500 ng/ml). Levels of viral reverse transcriptase activity in the culture supernatants were measured at different time points after infection. Results shown are the average of two independent infection experiments.

Measuring the infectivity of HIV-1 virions

TZM-bl cells were seeded in 24-well plates at 4×10^4 cells per well 1 day prior to infection with HIV-1 virions equivalent to 10 ng of HIV-1 p24 antigen. The infected cells were collected 40 h later and lysed in 100 μl of 1× passive lysis buffer (Promega). The lysates (10 μl) were mixed with 100 μl of firefly luciferase substrate solution (Promega) followed by reading luciferase activity in an luminometer (Promega).

CAT (chloramphenicol acetyl transferase) assay

HEK293T cells were seeded in six-well plates at 5×10^5 cells per well 1 day before transfection with 100 ng pDM128 and 50 ng pRev DNA. The TK-RL DNA (100 ng) was also used in transfection. Expression of *Renilla* luciferase was measured using luciferase assay kit (Promega) and the data were used as an internal control to normalize the levels of CAT activity. pDM128 carries the CAT gene and the Rev response element (RRE) and expresses CAT enzyme in a Rev-dependent manner (Hope et al., 1990). Cells were harvested and suspended in 200 μl of 250 mM Tris–HCl (pH 7.5). After three times of freeze and thaw, cells were pelleted at 1000×*g* at 4 °C for 10 min to remove the nuclei and cell debris. 10 μl of the cell lysates were mixed with a reaction buffer containing 250 mM Tris–HCl (pH 7.5), 0.3 mg/ml *n*-butyryl coenzyme A (Sigma), 30 μM [^{14}C]chloramphenicol (at 50 μCi/ml, Amersham), and incubated at 37 °C for 1 h. The substrates and the products were extracted with ethyl acetate, spotted on to the TLC plate and separated by chromatography using chlorophorm/methanol (190:10, v/v). [^{14}C]chloramphenicol and the products were visualized by exposure to X-ray films and their levels were quantified using a PhosphorImager (Storm 840; Amersham).

Immunomicroscopy

HeLa cells were seeded in a four-well chamber slide (40,000 cells per well) and transfected with the pRev-RFP (50 ng) and pFLAG-IMP1 (50 ng) DNA constructs. Cells were fixed with 4% paraformaldehyde (in 1× phosphate buffered saline) for 10 min at room temperature followed by permeabilization with 0.2% Triton X-100 for 10 min. After washing with 1× phospate buffered saline, cells were incubated with mouse anti-FLAG (1:500) and anti-Crm1 (1:200) antibodies for 2 h at room temperature, followed by incubation with Alexa Fluor 488-conjugated anti-mouse and Alexa Fluor 546-conjugated anti-rabbit secondary antibodies (Molecular Probes). Images were recorded using the PASCAL laser scanning confocal microscope (Zeiss).

Immunoprecipitation

HEK293T cells were transfected with the FLAG-IMP1 DNA and the pRev DNA. Cells were lysed in a buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and protease inhibitor cocktail (Roche). Following clarification by spinning at 10,000×*g* for 10 min at 4 °C, cell lysates of 1 mg were incubated with 30 μl slurry of agarose beads coated with anti-FLAG antibody (Sigma) for 2 h at 4 °C. After washing with a buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl and protease inhibitor cocktail (Roche), the beads were incubated with 150 ng/μl 3× FLAG peptides (Sigma) for 30 min to elute the FLAG-IMP1 and the associated proteins. The eluted materials were assessed by Western blotting using anti-FLAG or anti-Rev antibodies.

Acknowledgments

We thank Finn C Nielson and Jan Christiansen for providing the cDNA clones of IMP1, IMP2 and IMP3, and IMP1 antiserum, Joel K Yisraeli for providing the pEGFP-IMP1 and pEGFP-IMP1-ΔKH4 DNA constructs, and Bryn Cullen for the gift of DM128-ms2×4, MS2-Crm1 and MS2-TAP DNA constructs. The research was supported by funding from Canadian Institutes of Health Research (CIHR) and Canadian Foundation for AIDS Research (CANFAR). C.L. is the recipient of bourse de chercheur-boursier senior award from Fonds de la recherché en santé.

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