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**Nuclear and Cytoplasmic Effects of Human CRM1 on HIV-1 Production in Rat
Cells**

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Abstract

The human immunodeficiency virus type 1 (HIV-1) regulatory protein, Rev, mediates the nuclear export of unspliced *gag* and singly-spliced *env* mRNAs by bridging viral RNA and the export receptor, CRM1. Recently, rat CRM1 was found to be less efficient than human CRM1 in supporting Rev function in rats. In this study, to understand the role of CRM1 in HIV propagation, the mechanism underlying the function of human and rat CRM1 in HIV-1 replication was investigated in rat cells. The production of viral particles, represented by the p24 Gag protein, was greatly enhanced by hCRM1 expression in rat cells; however, this effect was not simply due to the enhanced export of *gag* mRNA. The translation initiation rate of *gag* mRNA was not increased, nor was the Gag protein stabilized in the presence of hCRM1. However, the processing of the p55 Gag precursor and the release of viral particles were facilitated. These results indicated that hCRM1 exports *gag* mRNA to the cytoplasm, not only more efficiently than rCRM1, but also correctly, leading to efficient processing of Gag proteins and particle formation.

Introduction

Appropriate animal models for viral infection allow the analysis of viral pathogenesis and oncogenesis, which in turn assist the development of therapeutic and preventative measures. Current animal models of HIV-1 (human immunodeficiency virus-1) disease have used non-human primates (Giuffre *et al.* 2003; Hazuda *et al.* 2004; Hu 2005; Veazey *et al.* 2005) and severe combined immunodeficiency (SCID) mice with transplanted fetal human tissues (Shultz *et al.* 2007; Watanabe *et al.* 2007). These models have made significant contributions to our understanding of lentiviral pathogenesis and have assisted in the development of therapeutic strategies. However, these models have significant shortcomings, such as the limited availability and high cost of nonhuman primates, the absence of or delay in disease progression, permissivity only for related retroviruses, and insufficient viral propagation. For these reasons, new animal models are needed. If viral infection is possible, it would be convenient to use small animal models, in particular mice and rats, since their inbred strains are well characterized and can be genetically manipulated.

Studies on rodent cell-specific defects in the HIV-1 life cycle have facilitated

the identification and characterization of the host cell gene products that are essential for viral replication, and these may provide a molecular basis for generating a fully permissive small-animal model. It has been suggested that the major block to HIV-1 replication is at the level of entry and that this hurdle could be overcome by introducing human CD4 and CCR5, which serve as receptors for HIV-1 (Keppler *et al.* 2001; Keppler *et al.* 2002). Transgenic (Tg) rats, expressing human CD4 and CCR5, have been reported to support some replication of HIV-1, albeit very poorly (Keppler *et al.* 2002; Goffinet *et al.* 2007). In contrast, Tg mice expressing human CD4/CCR5 do not allow the propagation of HIV-1 (Browning *et al.* 1997). These results support the rat as a promising small-animal model candidate.

Analyses have revealed novel interactions between rodent and viral factors. A notable example is CRM1 (chromosome region maintenance 1; exportin 1), a member of the karyopherin family of nucleocytoplasmic-transport factors (Neville *et al.* 1997; Cullen 2003) that is involved in nuclear export. In the nucleus, CRM1 binds its cargo in the presence of a GTP-bound form of the Ran GTPase (RanGTP), and following nuclear export, hydrolysis of GTP to GDP causes a conformational shift that

induces cytoplasmic cargo release, providing directionality for this export pathway (Fornerod *et al.* 1997; Neville *et al.* 1997; Nakielny & Dreyfuss 1999; Cullen 2003).

Human CRM1 cooperates with HIV-1 Rev and the human T cell leukemia virus type 1 (HTLV-1) Rex proteins to transport 4 kb of partially-spliced and 9 kb of unspliced mRNAs encoding viral structural proteins and genomes (Cullen 1998). Rev or Rex multimerizes on viral RNA to recruit several CRM1 molecules; furthermore, the recruitment of CRM1 enhances Rev or Rex interactions vice versa (Hakata *et al.* 2002).

Rat CRM1, which exhibits 98% amino acid identity to its human counterpart, is able to bind efficiently to Rex, but cannot induce the multimerization of Rex proteins on its cognate RNA. This results in the breakdown of HTLV-1 RNA transport in rat cells (Hakata *et al.* 2001). Thus, rat CRM1 represents a major factor in the human-rat species barrier to HTLV-1 (Zhang *et al.* 2006; Takayanagi *et al.* 2007). Further detailed comparisons of human and rat CRM1 proteins identified the domains essential for the induction of Rex multimerization and the binding of RanBP3, another component involved in RNA export (Hakata *et al.* 2003). These analyses suggested that CRM1 functions not only in RNA export out of the nucleus, but also in the formation of the

transport complex that includes viral RNA and various cellular components (Hakata *et al.* 2003).

In contrast to the definitive results obtained for rat CRM1-Rex interactions, the existence of a profound block in Rev function in rodent cells remains controversial, although a reduced level of the 9 kb transcript has been generally reported (Bieniasz & Cullen 2000; Keppler *et al.* 2001). Some studies have found impaired Rev activity (Marques *et al.* 2003), whereas others have ascribed the reduced transcript level to oversplicing or to the reduced stability of unspliced transcripts in rodent cells compared to human cells (Malim *et al.* 1991), a problem that is corrected by the expression of the human p32 protein (Zheng *et al.* 2003). These contradictory results seem to stem partly from the use of different cells and reporters for evaluating Rev activity and partly from the limited investigation of cellular cofactors. However, recently, we found that Gag production was increased by hCRM1 in rat macrophages in contrast to its marginal effect in rat T cells (Okada *et al.* 2009).

In this study, we have compared the molecular mechanisms underlying the function of human and rat CRM1 proteins in HIV propagation in rat cells. We show that

the expression of hCRM1 in rat cells not only enhances the export of HIV *gag* mRNA, but also restores the processing of the precursor Gag protein and release of viral particles, leading to efficient virus production.

Results

Enhanced p24 production in hCRM1-transfected rat cells

Recently, we found that hCRM1 enhanced Gag production in rat macrophages (Okada *et al.* 2009); therefore, it was decided to examine the molecular mechanism involved in this process using convenient rat cell lines. ER1/neo1 cells are particularly useful because they, like rat macrophages, support the efficient expression of HIV-1 genes without the need for human cyclin T1 (unpublished results; [Okada *et al.* 2009]). The initial step involved transfecting the hCRM1-expressing plasmid into ER1/neo1 cells along with the reporter pCRRE (Kimura *et al.* 1996), which harbors the HIV-1 genome, including the intact *gag* and *rev* sequences (Fig. 1A). Next, the production of the Gag protein, p24, in the medium was measured by ELISA. In addition, cells were simultaneously co-transfected with pCDM β -gal, which was used to normalize the transfection efficiency, and pH1-luc, which acted as a surrogate marker for total gene expression driven by the HIV-1 LTR. In comparison with untransfected rat cells, hCRM1-transfected cells produced increasing levels of p24 in the culture supernatant with increasing amounts of pSR α hCRM1. On the other hand, β -gal and luc activities

remained at similar levels in both hCRM1-transfected and untransfected rat cells, indicating similar transfection efficiencies and HIV LTR-mediated transcription, irrespective of hCRM1 expression (Fig. 1B). These results demonstrated the enhancement of p24 production following transfection with hCRM1.

Next, we compared the effect of hCRM1 and rCRM1 in HeLa and ER1/neo1 cells by measuring p24 production. Following transfection of either of the CRM1-expressing plasmids into HeLa cells, a small enhancement of p24 production was observed, although the differences between rat and human CRM1 were not significant (Fig. 1C). In addition, the same results were obtained in other human cell lines, including 293T and HOS cells (data not shown). In contrast, p24 production increased markedly in ER1/neo1 cells following transfection with the hCRM1-expressing plasmid, whereas expression of rCRM1 had no detectable effect (Fig. 1C). As expected, no significant changes in β -gal or luc activities were observed following transfection with human and rat CRM1-expressing plasmids (data not shown). In addition, hCRM1 enhanced expression of Gag in other rat cell lines (REF52, W31, FPM-SV, and NR8383 cells [Okada *et al.* 2009]), whereas rCRM1 did not (data not

shown). These results suggested that rCRM1 is a poor cofactor for Rev.

Effect of hCRM1 on the export of *gag* mRNA

The above results indicated that hCRM1 enhanced p24 production in rat cells, thus the next step was to examine the mechanism underlying this phenomenon. It is conceivable that hCRM1 may export *gag* mRNA more efficiently than rCRM1. To investigate this possibility, hCRM1- or mock-transfected cells were separated into their nuclear and cytoplasmic fractions, and *gag* mRNA levels in the cytoplasmic fractions were compared using quantitative RT-PCR. To confirm augmented levels of Gag protein in these hCRM1-transfected cells, the medium was collected and subjected to a p24 ELISA. In the cytoplasm of hCRM1-transfected rat cells, the increase in *gag* mRNA levels was two to three times that of the mock-transfectants. A similar increase in *gag* mRNA was also observed in the nuclear fraction in the presence of hCRM1 (data not shown). On the other hand, hCRM1 expression had no effect on *tat* mRNA levels, since CRM1 is not involved in that particular export pathway (Fig. 1D and E). No signals were detected in the samples that had not been subjected to a reverse

transcriptase (RT) reaction (data not shown). Efficient separation of the cytosolic and nuclear fractions was confirmed by amplification of *β-actin* pre-mRNA, which was detected exclusively in the nuclear fraction (data not shown). In contrast to the small increase in *gag* mRNA levels in the cytoplasm, at its peak, the production of p24 from hCRM1-transfected rat cells was nine times greater than in the mock-transfectants (Fig. 1E). p24 Gag production and the efficiency of *gag* mRNA export was decreased by transfection of 0.6μg hCRM1 expression plasmids (Fig. 1D and E). Too much hCRM1 may affect nuclear export adversely due to imbalance with other factors. These results revealed that hCRM1 modestly enhanced *gag* mRNA export in rat cells but greatly increased Gag protein production, suggesting that hCRM1 may function in the cytoplasm.

Stability of Gag protein

Next, we examined whether hCRM1 has an effect on Gag protein stability. A [³⁵S]-methionine/cysteine pulse-chase analysis of the Gag protein was performed in rat cells transfected with pCRRE. An inhibitor against HIV-1 protease was added to

prevent processing of the Gag precursor, which would hamper the assessment of Gag stability. Since Gag proteins are released as viral particles into the medium, the total amount of Gag protein in both the cell and the medium should be quantified to assess its stability. Thus, the total cell culture including both cell lysates and medium was dissolved with the detergent solution and then immunoprecipitated with an anti-p24 monoclonal antibody (mAb), followed by SDS-PAGE and autoradiography. The Gag protein was highly stable, irrespective of the presence or absence of hCRM1 (Fig. 1F). The stability of the Gag protein was ascertained using another plasmid, pNLmyr(-)pro(-)Flag, which exclusively expresses intracellular, nonmyristoylated p55 Gag due to a G2A mutation at the 5' region of the *gag* gene (Kawada *et al.* 2008) (Fig. 1G). These results excluded the involvement of hCRM1 in Gag protein stability.

The small increase of intensity of p55 Gag bands in the presence of hCRM1 (Fig. 1F and G) corresponded to the enhancement of gag mRNA in the cytoplasm (Fig. 1D and E), suggesting that the efficiency of gag mRNA translation was not elevated by hCRM1 expression. It is consistent with the results of polyribosome analysis that indicated a constant rate of translation initiation irrespective of hCRM1 expression

(Supplementary Materials Fig. S1).

Effect of hCRM1 on the export of *gag* mRNA and p24 production using pCMV Δ R8.2

Next, the effect of hCRM1 was examined further using the plasmid, pCMV Δ R8.2, which generates higher levels of Gag protein in the medium of ER1/neo1 cells, compared to other reporters, such as pCRRE. The results showed that pCMV Δ R8.2 synthesized Gag at 20- to 30-fold higher levels in the presence of hCRM1 than in its absence (Fig. 2A). As with the other reporters, a slight increase in cytoplasmic *gag* mRNA levels was observed in the presence of hCRM1 (Fig. 2B), while *tat* and *β -gal* mRNA levels were not affected (Fig. 2C and D). A similar increase in *gag* mRNA was also observed in the nuclear fraction in the presence of hCRM1 (data not shown). Efficient separation of the cytosolic and nuclear fractions was confirmed by the amplification of *β -actin* pre-mRNA, which was detected exclusively in the nuclear fraction (Fig. 2E), and *β -galactosidase* (as a cytoplasmic marker), of which approximately 95% was detected in the cytoplasmic fraction (Fig. 2F).

FISH analysis of *gag* mRNA

As part of our further investigation into the effect of hCRM1 on the export of *gag* mRNA in rat cells, the intracellular distribution of *gag* mRNA was analyzed using a fluorescence *in situ* hybridization assay (FISH). Since this method does not require the physical separation of the cytoplasmic and the nuclear fractions, it was possible to rule out the leakage of mRNAs from the nuclear fraction during the process. ER1/neo1 cells were cotransfected with pCMV Δ R8.2 and pSR α hCRM1, pSR α rCRM1 or the pSR α 296 control vector. The intracellular distribution of *gag* mRNA was visualized using a DIG-labeled minus strand DNA probe (nucleotides [nts] 964 to 798 of HIV-1_{NL4-3} provirus), which is complementary to the *gag* coding region. The *gag* mRNA was distributed throughout the nucleus and cytoplasm of some cells, but was confined to the nucleus of other cells, irrespective of hCRM1 or rCRM1 expression. Both patterns of *gag* mRNA distribution were observed in HeLa and ER1/hCRM1-7 cells, which constitutively express hCRM1 (Fig. 3A). Control experiments demonstrated that the signals detected were specific for HIV-1 *gag*

mRNAs; firstly, mock-transfected cells exhibited no signal after hybridization with the same probe, and secondly, hybridization with the plus strand DNA probe (nts 798 to 964 of the HIV-1_{NL4-3} provirus) failed to reveal any signals in the transfected cells (data not shown). Fig. 3B shows the percentage of cells positive for the nuclear or cytoplasmic accumulation of *gag* mRNA. An approximately three-fold increase in the cytoplasmic distribution of *gag* mRNA was observed in cells with constitutive or transient expression of hCRM1. These results were consistent with the quantification of cytoplasmic *gag* mRNA using RT-PCR (see Fig. 2B).

Effect of hCRM1 on the processing of Gag protein

We examined the processing of the Gag precursor and the release of viral particles using Western blot analysis. ER1/neo1 cells were transfected with pCMVΔR8.2, pCDMβ-gal and pSRαhCRM1-HA or pSRαrCRM1-HA as described above, and cell lysates and virus-like particle (VLP) fractions were prepared from the culture medium (Fig. 4A). The hCRM1 construct enhanced expression of the p55 Gag precursor approximately three-fold, whereas it had a remarkable effect on the efficient processing

of Gag (increasing it by approximately 10-fold) and on the formation of viral particles. The amount of VLP was evaluated by the p24 ELISA assay because the p24 bands of the VLP fractions, prepared from pSR α 296 and pSR α rCRM1-transfected cells, were too faint in the Western blot profile to be quantified. The ELISA revealed a 70-fold increase in VLP in response to the expression of hCRM1, while rCRM1 had no effect (data not shown). Less hCRM1 protein was expressed than rCRM1 protein (Fig. 4A), and it is possible that the hCRM1-HA protein may be unstable in ER1/neo1 cells, as shown previously (Okada *et al.* 2009). The slight increase in p55 Gag protein in the presence of hCRM1 is consistent with the slight increase in *gag* mRNA observed in the cytoplasm (see Figs. 1D, E and 2B). In addition, the remarkable increase in VLP is consistent with the increase in p24 detected by ELISA in the culture supernatant, as described above (Figs. 1B and 2A). In human HeLa cells, which support high levels of HIV-1 production, both efficient processing and particle release were observed (Fig. 4A).

Next, to investigate whether the amount of Gag protein in the cells affected the processing of Gag and the release of viral particles, cells were transfected with

various amounts of pCMV Δ R8.2, together with pSR α hCRM1-HA or pSR α rCRM1-HA. The proportions of the p55 and p24 Gag proteins were similar, irrespective of the amount of p55 in the hCRM1-transfected cells (Fig. 4B). In contrast, the ratio of p24/p55 in the rCRM1-transfected cells was less than in the hCRM1-expressing cells, even when the rCRM1-transfected cells contained more p55. Moreover, the bands of p24 were clearly detected in the VLP fractions that were prepared from hCRM1-transfected cells, but not in the fractions from rCRM1-transfected cells (Fig. 4B). The relative amounts of p24, as quantified by ELISA, were 4.6, 59 and 1 in the VLP fractions of 0.8 μ g pCMV Δ R8.2/pSR α hCRM1-, 1.6 μ g pCMV Δ R8.2/pSR α hCRM1-, and 0.8 μ g pCMV Δ R8.2/pSR α rCRM1-transfected cells, respectively (data not shown), consistent with the Western blotting profiles (Fig. 4B). These results suggested that the efficiency of Gag protein processing was enhanced by hCRM1-mediated *gag* gene expression but not by the amount of intracellular Gag protein.

Effect of hCRM1 on membrane trafficking and the release of p55 Gag

To examine the effect of hCRM1 on the destination of the Gag protein, in the absence of Gag processing, the p55 Gag protein was expressed using pNLmyr(-)pro(-)Flag and pNLmyr(+)-pro(-)Flag because these vectors express only uncleaved p55, while the FLAG tag supports clear images in immunofluorescent assays. Moreover, the amount of p55 expressed by pNLmyr(-)pro(-)Flag may reflect the amount of *gag* mRNA in the cytoplasm more accurately since it is not released into the medium due to the lack of myristoylation.

When pNLmyr(-)pro(-)Flag was transfected into ER1/neo1 cells, hCRM1 expression augmented the amount of intracellular p55 by 3.3- or 2.5-fold more than the empty vector or rCRM1 expression, respectively (Fig. 5A). This moderate increase in p55 Gag protein in the presence of hCRM1 reflected the small enhancement of *gag* mRNA observed in the cytoplasm (See Fig. 1D and E, and Fig. 2B), consistent with the results described in Fig. 1F, 1G, and S1. When pNLmyr(+)-pro(-)Flag was transfected into ER1/neo1 cells, hCRM1 expression enhanced the intracellular p55 level by two-fold compared to the empty vector or rCRM1 expression. In contrast, hCRM1 expression augmented VLP production by 19- or 8-fold compared to the empty vector

or rCRM1 expression, respectively (Fig. 5B). These results suggested that hCRM1 promotes the release of viral particles independent of the processing of p55.

Next, immunofluorescence was used to examine the localization of p55 Gag in the presence or absence of hCRM1. Unmyristoylated p55 was distributed diffusely throughout the cytoplasm of both HeLa and hCRM1-expressing ER1/neo1 cells transfected with pNLmyr(-)Gag-Flag (Fig. 5C panels a and c). On the other hand, myristoylated Gag was characterized by its punctate state on the plasma membranes of HeLa and ER1/neo1 cells transfected with pNLmyr(+)Gag-Flag, irrespective of the expression of human or rat CRM1 (Fig. 5C panels b, d, e, and f). The ratio (20 to 30%) of rat cells that showed the punctate Gag profile was not increased by the expression of hCRM1, whereas almost HeLa cells showed the punctate profiles. These results suggest that the membrane trafficking of p55 Gag occurred in the rat cells inefficiently compared to the human cells, and it was not restored by hCRM1.

Region of hCRM1 responsible for the upregulation of p24 production

To identify the region of hCRM1 responsible for the production of p24, we

compared the ability of the two chimeric CRM1s to produce p24: hrCRM1 consisted of the N-terminal half of hCRM1 (amino acids [aa] 1 to 679) and the C-terminal half of rCRM1 (aa 680 to 1072), while rhCRM1 was its reverse chimera (Fig. 6A). Expression of hrCRM1-HA increased p24 production, whereas there was little enhancement of p24 production by rhCRM1-HA. It should be noted that hCRM1, when expressed at the same level as rCRM1, augmented p24 production but that rCRM1 had no effect, which ruled out the possibility that overexpression of rCRM1 inhibited p24 production. No significant changes in β -gal activity were observed following transfection with CRM1-expressing plasmids (Fig. 6B). These results indicated that the N-terminal region of hCRM1 was involved in the enhanced production of p24.

Discussion

Although initial reports suggested that only fully-spliced viral transcripts are detectable and that the effects of Rev are diminished in murine cells (Trono & Baltimore 1990), these findings were not reproduced (Malim & Cullen 1991; Bieniasz & Cullen 2000; Keppler *et al.* 2001). Instead, it was proposed that excessive splicing was responsible for a significant reduction in the levels of viral unspliced RNA species and Gag protein in rodent cells (Zheng *et al.* 2003). Moreover, it has been reported that some rat cells exhibit largely unimpaired expression of Env, a Rev-dependent HIV-1 gene product that is translated from a partially spliced viral mRNA (Keppler *et al.* 2001). One reason for these inconsistent conclusions may have been due to lack to examine the cellular cofactors of Rev, such as CRM1. In both our recent studies and the present study, we found that expression of hCRM1, but not rCRM1, enhanced production of the Gag protein in various rat cells, including macrophages (Okada *et al.* 2009), supporting previous findings of poor Rev activity in these cells.

Although significant export of *gag* mRNA into the cytoplasm of rat cells was observed, we found that hCRM1 enhanced *gag* mRNA export two- to three-fold as

indicated by quantitative RT-PCR after cell fractionation and *in situ* hybridization. This increase was in sharp contrast to the 10- to 30-fold augmentation of the Gag protein in the medium. This phenomenon is quite interesting since it has been firmly established that CRM1 is an export factor for Rev, which bridges CRM1 and the viral *gag/env* mRNAs (Cullen 1998, 2003). The above results suggested that, in addition to simple nucleocytoplasmic transport, CRM1 is somehow involved in regulation of gene expression in the cytoplasm, which affects destination of the cognate protein.

To determine the step during which the activity of hCRM1 differs from that of rCRM1, we examined the stability of the Gag protein using a pulse label and chase experiment. However, the results suggested that hCRM1 does not play a role in prolonging the life of the Gag protein. Neither polyribosome profiling analysis nor the quantification of p55 Gag supported the enhancement of *gag* mRNA translation efficiency by hCRM1. Instead, the data showed that the p55 Gag precursor that was synthesized in the presence of hCRM1, but not rCRM1, underwent efficient processing to the mature Gag protein and budding of virus particles into the culture medium. The results that hCRM1 affects the efficient processing and budding of Gag proteins in rat

cells explain the marked enhancement of p24 accumulation in the medium in spite of the minor increase of *gag* mRNA in the cytoplasm in the presence of hCRM1.

Our finding is reminiscent of HIV-1 replication in murine cells where *gag* mRNA, exported through the CTE/Tap pathway, subsequently restored efficient trafficking of the Gag protein to the plasma membrane, thus underlying the efficient processing and budding of HIV Gag, whereas Gag, translated from mRNA exported via the RRE/Rev/CRM1 pathway, was not targeted to the plasma membrane (Swanson *et al.* 2004). The inefficiency of HIV-1 assembly in murine cells likely reflects a cellular deficiency in the RRE/Rev-dependent nuclear export pathway that is specifically linked to the activation of Gag membrane targeting by the regulatory MA globular-head domain (Sherer *et al.* 2009). Similarly, the trafficking of Gag to the plasma membrane was less efficient in rat cells than human cells, which was substantiated by the immunofluorescent localization of p55 Gag protein to the plasma membrane in rat cells, and was not restored by the presence of hCRM1. Thus, hCRM1 may improve budding process but not membrane trafficking of Gag protein in rat cells.

The results suggest that rCRM1 may export *gag* mRNA into the cytoplasm

improperly, resulting in the inefficient processing of Gag proteins. It is most likely that CRM1 has an indirect effect on *gag* mRNA in the cytoplasm, since, upon reaching the cytoplasm, it dissociates from the transport complex containing *gag* mRNA, Rev, Ran and other cellular factors via a mechanism in which RanGTP converts to RanGDP at the periphery of the nuclear pore (Fornerod *et al.* 1997; Fukuda *et al.* 1997; Kudo *et al.* 1997; Neville *et al.* 1997; Ossareh-Nazari *et al.* 1997; Stade *et al.* 1997). Another cellular factor, Rev-interacting protein (hRIP), also known as hRab or Hrb, which is likely bridged by CRM1, has been reported to function in releasing *gag* mRNA, which would otherwise be trapped at the nuclear periphery, to the cytoplasm (Sanchez-Velaz *et al.* 2004; Yu *et al.* 2005). Although, in our hands, *in situ* hybridization did not reveal improper localization of *gag* mRNA in the cytoplasm of rat cells, it is still possible that aberrant construction of the *gag* mRNA transport complex containing rCRM1 might prevent rat Rip and other cellular factors from functional interactions, finally leading to the inefficient processing of Gag proteins.

Alternatively, cytoplasmic effect of hCRM1 could be attributable to the properties of Gag proteins. Gag has capacities for nuclear entry and RNA binding

through its NC region, and RNA promotes multimerization of Gag proteins (D'Souza *et al*, 2005 ; Muriaux *et al*, 2001) that may be an important step of viral particle formation.

Thus it is also conceivable that aberrant RNA complex in rat nucleus may cause inefficient Gag multimerization, leading to reduced viral production.

It is possible that hCRM1 may be needed for correct formation of the complex, which can then interact with rat factors and/or Gag proteins appropriately. Our results indicated that hCRM1 exports *gag* mRNA not only more efficiently than rat CRM1, but also correctly, to the cytoplasm in rat cells, leading to the efficient processing of Gag proteins and HIV-1 particle formation. Our finding that HIV-1 production in rat cells was rescued efficiently by the expression of hCRM1 may have important implications for the development of rat animal models of HIV replication and pathogenesis.

Experimental procedures

Cells

Human cell lines, including HeLa, HOS and 293T, and the rat cell lines, ER1/neo1, ER1/hCRM1-7 (Zhang *et al.* 2006), REF52 and W31 (Kanki *et al.* 2000), were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) in the absence or presence of 300 $\mu\text{g/ml}$ G418 in 10% CO_2 at 37°C.

Plasmids

The following plasmids were used in this study: pCMV~~R~~R8.2 (Naldini *et al.* 1996); pCRRE (Kimura *et al.* 1996); pSR α hCRM1 and pSR α rCRM1 (Hakata *et al.* 1998; Hakata *et al.* 2001); pSR α 296 (Takebe *et al.* 1988); pSR α tat (Takebe *et al.* 1988); pCDM β -gal (Hakata *et al.* 1998); hCRM1-HA and rCRM1-HA, which carried CRM1, fused at the C terminus to an HA tag (Okada *et al.* 2009); pH1-luc (a kind gift from Dr. A. Adachi), which harbors the luciferase gene downstream of the HIV-1 LTR; and pNLmyr(-)pro(-)Flag and pNLmyr(+)pro(-)Flag (a kind gift from Dr. Y. Morikawa) (Kawada *et al.* 2008).

To construct pSR α hrCRM1-HA and pSR α rhCRM1-HA, the hrCRM1 and rhCRM1 coding regions in pSR α hrCRM1 and pSR α rhCRM1, respectively (Hakata *et al.* 2003), were amplified by PCR using the primer pair for hrCRM1-HA of 5'-GGT CAA TAC CCA CGT TTT TTG AGA GCT CAC TGG A-3' (the underlined sequence is a *SacI* site) and 5'-TAT GGT ACC TTA AGC ATA ATC AGG AAC ATC GTA TGG GTA GTC ACA CAT TTC TTC TGG GAT TTC-3' (the underlined sequence is a *KpnI* site), and the primer pair for rhCRM1-HA of 5'-CTG GAA TCA CTT GGC AGC TGA GCT CTA CAG AGA GAG TCC A-3' (the underlined sequence is a *SacI* site) and 5'-TAT GGT ACC TTA AGC ATA ATC AGG AAC ATC GTA TGG GTA ATC ACA CAT TTC TTC TGG AAT CTC-3' (the underlined sequence is a *KpnI* site.), which encodes the HA tag. The PCR reaction was performed using 10 ng of the plasmid as a template, which was denatured at 94°C for 2 min and was then followed by 20 cycles of amplification: denaturation (94°C, 30 s), annealing (62°C, 1 min), and extension (68°C, 2 min). A final extension was performed at 68°C for 10 min. The amplified DNA was digested with *SacI* and *KpnI*, and inserted into these sites in pSR α hrCRM1 or pSR α rhCRM1, as appropriate.

Real-time RT-PCR

To separate the cytoplasmic and nuclear fractions, lysis buffer (0.5% NP40, 10 mM Tris-HCl [pH 7.4], 0.14 M NaCl, 1.5 mM MgCl₂, 1 U/μl RNase Inhibitor (TOYOBO), 1 mM DTT) was added to the cell pellets and the lysates were centrifuged in a microcentrifuge at 3000 rpm for 5 min at 4°C. Total RNA in the cytoplasm (supernatant) and nucleus (pellet) was extracted using the Absolutely RNA Miniprep Kit (Stratagene). The ThermoScript RT-PCR System (Invitrogen) was used to synthesize cDNAs from 150 to 500 ng of RNA and the abundance was quantified using real-time PCR with a Light Cycler PCR machine (Roche). PCR reactions were performed using the following primer pairs: *gag* forward (5'-AGA GAA GGC TTT CAG CCC AGA AGT-3') and reverse (5'-GGA TTT GTT ACT TGG CTC ATT GCT-3'); *tat* forward (5'-GTG GAA GCA TCC AGG AAG TCA GCC-3') and reverse (5'-CTA TTC CTT CGG GCC TGT CGG GTC-3'); and *β-gal* forward (5'-GCG AAT ACC TGT TCC G-3') and reverse (5'-GCG TCA CAC TGA GGT T-3'). PCR mixtures (20 μl) were prepared in a capillary tube containing 1/16 of the volume of the RT reaction mixture, each primer pair (0.5 μM), and 1x Light Cycler-FastStart SYBR

Green PCR Master Mix. Following an initial Taq polymerase activation step (95°C, 15 min) the *gag* and *tat* mRNA amplification reactions were performed using 40 cycles of denaturation (95°C, 10 s), annealing (68°C, 10 s) and extension (72°C, 15 s), whereas the *β-gal* mRNA was amplified using 40 cycles of denaturation (95°C, 15 s), annealing (60°C, 5 s) and extension (72°C, 11 s). The abundance of each mRNA was estimated with a standard regression curve using the Light Cycler Software v. 3 (Roche). The standard curve was obtained by PCR amplification of 0.0064 to 500 pg of pCMVΔR8.2 (for *gag* mRNA), pSR α tat and pCDM β -gal.

To ascertain the efficiency of fractionation, *β-actin* pre-mRNA was amplified by PCR using the primers *β-actin*-F (5'-TCG ATC GCC TTT CTG ACT AGG TG-3') and *β-actin*-R (5'-GGT CAG GAT CTT CAT GAG GTA GTC TG-3'); the former targets the intron and the latter targets the exon of *β-actin* pre-mRNA. Reactions were performed with 25 cycles of denaturation at 94°C for 30 s, followed by annealing at 59°C for 1 min and extension at 72°C for 30 s.

Pulse chase assay

At 36 to 48 h post transfection, cells were metabolically labeled with an

[³⁵S]-methionine/cysteine mixture (GE Healthcare UK Ltd.) for 30 min. Cells were washed once and then chased for 0, 4, 8 and 24 hours in 1 ml of medium supplemented with 10 μ M methionine, 10 μ M cysteine and 100 nM KNI-272 (an HIV-1 protease inhibitor, the kind gift of Dr. Y. Kiso). After chasing, 0.25 ml of 5x detergent buffer containing 2.5% deoxycholate (DOC), 2.5% Triton X100, 250 mM Tris HCl (pH 7.6), 0.1 M NaCl, 0.1 M EDTA, 100 nM KNI-272, and a protease inhibitor cocktail (Roche) was added to the culture to dissolve Gag proteins in both the medium and cells. The lysates were centrifuged in a microcentrifuge at 12,000 rpm for 15 min and the supernatant was incubated with anti Gag mAb V107 (Ikuta *et al.* 1989) (a kind gift from Dr. K. Ikuta) for 1 hour at 4°C, followed by incubation with protein G Sepharose for 1 hour at 4°C. After three washes with detergent buffer, the precipitated complexes were analyzed by SDS-PAGE and autoradiography.

At 40 hours post transfection with pNLmyr(-)pro(-)Flag, cells were metabolically labeled with an [³⁵S]-methionine/cysteine mixture for 15 min, washed, and then chased for 0, 4 and 7 hours as described above. The detergent buffer (0.5 ml) was added to the cells and centrifuged in a microcentrifuge at 12,000 rpm for 15 min. The

supernatant was incubated with anti-Flag M2 antibody (Sigma) conjugated to protein G Sepharose for 3 hours at 4°C, followed by three washes with the detergent buffer.

***In situ* hybridization**

At 48 to 50 hours after transfection, cells were subjected to an *in situ* hybridization assay, which has been described previously (Tautz & Pfeifle 1989; Kimura *et al.* 1996). Probes were prepared by PCR amplification of a 167-bp fragment from HIV-1 *gag* using the primers T66 (5'-GAGAGCGTCGGTATTAAG-3'; HIV-1_{NL4-3} nts 798 to 815) and T67 (5'-GTCTACAGCCTTCTGATG-3'; HIV-1_{NL4-3} nts 964 to 947). The fragment generated was then used as a template for a second PCR, using either T67 or T66 primers and the digoxigenin (DIG)-nucleotide mixture (Roche), essentially as described by Tautz *et al.* (Tautz & Pfeifle 1989). Cells were examined under a confocal laser-scanning microscope (Olympus).

Western blotting

The transfected cells were dissolved in TMN buffer containing 10 mM Tris-HCl (pH 7.4), 2 mM MgCl₂, 0.5% NP40, and a protease inhibitor cocktail. After measuring the β-gal activity and protein levels, the proteins were solubilized with

sample buffer and resolved by SDS-PAGE, and then transferred to a nitrocellulose filter. V107, a rat anti-HA mAb (Roche), a rabbit anti- β -gal Ab, a mouse anti-actin mAb, and anti-FLAG M2 Ab were used as primary Abs. Horseradish peroxidase-conjugated anti-IgG Abs (Promega) were used as secondary Abs. Immunoreactive bands were visualized using ECL+plus (GE Health care) followed by the LAS-1000 Plus system (Fujifilm) and were evaluated by Image Gauge (version 3.4) software (Fujifilm).

Immunofluorescence

Subconfluent ER1/neo1 cells, seeded onto coverslips (Fisher Scientific) in six-well plates, were transfected using Polyethylenimine "Max" (PEI) (Polyscience) and fixed 18 hours post-transfection with 2% paraformaldehyde dissolved in PBS. The cells were then permeabilized with 0.5% NP40 in PBS and incubated with blocking solution (5% skim milk in PBS) for 1 hour at RT. The cells were stained with Alexa Fluor488-conjugated anti-HA-Tag (6E2) mouse mAb and Alexa Fluor® 555-conjugated anti DYKDDDDK (FLAG) Tag antibody (Cell Signaling Technology) for 1 hour at RT, washed, and then mounted with VECTASHIELD Hard set Mounting Medium (Vector Laboratories). The stained cells were examined under a confocal laser-scanning

microscope.

Preparation of VLP

To prepare the viral-like particle (VLP) fraction, medium from the cell cultures was centrifuged at 2000 rpm for 10 min and the supernatant was filtered through a Millex HV 0.45 μm Filter Unit (Millipore). The HIV-1 particles were pelleted through a 20% (W/V) sucrose solution by centrifugation at 27,000 rpm in an SW28 rotor for 2 hours at 4°C.

Statistical analysis

Comparisons between individual data points were made using a Student's *t*-test. Two-sided P values < 0.05 were considered statistically significant.

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Figure Legends

Figure 1. Effect of CRM1 on p24 production. (A) The structure of pCRRE. (B) ER1/neo1 cells (1×10^5) were transfected with 1.6 μg of pCRRE and pSR α hCRM1 as indicated, 0.1 μg of pCDM β -gal, and 0.3 μg of pH1-luc using TransIT-LT1 (Mirus) reagent. The total abundance of transfected plasmids was adjusted with pSR α 296. Two days after transfection, p24 was quantified from the cell culture medium using a RETRO-TEK HIV-1 p24 Antigen ELISA kit (ZeptoMetrix). Cytoplasmic β -gal activity was measured using standard colorimetric methods (β -gal assay) (Hakata *et al.* 2001), and luciferase activities were also quantified. (C) ER1/neo1 and HeLa cells were transfected with pCRRE, pCDM β -gal and pH1-luc along with pSR α hCRM1 or pSR α rCRM1 as described above. The amount of p24 in the medium was quantified as in (B). The value of the control sample without CRM1 was set at 1. The β -galactosidase and luciferase activities were similar in all samples (data not shown). These data are representative of three independent experiments. (D) The amount of mRNA in cytoplasmic fraction in ER1/neo1 cells was indicated. ER1/neo1 cells were

transfected with pCRRE, pSR α hCRM1, pCDM β -gal and pH1-luc, and then separated into nuclear and cytoplasmic fractions using an NP40 detergent-containing buffer, as described in Materials and Methods. Quantitative RT-PCR was used to determine the abundance of *gag*, *tat* and *β -gal* mRNAs in each fraction. The ratios of *gag* mRNA/ *β -gal* mRNA and *tat* mRNA/ *β -gal* mRNA were calculated. The value of the control sample without hCRM1 was set at 1. These values are the means of three independent sets of experiments and the SD was calculated. (E) Gag protein (p24) in the medium and *β -gal* activity were quantified and the Gag/ *β -gal* ratio was plotted and compared to the ratio of cytoplasmic *gag* mRNA/ *β -gal* mRNA. These data are representative of three independent experiments. (F) Stability of Gag protein in rat cells. At 48 hours post transfection, cells were pulse-labeled with [³⁵S]-methionine/cysteine and chased for 0, 4, 8 and 24 hours. The medium and cells were solubilized with detergent-containing buffer and combined. Then, Gag proteins were immunoprecipitated using anti-Gag antibodies and protein-G Sepharose, and analyzed by SDS-PAGE followed by autoradiography. (G) ER1/neo1 cells were transfected with pNLmyr(-)pro(-)Flag, pSR α hCRM1, and pCDM β -gal. At 40 hours

post transfection, cells were pulse-labeled with [³⁵S]-methionine/cysteine and chased for 0, 4 and 7 hours. Gag proteins in the cell lysates were immunoprecipitated using anti-FLAG antibodies and protein-G Sepharose.

Figure 2. Effects of rat and human CRM1 on the production of Gag protein and *gag* mRNA derived from pCMVΔR8.2 in rat cells. (A-D) ER1/neo1 cells were transfected with pCMVΔR8.2 (1.6 μg) and pCDMβ-gal (0.1 μg) along with pSRαhCRM1 (0.4 μg) or pSRαrCRM1 (0.4 μg). The level of p24 in the medium and cytoplasmic β-gal activity, *gag* mRNA, *tat* mRNA and β-gal mRNA were quantified. The average and SD were calculated based on three independent sets of experiments. (E) PCR fragments of β-actin pre-mRNA in the nucleus (lanes 1, 3 and 5) and cytoplasm (lanes 2, 4 and 6) of cells without (lanes 1 and 2) or with (lanes 3 and 4) hCRM1 and (lane 5 and 6) rCRM1. (F) β-gal activity in the nuclear and cytoplasmic fractions was quantified, and the ratios of the β-gal activities in each fraction were calculated.

Figure 3. Fluorescence *in situ* hybridization (FISH) profiles of *gag* mRNA distribution in cells. (A) ER1/neo1 cells (panels a-f) were transfected with

pCMV Δ R8.2, along with pSR α 296 (panels a and b), pSR α hCRM1 (panels c and d), or pSR α rCRM1 (panels e and f). ER1/hCRM1-7 (panels g and h) and HeLa cells (panels i and j) were transfected with ~~pCMV~~. Cells were subjected to *in situ* hybridization. In all panels, the upper left image is phase-contrast and the upper right shows DAPI-stained cells. The lower left image shows the FISH profile and the lower right is a merged image. (B) The number of cells (designated N) in which *gag* mRNA is localized exclusively to the nucleus and those (designated C), which contain *gag* mRNA distributed throughout the cytoplasm and nucleus. More than 150 cells were counted and their ratios are presented. These data represent the total number of cells counted in three independent experiments.

Figure 4. Western blotting of Gag proteins expressed in ER1/neo1 cells. (A) ER1/neo1 and HeLa cells were transfected with pCMV Δ R8.2 and pCDM β -gal along with the empty vector, pSR α 296, and pSR α hCRM1-HA or pSR α rCRM1-HA as described above. Fractions corresponding to approximately 1.5×10^4 and 5×10^5 ER1/neo1 cells were subjected to Western blotting to analyze Gag protein profiles in the

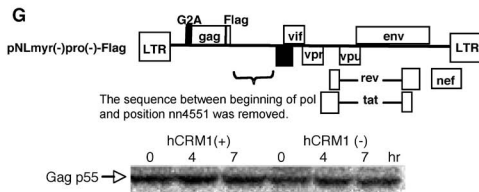
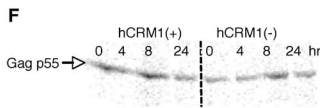
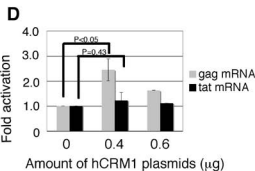
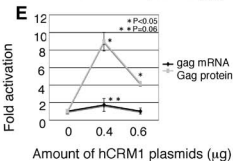
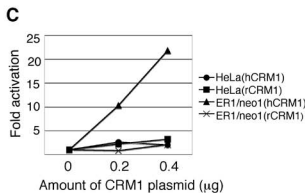
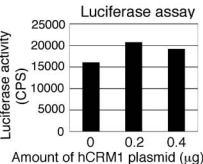
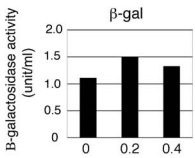
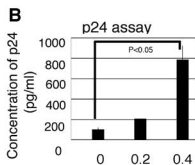
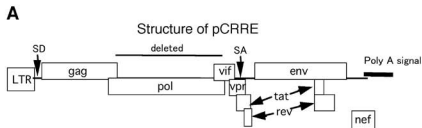
cell and VLP fractions, respectively. Fractions corresponding to approximately 5×10^3 and 5×10^4 HeLa cells were subjected to SDS-PAGE for analysis of the cell and VLP fractions, respectively. The intensities of the Gag proteins were quantified by LAS-1000. The amount of p55 Gag in pSR α 296-transfected ER1/neo1 cells was set at 1.0, and the relative amounts of Gag protein were calculated. The ratio of p24/p55 expressed in HeLa was also presented. (B) ER1/neo1 cells were transfected with pCMV Δ R8.2 (0.4 to 1.6 μ g) and pCDM β -gal (0.1 μ g) along with pSR α hCRM1-HA (0.4 μ g) or pSR α rCRM1 (0.4 μ g). Fractions corresponding to approximately 2×10^4 and 5×10^5 cells were subjected to Western blotting to analyze Gag protein profiles in the cell and VLP fractions, respectively. The intensity of the p55 Gag band in the lane representing ER1/neo1 cells, transfected with 0.8 μ g of pCMV Δ R8.2 and pSR α hCRRM1, was quantified and set at 1.0.

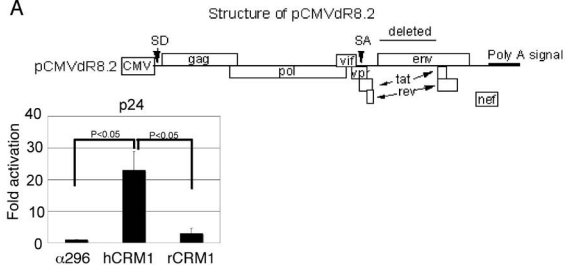
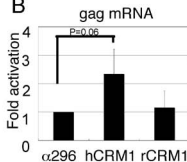
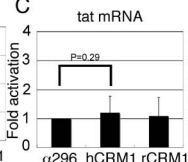
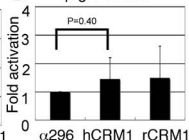
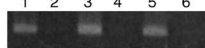
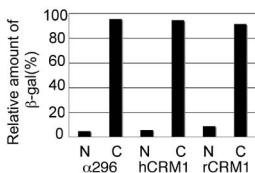
Figure 5. Effect of hCRM1 on membrane trafficking and release of virus particles using pNLmyr(-)pro(-)Flag and pNLmyr(+)-pro(-)Flag. Approximately 2×10^4 ER1/neo1 cells transfected with pNLmyr(-)pro(-)Flag along with pSR α 296, pSR α hCRM1 or

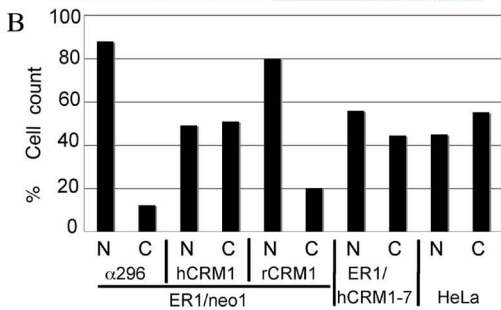
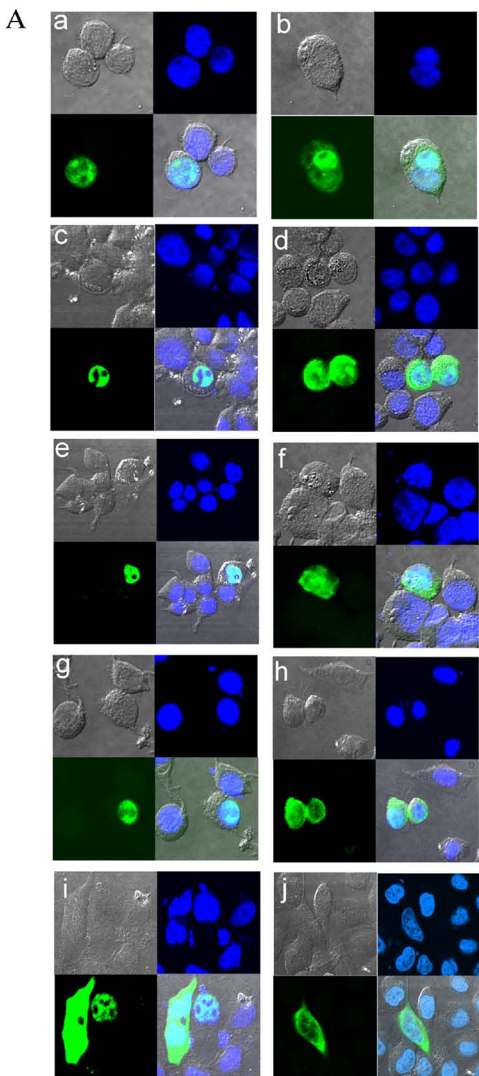
pSR α CRM1 were subjected to Western blotting (A). The cells and VLP fractions corresponding to approximately 5×10^3 and 5×10^5 cells transfected with pNLmyr(+)pro(-)Flag along with pSR α 296, pSR α hCRM1 or pSR α CRM1, were subjected to Western blotting, respectively (B). The band intensity of p55 Gag expression in pSR α 296-transfected cells was set at 1.0. (C) HeLa cells were transfected with pNLmyr(-)pro(-)Flag (panel a) or pNLmyr(+)pro(-)Flag (panel b). ER1/neo1 cells were transfected with pNLmyr(-)pro(-)Flag and pSR α hCRM1 (panel c), or pNLmyr(+)pro(-)Flag along with pSR α 296 (panel d), pSR α hCRM1 (panel e), or pSR α CRM1 (panel f). Cells were subjected to direct immunofluorescence assay. In all panels, the left image is a merged picture of DAPI (blue) and p55 Gag protein (red), and the right-hand panels show exogenous CRM1 (green).

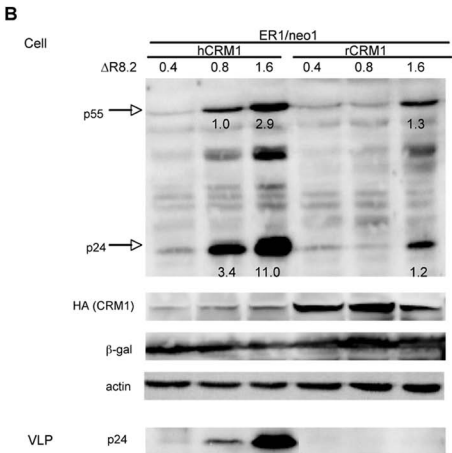
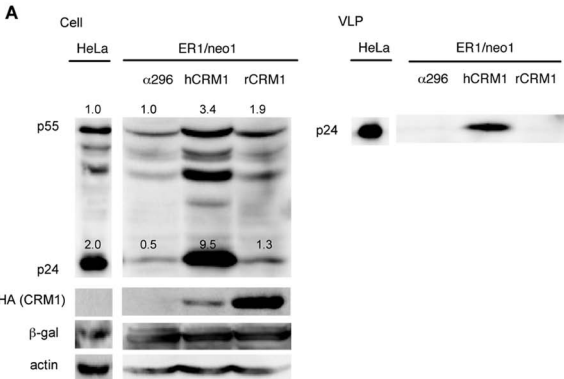
Figure 6. Analysis of the region of hCRM1 responsible for the upregulation of p24 production in ER1/neo1 cells. (A) Structure of CRM1. (B) ER1/neo1 cells were transfected with pCMV Δ R8.2, pCDM β -gal and various CRM1-expressing plasmids. HIV-1 p24 in the medium was quantified by ELISA. The protein expression of CRM1,

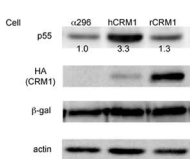
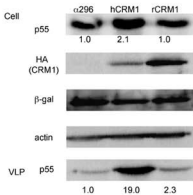
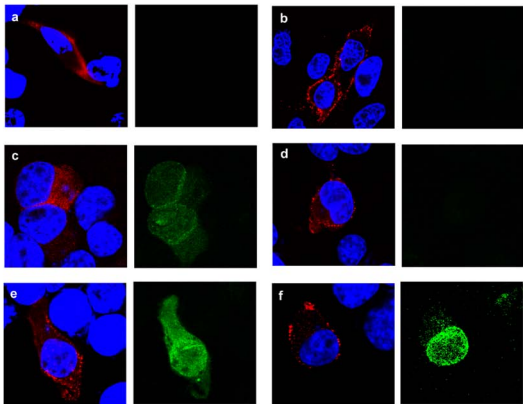
β -gal and actin was analyzed by Western blotting.



A**B****C****D****E****F**

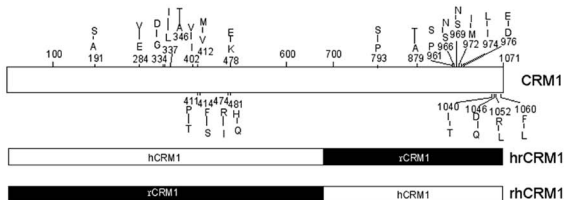




A**B****C**

A

Structure of CRM1

**B**