Mutation of the Rev-binding loop in the human immunodeficiency virus 1 leader causes a replication defect characterized by altered RNA trafficking and packaging

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An internal RNA loop, located within the packaging signal of human immunodeficiency virus 1, that resembles the Rev-responsive element (RRE) closely was identified previously. Subsequent in vitro studies confirmed that the loop, termed loop A, could bind Rev protein specifically. Its proximity to the major splice donor has suggested a role for Rev–loop A interaction supplementary to or preceding that of the Rev–RRE interaction. To investigate this further in a replication-competent provirus, loop A was mutated to decrease its affinity for Rev. Impairing the Rev–loop A interaction led to reduced nuclear export of viral genomic RNA. RNA packaging decreased by approximately 30%. Viral protein production and export of virus particles appeared normal; however, the virus was severely replication-deficient. The loop A sequence, which is 98% conserved amongst viral isolates, is implicated in several cis-acting functions critical to virus viability.

INTRODUCTION

The 5′-untranslated region of human immunodeficiency virus 1 (HIV-1) contains cis-acting sequences critical for the control and function of processes such as encapsidation of the genome, dimerization and translation. Encapsidation itself is a highly specific process, whereby an RNA structure termed psi (Ψ) is recognized by the Gag polyprotein against the background of spliced and cellular mRNAs. Ψ consists of a series of three to four stem–loops (Harrison et al., 1998). Stem–loop 1 contains the dimerization-initiation site (Laughrea & Jette, 1994; Skripkin et al., 1994) plus two internal loops termed loop A and loop B (Greatorex et al., 2002). These internal loops are highly conserved (Greatorex et al., 2002) and, importantly, so are the helices surrounding them, implying a role for the loops in the virus life cycle. The tertiary structure of loop A closely resembles that of the Rev-responsive element (RRE) (Fig. 1) and has been demonstrated to bind the Rev protein specifically (Gallego et al., 2003). A mutation of the loop changing adenine to guanine at positions 692 and 695 in the HIV-HXB2 sequence (Fig. 1), termed 4G, reduces Rev binding significantly (Gallego et al., 2003). The aim of this work was to examine the effect of this mutation against the background of an infectious molecular clone, to help to elucidate the role of this novel Rev-binding site in the context of virus replication.

METHODS

Plasmids. All constructs were derived from pSVC21, an infectious molecular clone of HIV-HXB2 (Fisher et al., 1985). The 4G mutation was introduced into pKS.BglII [described previously (Harrison et al., 1998)] and cloned back into pSVC21 by using Spe I and Sfo I to create pSVC4G. Plasmid pT319R-RRE was used to generate cold competitor RNA and has been described previously (Dangerfield et al., 2005).

Plasmid KSIIΨCS was used to generate the antisense probe for the ribonuclease-protection assays (RPAs) (Kaye & Lever, 1996).

RNA production. Templates for Ψ RNA were generated by using forward primer 5′-TATACCATGATAGGAGACCAGAGGAGC-3′ and reverse primer 5′-CTCTCTCCCTAGTGGCTCGC-3′. PCR and template purification were performed as described previously (Gallego et al., 2003). The template for the RRE RNA was generated by linearizing pT319R-RRE with EcoRI (Roche Molecular Biochemicals), cleaning the DNA using a QIAquick column (Qiagen) and ethanol precipitation. The template for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control probe came from the RPA II kit (Ambion).
DNA extraction.

Cytoplasmic RNA was extracted from COS-1 cells as described previously (Harrison et al., 1998). Briefly, DNA (90 nM) was heated to 95 °C for 2 min in binding buffer [250 mM Tris/HCl (pH 7.4), 250 mM KCl, 250 mM MgCl2] with tRNA and DEPC. RRE RNA was added prior to heating at concentrations ranging from equimolar to 1000-fold excess. The tRNA was snap-cooled, peptide (Bachem) was added to the mixture and the mixture was incubated at room temperature for 30 min. Following incubation, non-denaturating loading buffer (Ambion) was added to the mixture and the samples were analysed by electrophoresis on 2% native agarose gels.

Electrophoretic mobility-shift assays. Band-shift assays were performed as described previously (Galleo et al., 2003). Briefly, RNA (90 nM) was heated to 95 °C for 2 min in binding buffer [250 mM Tris/HCl (pH 7.4), 250 mM KCl, 250 mM MgCl2] with tRNA and DEPC. RRE RNA was added prior to heating at concentrations ranging from equimolar to 1000-fold excess. The tRNA was snap-cooled, peptide (Bachem) was added to the mixture and the mixture was incubated at room temperature for 30 min. Following incubation, non-denaturating loading buffer (Ambion) was added to the mixture and the samples were analysed by electrophoresis on 2% native agarose gels (Anachem).

Replcmentation studies. Equal numbers of virions, as judged by a reverse transcriptase (RT) assay, from transient DEAE/dextran transfections of COS-1 cells were used to infect 2 x 10⁶ Jurkat cells in 2 ml RPMI 1640 (Invitrogen) containing 10% fetal calf serum, 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹. After 24 h, cells were pooled to give a total volume of 10 ml. Every 4–5 days, the RNA was snap-cooled, and the mixture was incubated at room temperature for 30 min. Following incubation, non-denaturating loading buffer (Ambion) was added to the mixture and the samples were analysed by electrophoresis on 10% TBE/polyacrylamide gels (Anachem).

RNA extraction. Cytoplasmic RNA was extracted from COS-1 cells as described previously (Harrison et al., 1998). In brief, cells were lysed in chilled lysis buffer [50 mM Tris/HCl (pH 8), 100 mM NaCl, 5 mM MgCl2, 0.5% Nonidet P-40]. Debris and nuclei were removed by centrifugation for 1 min at 13 000 r.p.m. in a microcentrifuge (Heraeus Biofuge Fico). The supernatants were adjusted to 0.2% SDS and 125 μg proteinase K (Ambion) ml⁻¹, incubated at 37 °C for 15 min, extracted with phenol/chloroform and ethanol-precipitated. Nuclear RNA was purified post-lysis by using a Qiagen RNeasy kit. Extractions were performed following the manufacturer’s instructions.

Supernatants from transfected cells were passed through a 0.45 μm filter and the filtrate was precipitated overnight with 0.5 vol 30% PEG 8000 in 0.4 M NaCl at 4 °C. The precipitate was collected by centrifugation at 2000 r.p.m. in an MSE 43124-129 rotor at 4 °C and resuspended in 0.5 ml TNE [10 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% SDS, 100 μg proteinase K ml⁻¹, 100 μg tRNA ml⁻¹] for 30 min at 37 °C. Following this, the RNA was extracted with phenol/chloroform and ethanol-precipitated.

RREs. Nuclear and cytoplasmic RNA levels were normalized by RNA concentrations, with 5 μg being used per reaction for the cytoplasmic preparations. Virion RNA levels were normalized by RT count, as described above. The riboprobe was generated by using the KSIIPWS template described above, incorporating [α-32P]UTP (Amersham Biosciences). Probes were gel-purified and 10 μl was added to 2 ml scintillant to determine the number of counts μl⁻¹.

Reagents for the RPA were obtained commercially (RPA II kit; Ambion) and the assay was performed following the manufacturer’s instructions. Each reaction used 2 x 10⁵ counts of probe. Reactions were analysed on 6% polyacrylamide gels and the relative quantities of viral RNA in each sample were counted by using a Packard Real-time Instant Imager or the NIH Image program. The probe that was used protected fragments of the following sizes: DNA, 517 and 332 nt; genomic RNA, 375 and 238 nt; spliced RNA, 289 nt.

Protein production. Equal numbers of cells were harvested at 72 h post-transfection (or later). For cytoplasmic-protein analysis, cells were washed twice with ice-cold PBS, trypsinized, resuspended in 1 ml PBS and spun gently. Cells were resuspended in 100 μl PBS and 200 μl loading buffer [6-25 mM Tris/HCl (pH 6.8), 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.02% bromophenol blue]. Protein from virions was prepared as follows: virions were harvested as described above. Following centrifugation at 2000 r.p.m. in an MSE 43124-129 rotor, the pellet was resuspended in 100 μl PBS and 200 μl loading buffer. Samples were subjected to sonication and the RRE RNA in each sample were counted by using a Packard Real-time Instant Imager or the NIH Image program. The probe that was used protected fragments of the following sizes: DNA, 517 and 332 nt; genomic RNA, 375 and 238 nt; spliced RNA, 289 nt.

Northern blotting. Virion RNA was prepared and levels were normalized as described above. RNA samples were electrophoresed on 1% agarose gels (Ambion), transferred to nitrocellulose overnight at room temperature and the RNA was detected by using commercially available reagents (Northern Max-Gly and BrightStar BioDetect; Ambion).

RESULTS

RRE RNA can outcompete loop A for binding of the Rev peptide

We have shown previously that loop A RNA binds the Rev protein specifically. Analysis of replicate experiments using ImageJ software calculated the binding affinity as 160 μM. As might be expected given the affinity of the interaction, the Rev peptide-binding loop A could be displaced by the
addition of cold competitor RRE RNA at >10-fold excess (Fig. 2, lanes 6 and 7).

**Replication of viruses containing the 4G mutation is impaired**

The 4G mutation was introduced into an infectious molecular clone of HIV-1, termed pSVC21 (Fisher et al., 1985), to generate pSVC4G. The two plasmids were transfected into COS-1 cells and virus was harvested at 48–72 h post-transfection. Virion levels were normalized by RT assay and used to infect Jurkat T cells. Following infection, RT assays were performed at regular time points and cells were split either 1:4 every 7–8 days (Fig. 3a) or 1:2 every 5 days (Fig. 3b). Under both sets of conditions, the 4G-mutated virus showed a replication defect. Peak viral production was delayed and never reached wild-type levels, as judged by RT assays.

**Protein production is unaffected by the 4G mutation**

Protein production by SVC4G was compared with that of the wild-type virus by Western blotting following transfection and transduction. Protein from equal numbers of transfected COS-1 cells was harvested from cytoplasmic and virion samples, analysed by SDS-PAGE, transferred to nitrocellulose membranes and detected by using an anti-p24 antibody. No difference could be discerned between the wild-type and mutant protein levels (Fig. 4a, compare lanes 1 and 4 with lanes 2 and 5). Once Jurkat cells had been infected and virus was being produced (as shown by a rise in RT values), protein was harvested as before from equal numbers of cells and protein levels were compared. Total viral protein levels in the Jurkat cells were marginally reduced, consistent with a replication defect, but there was no marked qualitative difference in protein production or processing (Fig. 4b).

**Fig. 2.** RRE RNA competes with loop A-containing Y RNA for binding to the Rev peptide. Lane 1, Y RNA and peptide (Rev); lanes 2–7, increasing concentrations of cold competitor RRE RNA. Arrows indicate the bound complex (C) and unbound Y RNA (Y). The far-right lane contains the size marker.

**Fig. 3.** Replication of the 4G virus. Jurkat cells were infected with normalized quantities of virus (wild-type or 4G mutant) and replication was assessed by RT production. Cells were split 1:4 every 7–8 days (a) or every 5 days (b). Symbols: black, wild-type virus; dark grey, mutant virus; light grey, mock infection.
The 4G mutation affects viral RNA packaging

Post-transfection, cytoplasmic and virion RNA was harvested from the COS-1 cells. Packaging was assessed by RPA. Cytoplasmic RNA levels were normalized so that 5 μg of each RNA was used in the assay; virion RNA levels were normalized by RT count. The probe used detected both RNA and DNA and this ensured, after adjustment for contaminating DNA, that RNA values were not affected (Fig. 5, lanes 1–6). A GAPDH probe was also used in separate assays (data not shown) to confirm equivalent cytoplasmic RNA loading. A consistent difference in RNA packaging was seen between the wild-type and 4G-mutant virus. The latter packaged RNA at 65–70% of wild-type levels (Fig. 5, compare lanes 11 and 12 with lanes 9 and 10). Experiments were performed independently three times.

The 4G mutation affects dimerization of the RNA in vitro, but not in vivo

A possible cause of the packaging defect might have been that the introduced mutation affected dimerization of the viral genome. To investigate this, the electrophoretic mobility of the Y RNA with and without the 4G mutation was assessed on non-denaturing agarose gels. The RNA containing the 4G mutation had marginally less stability as a dimer than did the wild-type RNA. The latter was wholly dimeric, even after heating at 90°C (Fig. 6). Unheated RNA containing the 4G mutation was 100% dimeric (Fig. 6, lane 5), but heating revealed a lower dimer stability and the monomer was detected following heating at all temperatures tested (Fig. 6, lanes 6–8).

Northern blotting was performed to assess the nature of the RNA contained within virus particles. Virions were harvested post-transfection as before and RNA levels were normalized by RT count. The RNA was electrophoresed on non-denaturing agarose gels, transferred to nitrocellulose and detected by using a biotinylated antisense RNA probe. Both wild-type and 4G mutation-containing RNAs were dimeric (Fig. 7). However, in agreement with the RPA data, the 4G-mutant virus packaged less RNA.

Fig. 4. (a) Viral protein production following transient transfection. Levels of protein were determined in the cytoplasm (lanes 1–3) and in virions (lanes 4–6). Lanes: 1 and 4, wild-type virus; 2 and 5, 4G-mutant virus; 3 and 6, mock transfections. (b) Viral protein production post-infection (protein harvested at > 20 days post-infection). Protein production was analysed in Jurkat cells (lanes 1–3) and in virion particles (lanes 4–6). Lanes: 1 and 4, mock infections; 2 and 5, 4G-mutant virus; 3 and 6, wild-type virus.

Fig. 5. RPA of RNA extracted from the cytoplasm of infected cells (lanes 1–6) and virion particles post-transfection (lanes 9–14). Undigested and digested probe are shown in lanes 7 and 8, respectively. Lanes: 1, 2, 9 and 10, wild-type virus RNA; lanes 3, 4, 11 and 12, 4G-mutant virus RNA; lanes 5, 6, 13 and 14, mock transfection. Century-Plus Marker (Ambion) was used as a size marker; fragment sizes (bp) are indicated on the left of the gel.

Fig. 6. Dimerization of wild-type and 4G-mutant RNA. RNA was produced by in vitro transcription from a DNA template and resuspension in DEPC-treated water. The RNA was heated for 2 min and incubated for 60 min at 37°C. The dimer and monomer bands are indicated. Heating temperatures were: room temperature (lanes 1 and 5); 90°C (lanes 2 and 6); 50°C (lanes 3 and 7); 70°C (lanes 4 and 8). Lane M, Century-Plus marker (Ambion).
Defective nuclear export of 4G mutant RNA

Cellular trafficking and segregation of RNA during the replication and encapsidation process was monitored by RPA of nuclear and cytoplasmic RNA. RPAs were carried out as described above, with normalization as before.

At 72 h post-transfection, a difference in RNA trafficking could be detected (Table 1). The percentages shown in Table 1 represent the level of each nucleic acid detected (the probe also detects DNA, hence the total RNA does not reach 100%). More full-length RNA was retained in the nucleus following transfection of the 4G-mutant construct compared with the wild type. The wild-type virus showed preferential export of genomic RNA, with twice as much genomic as spliced message in the cytoplasmic fraction. Actual levels of spliced RNA were higher in the cytoplasm of cells transfected with the mutant than in those transfected with wild type; however, due to the nature of the overexpression system used, these results must be interpreted with caution.

**DISCUSSION**

The Rev protein of HIV-1 is responsible for the temporal control of gene expression. Produced from spliced mRNA, it is transported back into the cell nucleus and facilitates transport of the full-length genomic RNA and singly spliced mRNAs from that compartment (Cullen, 1992). It has also been suggested that Rev has other effects, such as enhancement of viral encapsidation and translation (D’Agostino et al., 1992; Kaye et al., 1995).

We have demonstrated previously that the packaging signal of HIV-1 contains within it a Rev-binding motif. Mutation of adenines at positions 692 and 695 within this motif was demonstrated to affect Rev binding (Gallego et al., 2003). It was not known, however, whether this RNA–protein interaction was essential for virus replication.

We first investigated the relative affinity of the loop A–Rev interaction compared with that of RRE and Rev. We demonstrated that an excess of cold competitor RRE RNA could outcompete loop A for Rev interaction. The RRE competed successfully even at equimolar concentrations, suggesting that it had a higher affinity for the protein.

The mutation described above was introduced into an infectious molecular clone, HIVHXRD, and its protein production, RNA composition and replication ability were compared with those of the wild-type virus.

Replication of the virus was severely compromised. The substitution of two adenine residues for two guanines produced a virus with significantly delayed replication, which never reached wild-type levels.

Viral protein production of the mutant appeared to be normal, as assessed by both transient transfection of cells and virus infection of cells. There was no significant difference between the mutant and wild-type viruses.

The mutant displayed a modest genomic RNA-packaging defect. This result was confirmed by Northern blotting (Fig. 7). One possible cause of decreased packaging would be a change in RNA structure due to the mutations that were introduced into the stem–loop 1 RNA. RNAs were generated in vitro from wild-type and mutant templates and their stability and electrophoretic characteristics were compared under non-denaturing conditions. Whilst both RNAs formed dimers, there was a difference in dimer stability, the mutant being less stable than the wild type. However,
genomic RNA in the virions from the mutant was dimeric, suggesting that this was unlikely to be responsible for either the packaging defect or the striking effect on replication unless such a minor dimerization defect has a significant impact on post-entry stages of replication.

We observed a reproducible reduction in the export of genomic RNA from the nucleus in the mutant virus. Whilst it was less marked than published effects on transport caused by mutation of the RRE (Cullen, 1998), it supported the possibility that Rev has more than one point of action on the RNA. The reduced export of genomic RNA was modest but reproducible compared with that seen in the wild-type virus. Spliced mRNA was exported very efficiently in the mutant, implying that there was no reduction in availability of Rev protein to facilitate export of the other RNA species. The level of spliced RNA seen in the 4G mutant may also be consistent with the mutation favouring splicing; however, the fact that viral protein production was equivalent in the mutant also suggests that sufficient unspliced RNA was arriving at cytoplasmic ribosomes to fulfil the translation function and to produce virion particles.

It has been demonstrated that, in lentiviruses, translation to produce Gag/Pol and encapsidation can occur from the same unspliced RNA pool (Dorman & Lever, 2000). However, RNA trafficking may ultimately influence the destiny of an mRNA. Swanson et al. (2004) described how the route that an mRNA takes affects the function and therefore the fate of the encoded protein. In addition, Poole et al. (2005) showed in HIV-1 that, for efficient packaging to occur, RNA and Gag had to interact at a certain point within the cell. Alterations to RNA transport could therefore affect when and where (or whether) these interactions occur and consequently result in a defect in virus encapsidation. Thus, a relatively modest global export defect may conceal a larger effect on trafficking of some of the RNA to a critical site where encapsidation may occur.

Interaction of the Rev protein with the loop A motif in the packaging signal of HIV-1 appears to be essential for optimal virus replication. In the context of the virus, a mutation in the RNA loop that caused impaired Rev binding in vitro led to a replication defect, characterized by a decrease in and loss of specificity of viral RNA encapsidation. The accompanying alteration in nuclear export of genomic RNA suggests that this defect resulted from altered RNA trafficking.

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REFERENCES


