Reduced Mobilization of Rev-Responsive Element-Deficient Lentiviral Vectors

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Infection of cells transduced with a lentiviral vector by human immunodeficiency virus (HIV) could lead to packaging of the lentiviral vector RNA into HIV particles and unintended transfer of the vector. To prevent this, the Rev-responsive element (RRE) of an HIV-1 vector was functionally replaced by a heterologous RNA element (MS2). Providing Rev fused to an MS2 binding protein allowed efficient vector production. Mobilization of the vector from infected target cells was below the level of detection and at least 10^3 - to 10^4 -fold lower than for the RRE-containing vector. Thus, RRE-deficient lentiviral vectors provide a novel approach to reduce the risk of vector mobilization.

Lentiviral vectors based on human immunodeficiency virus (HIV) (3, 18, 21), simian immunodeficiency virus (SIV) (14, 22), feline immunodeficiency virus (10, 19), and equine infectious anemia virus (16) can transduce nondividing cells and thus allow efficient gene transfer into terminally differentiated cells of various tissues. However, coinfection of cells transduced with a primate lentiviral vector and HIV could lead to packaging and transfer of the lentiviral vector. Currently, self-inactivating vectors are used to reduce the risk of vector mobilization. Deletion of the 3' U3 region of the lentiviral vector leads to a promoter-less 5' long terminal repeat (LTR) after one round of reverse transcription. Therefore, packaging sequences of the lentiviral vector integrated in the genome of the target cell should be poorly transcribed. Although self-inactivating vectors reduce vector mobilization, a low level of transcription of 5' lentiviral vector sequences and/or mobilization has been observed despite deletion of the TATA box and enhancer elements from the 3' U3 region (8, 17, 22, 25, 26). Recently, Logan et al. (11) observed that residual promoter activity of an HIV-1 SIN vector resides in the 5' leader sequence of HIV-1 overlapping with packaging sequences. Although downstream of the transcriptional initiation site, this element is capable to initiate transcription at the 5' end of R, which should lead to the synthesis of a transcript that can be packaged and transferred. Additional mechanisms that might contribute to mobilization of SIN vectors are promoter trapping by integration of the lentiviral vector downstream of an active cellular promoter and recombination events leading to the reconstitution of a transcriptional active 3' U3 region of the lentiviral vector during vector production. Mutation of the primer binding site (PBS) of an SIV vector and complementation of the PBS mutations during vector production by a matched artificial tRNA provided an independent approach to reduce the risk of SIV vector mobilization by HIV-1 (8, 9). However, mobilization of the SIV vector by homologous SIV was reduced to a lesser extent.

Therefore, the aim of the study was to develop and evaluate a third approach to prevent lentiviral vector mobilization that is also active against homologous lentiviruses. During transcription of HIV the viral Rev proteins binds to the Rev-responsive element (RRE) sequence tethering the viral RNA to the CRM-1 export pathway (6, 20). In the absence of Rev or RRE the genomic RNA of HIV is either spliced or degraded and does not efficiently reach the cytoplasm, a prerequisite for packaging. However, direct interaction of Rev with the RRE is not required, since tethering Rev as a fusion protein to a heterologous RNA element introduced into the unspliced HIV RNA also mediates efficient export of the unspliced RNA and subsequent translation of the gag-pol precursor protein (15, 23). While these studies were performed with constructs containing large fragments of the HIV genome, the genomic RNA of lentiviral vectors in the cytoplasm was recently shown to be only marginally enhanced by Rev despite the presence of the RRE (2). In contrast to the minor effect on cytoplasmic RNA levels, Rev increased vector titer and infectivity at least 10-fold (2). We now replaced the RRE of an HIV-1 vector by the MS2 stem loop and rescued the RRE defect during vector production by fusing Rev to an MS2 binding protein. Although genomic vector RNA levels were readily detectable in the cytoplasm of cells transduced with the RRE-deficient vector, mobilization of the RRE-deficient vector was severely reduced in several assays.

Construction and characterization of RRE-deficient lentiviral vectors. A map of the four different vector constructs used in this study is shown in Fig. 1A. HIV-CL-CG and its self-inactivating counterpart HIV-CS-CG are RRE-containing prototype HIV vectors previously described (17). In HIV-CS-CG-MS and HIV-CL-CG-MS, the RNA binding site of the coat protein of the bacteriophage MS2 replaces stem loop II of the RRE as previously described (23).

All vectors were cotransfected with a codon-optimized expression plasmid for HIV-1 gag-pol (24) and expression plasmids for VSV-G (7) and Tat (13) in the presence or absence of expression plasmids for Rev (13) or the Rev/MS-C fusion protein (23). Transfection of 293T cells and vector titration was performed as previously described (8). Cotransfection of wild-type HIV-CL-CG and HIV-CS-CG with Rev and Rev/MS-C

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FIG. 1. Map of HIV-1 vector constructs (A) and vector titers (B). (A) The Rev-responsive element (RRE) and the MS2 stem-loop (MS) are shaded, the black triangles represent a deletion of the TATA box and enhancer elements from the 3'U3 region. (B) 293T cells were transfected with the different vector constructs and expression plasmids for codon-optimized gag-pol (Hgp^{syn}), VSV-G (pHIT-G), and Tat (pcTat) in the presence or absence of plasmids encoding Rev or Rev/MS-C fusion protein. Vector titers in the supernatant of transfected cells were determined on 293A cells. The means of three independent experiments and the standard deviations are shown. GFU, GFP-forming units.

FIG. 2. Cytoplasmic vector RNA levels (A) and mobilization by transfection of packaging plasmids (B). 293T cells transduced with the indicated vectors were transiently transfected with Hggsyn and pHIT-G in the presence or absence of expression plasmids for Tat, Rev, or Rev/MS-C fusion protein. (A) Northern blot analysis from cytoplasmic RNA with a probe spanning the R-region and parts of the 5' untranslated region reveals the genomic vector RNA, the spliced vector RNA, and the transcript originating from the internal cytomegalovirus promoter. (B) Vector titers in the supernatant of transfected cells were determined on 293A cells. The means of three independent experiments and the standard deviations are shown. GFU, GFP-forming units.

led to an approximately 30- to 200-fold increase in vector titers (Fig. 1B). In contrast, only cotransfection of Rev/MS-C, but not Rev, resulted in high titers of HIV-CS-CG-MS and HIV-CL-CG-MS, indicating successful tethering of the vector RNA to the CRM-1 export pathway by the Rev/MS-C fusion protein. The vector titers obtained with the RRE-deficient vectors in the presence of Rev-MS2 are comparable to the titers obtained with the parental vector.

Mobilization of vectors from transduced cells by infection with HIV-1. To study the effect of the RRE deficiency on vector mobilization, the CD4 receptor and coreceptor-positive P4CCR5 indicator cells (4) were transduced with the same infectious dose of HIV-CL-CG and HIV-CL-CG-MS vector particles prepared by transient cotransfection with expression plasmids encoding codon-optimized gag-pol, VSV-G, and Tat, and Rev or Rev/MS-C, respectively. After expansion, fluorescence-activated cell sorter analyses revealed that approximately 60% of P4CCR5 cells were green fluorescent protein (GFP) positive (data not shown). These cells were then infected with an HIV-1 stock, and supernatants of these vector-transduced and HIV-infected cultures were analyzed for vector titer and HIV-1 titer (Table 1). While the HIV-1 titer was comparable in HIV-CL-CG- and HIV-CL-CG-MS-transduced

cultures, a vector titer (1.6×10^3 GFP-forming units [GFU]/ml) was only obtained in the HIV-CL-CG transduced cells but not in the cells transduced with the RRE-deficient lentiviral vector HIV-CL-CG-MS (<1 GFU/ml). Thus, mobilization of the RRE-deficient lentiviral vector by HIV-1 is reduced at least 1,000-fold. The self-inactivating vectors HIV-CS-CG and HIV-CS-CG-MS, analyzed in parallel, could not be mobilized by HIV-1 either (Table 1).

TABLE 1. Vector mobilization by infection with HIV-1^a

Vector P4CCR5 cells were transduced with	Titer	
	HIV-1 (BFU/ml)	Vector (GFU/ml)
HIV-CL-CG	$9.74 \times 10^4 \pm 4.11 \times 10^4$	$1.55 \times 10^3 \pm 8.36 \times 10^2$
HIV-CL-CG-MS	$4.71 \times 10^4 \pm 9.72 \times 10^3$	<1
HIV-CS-CG	$9.75 \times 10^4 \pm 1.11 \times 10^4$	<1
HIV-CS-CG-MS	$7.65 \times 10^4 \pm 1.49 \times 10^4$	<1

^a P4CCR5 indicator cells were transduced with the indicated vectors and superinfected with the HIV-1 molecular clone NL4-3 (1). HIV-1 and vector titers were determined after 2 days in the supernatant of transduced and infected cells. The means of three independent experiments and the standard deviations are shown. BFU, β-galactosidase-forming units; GFU, GFP-forming units.

TABLE 2. Vector mobilization by transfection of an *env* deletion mutant of HIV^a

Vector 293T cells were transduced with	Vector titer (GFU/ml) with:	
	Rev/MS-C	None
HIV-CL-CG	$4.05 \times 10^3 \pm 9.19 \times 10^2$	$7.35 \times 10^3 \pm 3.32 \times 10^3$
HIV-CL-CG-MS	$3.45 \times 10^3 \pm 9.19 \times 10^2$	<1
HIV-CS-CG	<1	<1
HIV-CS-CG-MS	<1	<1

^a 293T cells were transduced with the indicated vectors and transfected with an HIV-1 *env* deletion mutant (5) of pNL4-3, pHIT-G, and with or without Rev/MS-C fusion protein. Vector titers in the supernatant were determined 2 days after transfection. The means of three independent experiments and the standard deviations are shown. GFU, GFP-forming units.

Mobilization of vectors by transient transfection of packaging plasmids. The vector titers obtained by infection of HIV-CL-CG transduced P4CCR5 cells with HIV-1 were only approximately 1,000-fold above the level of detection. Therefore, it was only possible to conclude that the RRE-deficient vector is mobilized at least 1,000-fold less efficient than the parental control vector. To increase the sensitivity of the mobilization assay, 293T cells were transduced with HIV-CL-CG, HIV-CL-CG-MS, HIV-CS-CG, or HIV-CS-CG-MS vector particles as described for P4CCR5 cells. Transduced 293T cells were then cotransfected with an HIV-1 *env* deletion mutant and a VSV-G expression plasmid in the presence or absence of a plasmid encoding Rev/MS-C. The vector titers obtained in HIV-CL-CG transduced 293T were around 5×10^3 GFU/ml (Table 2) independent of cotransfection of Rev/MS-C, since the HIV-1 *env* deletion mutant encoded Rev. In contrast, HIV-CL-CG-MS could not be mobilized in the absence of Rev/MS-C. Lack of mobilization was not due to inefficient vector transduction, since cotransfection with Rev/MS-C plasmid led to mobilization of the HIV-CL-CG-MS vector at a titer of 3.4×10^3 GFU/ml. Mobilization of the self-inactivating vectors remained undetectable.

To investigate whether prevention of mobilization of the RRE-deficient vectors is attributable to the well-described Rev transport function, Northern blot analysis was performed to evaluate expression of genomic vector RNA in the cytoplasm of cells transduced with non-self-inactivating vectors HIV-CL-CG and HIV-CL-CG-MS (Fig. 2A). To induce transcription from the HIV-1 LTR of the vector, cells were cotransfected with a Tat expression plasmid in the presence or absence of Rev or Rev/MS-C. Bands corresponding to the genomic vector RNA (3.8 kb) and the spliced transcript (2.8 kb) were only detectable in the presence of Tat, while the transcript driven by the internal cytomegalovirus promoter (1.9 kb) was detected in all samples. Cotransfection of Rev increased the genomic RNA levels of HIV-CL-CG but not HIV-CL-CG-MS, while Rev/MS-C had no detectable effect on genomic RNA levels. Since the same cells had also been cotransfected with a codon-optimized HIV-1 gag-pol expression plasmid and a VSV-G expression plasmid, the influence of Rev and Rev-MS/C on vector mobilization could be determined in parallel. Titers obtained after mobilization of HIV-CL-CG in the presence of Rev or Rev-MS/C exceeded 10^4 GFU/ml (Fig. 2B). For the RRE-deficient vector, mobilization titers were 6.3×10^3 GFU/ml in the presence of Rev/MS-C but less than 1

GFU/ml in its absence or in the presence of Rev. Mobilization of the self-inactivating vector and the vector which is RRE-deficient and self-inactivating remained undetectable, indicating that both approaches reduce vector mobilization by more than a factor of 10^4 .

Despite similar levels of genomic vector RNA in the cytoplasm, the mobilization titer of HIV-CL-CG-MS is more than 6,000-fold higher in the presence of Rev/MS-C than Rev. Our study thus confirms a recent study by Anson et al., showing poor correlation of cytoplasmic vector RNA levels exported by constitutive transport elements and vector titers (2). Both studies support the notion that delivery of genomic vector RNA to the cytoplasm is not sufficient for vector infectivity and suggest that Rev function extends beyond transport of viral RNA from the nucleus to the cytoplasm. Furthermore, our results indicate that binding of Rev to the genomic vector RNA is also required for the extended Rev function. Although the precise molecular mechanism warrants further investigation, deletion of the RRE clearly provides a novel approach to reduce lentiviral vector mobilization.

Mobilization of the self-inactivating vector and the RRE-deficient vector were both reduced by more than a factor of 10^4 . The increase in vector titer by providing Rev function is much stronger in vector-transduced cells than in transiently transfected 293T cells. This is probably the result of overexpression of vector RNA in transiently transfected cells and underlines the importance of checking vector mobilization from transduced cells. Since RRE-deficient vectors reduce mobilization by a posttranscriptional mechanism while SIN vectors block transcription, both approaches should act synergistically. Despite extensive efforts, mobilization of the RRE-deficient vector and the SIN vector remained undetectable. Thus, it was not possible to prove the synergistic effect.

It was recently shown that mobilization of a different HIV-1 SIN vector was readily detectable, but mobilization efficiency had not been precisely quantified relative to the parental vector (11). Nevertheless, the HIV-1 SIN vector used in the previous study seemed to be more efficiently mobilized, since mobilization of the SIN vector and the wild-type vector resulted in transduction of 0.7% and 9.4% of the target cells, respectively. Transduction of cells with the same multiplicity of infection, and a side by side comparison in the same mobilization assay will be necessary to reveal whether the HIV SIN vectors used indeed differ in mobilization efficiency. Similarly, deletion of the U3 region of an SIV vector reduced mobilization by a factor of 2.6×10^5 , but mobilization of the SIV SIN vector was clearly detectable (8). Thus, increasing evidence suggests that deletion of the U3 region in lentiviral vectors is not sufficient to prevent vector mobilization under all circumstances. The RRE-deficient lentiviral vectors provide a highly effective alternative approach that could also be used to reduce vector mobilization of lentiviral vectors transcriptionally targeted by tissue-specific promoters in the 3'/U3 region (12). Combining the SIN approach with RRE-deficient lentiviral vectors should further improve the safety of lentiviral vectors, which might be particularly relevant for gene transfer into hematopoietic cells.

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