**Insulin-Like Growth Factor II mRNA Binding Protein 1 Associates with Gag Protein of Human Immunodeficiency Virus Type 1, and Its Overexpression Affects Virus Assembly**

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Received 27 January 2008/Accepted 21 March 2008

Human immunodeficiency virus type 1 (HIV-1) is a complex retrovirus and encodes nine viral proteins, including Gag, Gag-Pol, Env, Tat, Rev, Nef, Vif, Vpr, and Vpu (17). Gag protein is the major structural component of HIV-1 particles and orchestrates the process of virus assembly (26). This function of Gag is ascribed to its self-multimerization property but also depends on its interaction with a number of cellular factors such as TSG101 and ALIX/AIP1 that promote virus budding and release from cell surfaces. However, interaction with Gag also allows some cellular factors such as APOBEC3G and Trim5α to access viral replication machinery and block viral replication. In this study, we report a new HIV-1 Gag-binding factor named insulin-like growth factor II mRNA binding protein 1 (IMP1). Gag-IMP1 interaction requires the second zinc finger of the nucleocapsid (NC) domain of Gag and the KH3 and KH4 domains of IMP1. A fourfold reduction of HIV-1 infectivity was seen with overexpression of the wild-type IMP1 and its mutant that is able to interact with Gag but not with overexpression of IMP1 mutants exhibiting Gag-binding deficiency. The decreased viral infectivity was further shown as a result of diminished viral RNA packaging, abrogated Gag processing on the cellular membranes, and impeded maturation of virus particles. Together, these results demonstrate that IMP1 interacts with HIV-1 Gag protein and is able to block the formation of infectious HIV-1 particles.

Human immunodeficiency virus type 1 (HIV-1) particles are driven by viral Gag protein. This function of Gag not only benefits from its self-multimerization property but also depends on its interaction with a number of cellular factors such as TSG101 and ALIX/AIP1 that promote virus budding and release from cell surfaces. However, interaction with Gag also allows some cellular factors such as APOBEC3G and Trim5α to access viral replication machinery and block viral replication. In this study, we report a new HIV-1 Gag-binding factor named insulin-like growth factor II mRNA binding protein 1 (IMP1). Gag-IMP1 interaction requires the second zinc finger of the nucleocapsid (NC) domain of Gag and the KH3 and KH4 domains of IMP1. A fourfold reduction of HIV-1 infectivity was seen with overexpression of the wild-type IMP1 and its mutant that is able to interact with Gag but not with overexpression of IMP1 mutants exhibiting Gag-binding deficiency. The decreased viral infectivity was further shown as a result of diminished viral RNA packaging, abrogated Gag processing on the cellular membranes, and impeded maturation of virus particles. Together, these results demonstrate that IMP1 interacts with HIV-1 Gag protein and is able to block the formation of infectious HIV-1 particles.

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In conclusion, our study demonstrates that IMP1 interacts with HIV-1 Gag protein and is able to block the formation of infectious HIV-1 particles. This interaction inhibits HIV-1 infection by preventing virus budding and release from cell surfaces. This mechanism may provide a potential target for developing antiviral therapies.
tions into viral cDNA or by diminishing the efficiency of viral reverse transcription (12, 30, 40, 42, 43, 61, 67). Another important anti-HIV factor is Trim5α that recognizes the CA protein of the incoming HIV-1 cores immediately after virus entry and demolishes the viral reverse transcription complex (65, 64).

We have recently performed proteomic analysis to search for new cellular factors that interact with HIV-1 Gag (55). Among the candidates is the insulin-like growth factor II mRNA binding protein 1 (IMP1) that is evolutionarily conserved and regulates RNA trafficking and local translation (66). In the present study, we have characterized the interaction of IMP1 with HIV-1 Gag protein and demonstrated the specific incorporation of IMP1 into HIV-1 particles. More importantly, the results show that overexpression of the wild-type IMP1, but not IMP1 mutants that have lost the Gag-binding ability, impedes HIV-1 assembly and severely reduces the infectivity of HIV-1 virions.

**MATERIALS AND METHODS**

**Plasmids and antibodies.** The HIV-1 infectious proviral DNA clone BH10 was obtained from the National Institutes of Health AIDS Reagent and Reference Program. The pEGFP-IMP1 DNA construct was kindly provided by Jan Christiansen (47). The pVRC-3900 DNA construct was obtained from Gary J. Nabel's lab (32). The following DNA constructs were also used in the present study and were described previously: pGag-TAP DNA (55), pN-TAP DNA (55), and pVRC-3900 DNA construct. The pEGFP-IMP1 DNA construct was kindly provided by Jan Christiansen (32). The following DNA constructs were also used in the present study and were described previously: pGag-TAP DNA (55), pN-TAP DNA (55), and pVRC-3900 DNA construct.

**Purification of HIV-1 particles and subtilisin digestion assay.** The 293T cells were transfected with HIV-1 cDNA clone BH10. The culture supernatants were first centrifuged by ultracentrifugation at 3,000 rpm for 4°C for 30 min in a GR-68 Beckman centrifuge, followed by ultracentrifugation at 100,000 × g for 1 h at 4°C. Viruses were pelleted at the top of a 15%/65% sucrose step gradient and were spun at 100,000 × g for 1 h. The virus particles were recovered from the interface of the 15%/65% sucrose layers and further concentrated by ultracentrifugation at 100,000 × g for 1 h. The subtilisin digestion was performed as described previously with minor modifications (50, 51).

**Cell culture and transfection.** 293T and HeLa cells were grown in complete Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Transfection was performed with Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instructions.

**Immunoprecipitation and Western blotting.** The pFLAG-IMP1 (2 μg) and the pVRC-3900 (2 μg) DNA constructs were cotransfected into 293T cells that were seeded in 100-mm dishes. The pVRC-3900 DNA expresses HIV-1 Gag protein (32). After 48 h, cells were lysed on ice 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) for 30 min before centrifugation at 10,000 × g for 15 min at 4°C.

**Immunofluorescence staining.** HeLa cells were seeded in a four-well chamber slide (4 × 10^4 cells/well) 1 day prior to transfection with BH10 DNA. After 40 h, the cells were washed twice with 1× phosphate-buffered saline, fixed with 3.7% formaldehyde (in 1× phosphate-buffered saline) for 15 min at room temperature, and permeabilized with 0.2% Triton X-100 for 10 min. After a washing step with 1× phosphate-buffered saline, cells were incubated with mouse anti-HIV-1 p24 antibody (1:200; Fitzgerald) and rabbit anti-IMP1 antibodies (1:200, provided by Jan Christiansen) for 2 h at 37°C, followed by a 1-h incubation at 37°C with Alexa Fluor 488-conjugated anti-mouse and Alexa Fluor 546-conjugated anti-rabbit secondary antibodies (Molecular Probes). Images were recorded by using an LSM Pascal confocal microscope (Zeiss).

**Viral RNA analysis.** Levels of viral RNA associated with HIV-1 virions were measured by native Northern blotting (57). Briefly, HIV-1 particles were pelleted by ultracentrifugation and suspended in TN buffer. RNA was prepared from virus particles containing 200 ng of p24(CA) antigen by proteinase K digestion (100 μg/ml), phenol-chloroform extraction, and precipitation in 70% ethanol. After electrophoresis in 0.9% agarose gel in 1× Tris-borate-EDTA buffer at 4°C and 100 V for 3 h, RNA was transferred to Hybond N membrane and detected with α-32P-labeled HIV-1 DNA probes (HIV-1 nucleotide positions 1 to 2000 in BH10). The amounts of viral RNA were quantified by using a PhosphorImager (Storm 840; Amersham).

**Membrane flotation assay.** The transfected 293T cells were harvested and Dounce homogenized on ice in TN buffer containing 10% sucrose. After clarification in a Beckman benchtop centrifuge at 3,000 rpm for 30 min at 4°C, the cell lysates were mixed with 85% sucrose to a final concentration of 73% and loaded at the bottom of a 5-ml ultracentrifuge tube (Beckman). Two more layers of sucrose solutions (1 ml of 10% and 2.5 ml of 65%) were added before ultracentrifugation at 100,000 × g for 16 h at 4°C. Eight 625-μl fractions were collected from the top of the sucrose gradient. The samples were examined by Western blotting for HIV-1 Gag protein and the membrane marker TIR.

**Transmission electron microscopy.** After ultracentrifugation, 3,000 pellets were collected and pelleted in the GS-6R Beckman benchtop centrifuge at 10,000 rpm (approximately 1,000 × g) for 10 min at 4°C. The pellets were fixed in 2.5% glutaraldehyde for 20 min at room temperature and underwent routine processing and embedding procedures. The thin-sectioned samples were stained with dodecyl sulfate–10% polyacrylamide gels. After being transferred onto polyvinylidene difluoride membranes (Roche), the FLAG-IMP1 protein and the Gag protein were probed with anti-FLAG (Sigma) and anti-p24(HIV-1) (ID Labs, Inc.) antibodies, respectively. The protein signals were visualized by incubation with ECL reagents (Perkin-Elmer) and exposure to X-ray film (Kodak). Purification of the FLAG-TAP protein from transfected 293T cells was performed using immobilized G (IgG)-Sepharose and calmodulin-Sepharose (Amersham) as previously described (55).

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lead citrate and uranyl acetate and examined with a JEOL JEM-2000 FX transmission electron microscope equipped with a Gatan 792 Bioscan 1,024-by-1,024-byte wide-angle multiscan charge-coupled device camera.

RESULTS

IMP1 associates with HIV-1 Gag and is packaged into virus particles. We first tested whether IMP1 interacts with HIV-1 Gag in cells. The Gag-TAP DNA construct was used in the following interaction studies (55). This plasmid DNA expresses HIV-1 Gag protein that has two tags attached at its C terminus, namely, the calmodulin-binding domain and the protein A motif, which permit efficient isolation of Gag protein from cell lysates to a high purity (Fig. 1A) (55). We transfected the Gag-TAP DNA into 293T cells and purified Gag protein by using IgG-Sepharose and calmodulin-Sepharose as previously described (55). Presence of the IMP1 protein in the immunoprecipitated Gag complex was assessed by Western blotting with anti-IMP1 antibody. The copurified IMP1 protein was detected by Western blotting with anti-IMP1 antibody. The pN-TAP DNA expresses the TAP protein only and is used in the transfection and purification experiments as a control (55). The lysates of 293T cells were tested in the Western blot as a control to indicate the size of endogenous IMP1.

FIG. 1. The NC sequence is important for Gag to bind IMP1. (A) Domain structures of Gag-TAP protein and Gag mutants. The amino acid number refers to the first residue of Gag. The zinc finger motifs in NC are highlighted by filled boxes. CBD, calmodulin-binding domain; TEV, tobacco etch virus protease cleavage site. (B) Gag interacts with IMP1. Gag protein was purified by using IgG-Sepharose and calmodulin-Sepharose as previously described (55). Levels of Gag and its mutants were assessed by Western blotting with anti-p24 antibody. The copurified IMP1 protein was detected by Western blotting with anti-IMP1 antibody. The pN-TAP DNA expresses the TAP protein only and is used in the transfection and purification experiments as a control (55). The lysates of 293T cells were tested in the Western blot as a control to indicate the size of endogenous IMP1.

VOL. 82, 2008 ROLE OF IMP1 IN HIV-1 REPLICATION 5685

FIG. 2. The KH3 and KH4 domains are essential for IMP1 to interact with Gag. (A) Illustration of the IMP1 domain structure. The FLAG tag is attached to the N terminus of IMP1 and its mutants. (B) IMP1 binds to Gag. The FLAG-IMP1 DNA or its mutants were cotransfected with the pVRC-3900 DNA into 293T cells. The FLAG-IMP1 protein and its mutants were immunoprecipitated with anti-FLAG antibody, and the precipitates were assessed by Western blotting with anti-FLAG or anti-p24 antibodies. Expression of FLAG-IMP1 protein and Gag protein from pVRC-3900 DNA in cells was also determined by Western blotting with anti-FLAG and anti-p24 antibodies. The levels of β-actin were measured as the internal control.
IMP1 bears two copies of RRM (for RNA recognition motif) and four copies of KH (for hnRNP K homology) domains (Fig. 2A) (46). To determine which domains are involved in IMP1-Gag interaction, we generated three IMP1 mutants—ΔKH(3-4), ΔKH(1-4), and ΔRRM(1-2)—that lack the KH3 and KH4 domains, the KH1 to KH4 domains, and the RRM1 and RRM2 domains, respectively (Fig. 2A). As opposed to the efficient coimmunoprecipitation of Gag by the ΔRRM(1-2) mutant, the ΔKH(3-4) mutant exhibited a residual level of Gag-binding activity, whereas the ΔKH(1-4) mutant lost the interaction with Gag (Fig. 2B). Thus, the KH3 and KH4 domains are essential for IMP1 to interact with Gag.

We next studied whether Gag and IMP1 colocalize in cells. Accordingly, HeLa cells were transfected with the BH10 DNA, followed by immunofluorescence staining for HIV-1 Gag protein with mouse anti-p24 antibody and for the endogenous IMP1 protein with rabbit anti-IMP1 antibody. The results of confocal microscopy revealed significant overlapping of the fluorescence signals for Gag (Red) and IMP1 (Green), especially at the cell edges (Fig. 3). This observation, together with the immunoprecipitation data in Fig. 1 and 2, supports the specific association of the IMP1 protein with HIV-1 particles.

Interaction of IMP1 with Gag prompted us to investigate whether IMP1 is incorporated into HIV-1 particles. Since extracellular microvesicles are often found to pellet together with HIV-1 particles and thus contaminate virus preparation (6), we used the BH10-LZ mutant virus as a control. This HIV-1 mutant has the NC domain of Gag replaced with the leucine zipper sequence from the yeast transcription factor GCN4 (28). The TAP tag was attached to the C termini of Gag and Gag(LZ) in the context of HIV-1 proviral DNA BH10-PR− (protease negative). After affinity purification using IgG-Sepharose and calmodulin-Sepharose, the Gag and Gag(LZ) precipitates were assessed by Western blotting with either anti-p24 antibody or anti-IMP1 antibody. The pN-TAP DNA was used in the experiments as a control. (B) IMP1 was detected in HIV-1 particles. The BH10-PR− and BH10-LZ virus particles were purified by ultracentrifugation through a 15%/65% sucrose step gradient. The amount of virus particles was assessed by Western blotting with anti-p24 antibody. Virion-associated IMP1 protein was detected by Western blotting with anti-IMP1 antibody. (C) Subtilisin treatment of virus samples. The protease-negative HIV-1 particles were purified as described above and subjected to subtilisin digestion. After ultracentrifugation, the pelleted viruses were assessed in Western blot with antibodies to either IMP1, HIV-1 p24, or viral gp120.

We further investigated the association of IMP1 with HIV-1 particles by treating the BH10-PR− or the BH10-LZ DNA into 293T cells and purified virus particles by ultracentrifugation through a 15%/65% sucrose step gradient. The amounts of virus particles were first measured by Western blotting with anti-p24 antibody, and then the BH10-PR− and the BH10-LZ viruses containing the same amount of Gag were tested by Western blotting for the presence of IMP1 protein using anti-IMP1 antibody. The results showed substantially higher levels of IMP1 protein in the BH10-PR− virus sample than in the BH10-LZ virus sample (Fig. 4B), suggesting a specific enrichment of IMP1 in HIV-1 particles formed by the wild-type Gag. The residual amount of IMP1 protein seen in the BH10-LZ virus sample may result from a possible contamination from the extracellular microvesicles or a weak interaction of IMP1 with the mutated Gag(LZ) protein.

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Exogenously expressed FLAG-IMP1 protein severely diminishes HIV-1 production. One major function of Gag protein is to drive the assembly of HIV-1 particles. We thus sought to
determine whether, by association with Gag, IMP1 exerts any effect on the production of HIV-1 particles. To this end, we transfected different amounts of the FLAG-IMP1 DNA (0.2, 0.5, and 1 μg), together with the HIV-1 proviral DNA clone BH10 (0.2 μg), into 293T cells and assessed the levels of viral Gag protein in cells and in the culture fluids by Western blotting and enzyme-linked immunosorbent assay (ELISA). The results showed that production of HIV-1 particles was markedly inhibited by expression of the FLAG-IMP1 protein, with the highest reduction detected when 1 μg of the FLAG-IMP1 DNA was used for cotransfection (data not shown). We next performed detailed analyses to assess the effects of the FLAG-IMP1 protein and its mutants on HIV-1 production by using 1 μg of these DNA constructs in the transfection experiments. The data are summarized in Fig. 5.

Results of Western blot showed that expression of the FLAG-IMP1 protein did not markedly affect the total levels of Gag protein in cells (Fig. 5A, lanes 1 and 2). Interestingly, a marked accumulation of the p40 protein, an intermediate product of Gag processing, was observed (Fig. 5A, lane 2). This suggests a negative effect of FLAG-IMP1 expression on Gag cleavage. We next pelleted virus particles from the culture supernatant and determined the levels of virion p24(CA) and virion RT by HIV-1 p24 ELISA and Western blotting. The results showed that expression of the FLAG-IMP1 protein led to a fivefold reduction of virion p24(CA) and a tenfold decrease of virion RT proteins (Fig. 5A and B), indicating a significant suppression of virus production. A greater degree of reduction in virion RT than virion p24(CA) suggests a relatively low level of Gag-Pol incorporation into virus particles as a result of FLAG-IMP1 overexpression.

We next assessed the effects of the ΔRRM(1-2), ΔKH(1-4), and ΔKH(3-4) IMP1 mutants on HIV-1 production with the aim to define which domains of IMP1 protein are important for inhibiting HIV-1 production. None of the three IMP1 mutants affected Gag expression to a significant extent (Fig. 5A). The amounts of viruses in supernatants were moderately decreased with expression of the ΔRRM(1-2) or the ΔKH(3-4) protein and were modestly increased with ΔKH(1-4) expression (Fig. 5A and B). Therefore, IMP1 needs both the two RRM domains and the four KH domains to effectively suppress HIV-1 production.

Expression of the FLAG-IMP1 protein decreases the infectivity of HIV-1 virions and inhibits viral RNA packaging. We next measured the infectivity of HIV-1 virions that were produced from 293T cells that had been transfected with the BH10 DNA and the wild-type FLAG-IMP1 or its mutated DNA. To this end, an amount of HIV-1 equivalent to 5 ng of p24 antigen was used to infect TZM-bl indicator cells that express the CD4 receptor and are stably transfected with the HIV-1 LTR-Luc reporter DNA. Cells were collected 40 h after infection, and the luciferase activity was measured. The results showed that expression of FLAG-IMP1 or the ΔRRM(1-2) mutant reduced virus infectivity by as much as fourfold, whereas the ΔKH(1-4) and ΔKH(3-4) mutants did not exert any effect in this regard (Fig. 5C). It is noteworthy that the effects of FLAG-IMP1 and its mutants on HIV-1 infectivity correlate with their abilities to interact with viral Gag protein, i.e., both the FLAG-IMP1 and the ΔRRM(1-2) proteins efficiently associated with Gag, as opposed to the ΔKH(1-4) and the ΔKH(3-4) mutants that were Gag binding deficient (Fig. 2; summarized in Table 1).

To identify the defect causing the decreased viral infectivity, we prepared RNA from virus particles that were equivalent to 200 ng of p24 antigen and assessed the levels of viral RNA and their dimerization status by native Northern blotting. The results showed that viral RNA was detected as dimers in both the control virus sample and the virus samples obtained with expression of FLAG-IMP1 or its mutants (Fig. 5D). By incubating viral RNA at gradually increasing temperatures, we sought to determine the temperature at which viral RNA dimers dissociate, which indicates the thermostability of the dimeric RNA molecules. The results in Fig. 5E show that expression of the FLAG-IMP1 protein evidently did not change the melting temperature of viral RNA dimers, which was measured to the 50 to 55°C range. These data suggest that FLAG-IMP1 expression does not affect the dimerization of HIV-1 RNA. However, expression of FLAG-IMP1 or the ΔRRM(1-2) mutant led to a decrease in the levels of virion-associated viral RNA, as opposed to the negligible effects from expression of the ΔKH(1-4) and ΔKH(3-4) mutants (Fig. 5D). These reductions were consistently seen in three independent Northern blots with viruses harvested from three different transfection experiments. Together, the data suggest that the expression of the FLAG-IMP1 protein or the ΔRRM(1-2) mutant inhibits viral RNA packaging, a defect that, at least partially, correlates with the decreased viral infectivity. However, considering the 20 to 40% reduction in viral RNA packaging, the fourfold decrease of viral infectivity should be attributed to some major defects yet to be identified.

Expression of the FLAG-IMP1 protein impedes the assembly and processing of Gag protein on cellular membranes. Since Gag protein produces virus particles on cellular membranes (2, 26), we were thus interested in whether the expression of the FLAG-IMP1 protein inhibits HIV-1 production by blocking the association of Gag with membranes. Toward this end, cellular membranes and membrane-associated proteins were isolated by the membrane flotation assay in which cell lysates were loaded as the 73% sucrose fraction of the 15%/65%/73% sucrose step gradient and centrifuged at 100,000 × g for 16 h. Membranes migrated to the interface of the 15 and 65% sucrose layers as shown by the location of membrane marker TIR in the sucrose gradient (Fig. 6A). In the control experiment performed with the BH10 DNA alone, 42% of the total Gag protein was recovered as membrane bound (Fig. 6). Expression of the FLAG-IMP1 protein increased the percentage of membrane-bound Gag to 61%. Expression of the ΔRRM(1-2), ΔKH(1-4), and ΔKH(3-4) IMP1 mutants also led to higher levels of membrane-bound Gag, albeit not as significant as that observed for the wild-type FLAG-IMP1 protein (Fig. 6). This increase in membrane-bound Gag either reflects an elevated efficiency of Gag-membrane binding or is a result of delayed release of virus particles from cell surfaces.

More interestingly, the results of the membrane flotation experiments revealed an adverse effect of FLAG-IMP1 expression on Gag processing (Fig. 6). When only the HIV-1 proviral DNA clone BH10 was used in transfection, the mature p24(CA) protein of Gag was mainly seen in membrane fractions 2 and 3 with a limited amount detected in cytosol frac-
FIG. 5. Effects of IMP1 overexpression on HIV-1 production and virus infectivity. (A) The HIV-1 proviral DNA clone BH10 was cotransfected with the wild-type FLAG-IMP1 DNA or the IMP1 mutated DNA into 293T cells. Expression of FLAG-IMP1 and its mutants was determined by Western blotting with anti-FLAG antibody. The levels of Gag protein in cells and in virus particles were assessed by Western blotting with anti-p24 antibody. The amounts of RT proteins in virus particles were also measured by Western blotting with anti-RT(HIV-1) antibody. (B) The amounts of virion p24(CA) were determined by p24 ELISA. The results are summarized in the bar graph, with the amount in the control experiment arbitrarily set as 100. The levels of virion RT products were quantified from the Western blot results by using ImageJ software. The data shown are averages of three independent transfection experiments. *, P < 0.05. (C) Infectivity of HIV-1 virions was measured by infecting TZM-bl indicator cells with 5 ng of p24 of viruses. The results shown represent those from three independent infections using viruses from three different transfection experiments. *, P < 0.05. (D) Native Northern blot to evaluate viral RNA dimerization and level of virion-associated viral RNA. The bar graph shows the average of viral RNA amounts from three independent native Northern blot assays. *, P < 0.05. (E) The stability of viral RNA dimers was measured by incubating viral RNA samples at 40, 45, 50, or 55°C for 10 min in a buffer containing 150 mM NaCl prior to native Northern blot analyses.
Virus production assuming equal variances). FLAG-IMP1 and BH10 DNA (Fig. 7, BH10 seen in cell samples that had been cotransfected with an electron-dense layer under the virus envelope were detected mainly as mature p24(CA) and p51/p66, respectively, in viruses that were collected from culture supernatants (Fig. 5A). This indicates that viruses did become mature, albeit at a reduced rate. The delayed virus maturation is likely a result of less incorporation of Gag-Pol protein into viruses, since the reduction in virion RT products (p51/p66) is more prominent than virion p24(CA) protein under the overexpression of IMP1 or the ΔRRM(1-2) mutant (Fig. 5A and B). Alternatively, the immature morphology of the virus particles seen with the IMP1 overexpression could also result from the deleterious effect of IMP1 on the core protein rearrangement with a consequence of impaired formation of a mature core structure.

Inhibition of HIV-1 infection by IMP1 depends on its ability to interact with viral Gag protein. This conclusion is not merely based on the evidence that IMP1 and Gag proteins interact with each other but is also supported by the results from studying a panel of IMP1 mutants. We generated the ΔRRM(1-2), ΔKH(1-4), and ΔKH(3-4) mutants and assessed their abilities to interact with Gag and to suppress HIV-1 infectivity as well (Table 1). For instance, the ΔKH(1-4) proteins leads to the accumulation of immature virus particles on cell surfaces. This implicates a defect in virus maturation and accounts, probably to a large extent, for the decreased viral infectivity.

**DISCUSSION**

Our results show that overexpression of IMP1 interferes with HIV-1 assembly by inhibiting viral RNA packaging, blocking Gag cleavage, and impeding virus maturation. Of these defects, the extent of the reduction in virion-associated viral RNA is moderate compared to the abrogated Gag processing on cellular membranes and the impeded virus maturation. It is noted that virus maturation was not completely blocked by FLAG-IMP1 expression because Gag and RT proteins were detected mainly as mature p24(CA) and p51/p66, respectively, in viruses that were collected from culture supernatants (Fig. 5A). This indicates that viruses did become mature, albeit at a reduced rate. The delayed virus maturation is likely a result of less incorporation of Gag-Pol protein into viruses, since the reduction in virion RT products (p51/p66) is more prominent than virion p24(CA) protein under the overexpression of IMP1 or the ΔRRM(1-2) mutant (Fig. 5A and B). Alternatively, the immature morphology of the virus particles seen with the IMP1 overexpression could also result from the deleterious effect of IMP1 on the core protein rearrangement with a consequence of impaired formation of a mature core structure.

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**TABLE 1. Effects of IMP1 overexpression on HIV-1 assembly and infectivity**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>FLAG-IMP1</th>
<th>FLAG-ΔRRM(1-2)</th>
<th>FLAG-ΔKH(1-4)</th>
<th>FLAG-ΔKH(3-4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction with Gag</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
<td>Virus production</td>
<td>100</td>
<td>17.5 ± 2.9*</td>
<td>65.25 ± 5.3</td>
<td>128.25 ± 22.7</td>
<td>63.7 ± 10.7</td>
</tr>
<tr>
<td>Vial infectivity</td>
<td>100</td>
<td>26.6 ± 3.4*</td>
<td>26.8 ± 1.6*</td>
<td>121.9 ± 19.0</td>
<td>99.3 ± 24.0</td>
</tr>
<tr>
<td>Viral RNA packaging</td>
<td>100</td>
<td>56.5 ± 5.8*</td>
<td>77.6 ± 7.9*</td>
<td>111.9 ± 9.6</td>
<td>109.4 ± 12.3</td>
</tr>
<tr>
<td>Gag-membrane binding</td>
<td>41.8 ± 0.33</td>
<td>61.0 ± 1.7*</td>
<td>49.3 ± 4.8</td>
<td>50.2 ± 0.63</td>
<td>51.6 ± 4.1</td>
</tr>
<tr>
<td>Gag processing on membrane</td>
<td>47.7 ± 13.3</td>
<td>1.5 ± 0.19*</td>
<td>6.6 ± 3.1*</td>
<td>39.2 ± 11.3</td>
<td>30.6 ± 5.4</td>
</tr>
<tr>
<td>Virus morphology</td>
<td>Mature</td>
<td>Immature</td>
<td>Immature</td>
<td>Mature</td>
<td>Mature</td>
</tr>
</tbody>
</table>

* The consistent impacts from expression of the FLAG-IMP1 and the FLAG-ΔRRM(1-2) proteins on viral infectivity, viral RNA packaging, Gag processing, and virus morphology are indicated in boldface.

Values are means ± the SEM except as noted. Asterisks denote statistical significance (P < 0.05), which was calculated by using a two-sample t test (two-tailed, assuming equal variances).

The results refer to the data shown in Fig. 2.

The results refer to the HIV-1 p24 ELISA data shown in Fig. 5B.

The results refer to the data shown in Fig. 2.

The results refer to the data shown in Fig. 5C.

Refer to Fig. 5D.

Refer to Fig. 6B.

Refer to Fig. 7.
aging, Gag processing, and virus maturation, all of which correlate well with their abilities to bind Gag and to diminish the infectivity of HIV-1 virions (Table 1). One exception though is that the RRM(1-2) mutant binds to Gag as efficiently as the wild-type IMP1 and yet inhibits HIV-1 production only to a moderate extent. One implication of this result is that IMP1 requires both the RRM and the KH domains to maximally suppress the production of HIV-1 particles.

It is interesting that IMP1 overexpression leads to a decrease in virus production and accumulation of immature virus particles on the cell surface, a phenotype that is reminiscent of defective late-domain function. This suggests that IMP1 may impede HIV-1 production by interfering with the function of viral late domain. HIV-1 late domain consists of two motifs: the PTAP motif and the LXXLF motif (19). The PTAP motif is located at the N terminus of the p6 region within Gag and is bound by the TSG101 protein (25, 27, 31). The LXXLF motif resides at the C terminus of p6 and is recognized by the ALIX/AIP1 protein (20, 62). Since IMP1 associates with the C-terminal region of NC that is adjacent to p6, an excessive amount of IMP1 protein may interfere with the binding of TSG101 and LXXLF to the p6 domain and thereby blocks recruiting the ESCRTs to the virus assembly site. Alternatively, the overexpressed IMP1 protein may have negatively modulated the function of the ESCRTs themselves. It is also noted that the inhibitory effect of IMP1 on HIV-1 production has been observed with the overexpressed IMP1 protein. The physiological role of IMP1 in HIV-1 replication, including the virus assembly step, requires further investigation.

A simple mechanism underlying the adverse effect of IMP1 overexpression on HIV-1 RNA packaging involves a possible binding of IMP1 to viral RNA. IMP1 is a member of the VICKZ (for Vg1RBP/Vera, IMP-1,2,3, CRD-BP, KOC, ZBP-1) protein family that comprise Vg1RBP/Vera in *Drosophila* and *Xenopus*; ZBP-1 in chickens; CRD-BP in mice; and IMP1, IMP2, and IMP3 in humans (66). The VICKZ proteins are highly conserved between species, all bear two copies of RRM domains and four copies of KH domains. IMPs in humans were

![FIG. 6. Effects of IMP1 overexpression on Gag-membrane association and Gag processing. (A) The FLAG-IMP1 DNA or its mutants was cotransfected with the BH10 DNA into 293T cells. Membrane-associated Gag protein was isolated by membrane flotation assay and detected by Western blotting with anti-p24 antibodies. Membrane fractions 2 and 3 were indicated by the membrane marker TfR. (B) The levels of membrane-bound Gag protein (including pr55, p40, and p24) were quantified from the Western blot data by using ImageJ software and are expressed as the percentage of the total Gag protein in all eight fractions. The amounts of mature p24(CA) protein in the membrane fractions (2 and 3) were also determined and are expressed as the percentage of the total membrane-associated Gag protein (including pr55, p40, and p24). The results shown are the average of three independent flotation experiments. *P < 0.05.*](http://jvi.asm.org/)

![FIG. 7. Effect of IMP1 overexpression on the morphology of HIV-1 particles. 293T cells were either transiently transfected with the BH10 DNA alone or together with the FLAG-IMP1 DNA or its mutants. Cells were examined by electron microscopy. Two images are shown for the IMP1 overexpression experiment. Scale bar, 200 μm.](http://jvi.asm.org/)
originally discovered because of their ability to bind the 5’ leader region of the insulin-like growth factor II mRNA and to regulate the translation of insulin-like growth factor II (48). The RNA targets currently known for IMP1 also include c-leader region of the insulin-like growth factor II mRNA and to originally discovered because of their ability to bind the 5’-CAAAAAAA AAAUUU-3’ RNA sequence (52). Notably, the 5’ untranslated region of HIV-1 RNA bears the 5’-CGCCAAAAAAUUU U-3’ sequence that resembles the core element of the IMP1 binding site in the PABP mRNA. Thus, IMP1 may associate with HIV-1 RNA by recognizing this 5’-CGCCAAAAAAUUU U-3’ RNA sequence. Since the 5’-CGCCAAAAAAUUU U-3’ sequence is located between the SL2 and SL3 RNA structures that represent the major signals for HIV-1 RNA packaging (3, 5, 16), binding of IMP1 to this short RNA fragment may either change the folding of SL2 and SL3 or simply interfere with recognition of these RNA packaging signals by viral Gag protein.

IMP1 together with the other members of its family belong to a large group of proteins known as zipcode-binding proteins. These proteins recognize cis-acting RNA elements named “zipcodes” and regulate the transport, subcellular distribution, and local translation of mRNA (33). In addition to IMP1, another zipcode-binding protein called Staufen 1 has also been reported to interact with HIV-1 Gag and is packaged into virus particles (11, 44). The results of recent proteomic studies suggest that IMP1 and Staufen 1 form discrete RNA complexes (21, 34). This finding reflects the different nature of RNA-binding domains that are harbored by these two proteins. IMP1 bears the RRM and KH domains that mainly recognize single-stranded RNA, whereas Staufen 1 contains five copies of double-stranded RNA-binding domains. It is plausible that IMP1 and Staufen 1 recognize different RNA targets and assemble into discrete RNA granules. This possibility underlies the different effects of IMP1 and Staufen 1 on the production of HIV-1 particles. In contrast to the adverse effect of IMP1 overexpression on HIV-1 production seen in the present study, increasing the level of Staufen 1 augments production of HIV-1 particles (11). We propose that, by associating with Gag, IMP1 and Staufen 1 recruit distinct sets of cellular factors to the viral assembly machinery and thus modulate virus production with opposite consequences.

We have tried to downregulate the level of endogenous IMP1 protein using the small interfering RNA method and assessed the effects of this treatment on HIV-1 production and viral infectivity. Two small interfering RNA oligonucleotides were designed to target the IMP1 mRNA (NM.006546) at nucleotide positions 1654 to 1672 and positions 1930 to 1948, respectively. Both oligonucleotides decreased IMP1 expression by more than 70%, but neither led to a measurable effect on the production of HIV-1 particles and the infectivity of HIV-1 virions (data not shown). We interpret this negligible effect of IMP1 knockdown as a result of the existence of two homologues of IMP1: IMP2 and IMP3 (48). These three IMPs share 59% sequence identity and are highly homologous in the four KH domains which are essential for the proteins’ function (48). It is likely that IMP2 and IMP3 are able to compensate for the loss of IMP1 by inhibiting HIV-1 infection.

In summary, our results demonstrate that IMP1 represents a new Gag-binding protein and is packaged into HIV-1 particles. IMP1 has the ability to interfere with HIV-1 assembly and exhibits potent anti-HIV activity when it is overexpressed.

ACKNOWLEDGMENTS

We thank Gary J. Nabel and Jan Christiansen for providing DNA constructs and antibodies. We also thank Susan P. Colby-Germaino for careful reading of the manuscript.

This research was supported by grants from the Canadian Institutes of Health Research.

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