Effects of the ligand sequence modifications on the retargeted transduction by the retroviral vector having a ligand-chimeric Env protein

Kei Miyakawa,† Rika Fujita,† Masumi Katane,† Yoshinao Kubo‡ and Hiroshi Amanuma†

†Discovery Research Institute, RIKEN, Wako, Saitama 351-0192, Japan
‡Department of AIDS Research, Institute of Tropical Medicine, Nagasaki University, Nagasaki 852-8523, Japan

Correspondence
Hiroshi Amanuma
h-amanuma@nihs.go.jp

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INTRODUCTION

Replication-defective retroviral vectors have been valuable tools in both molecular biology research and human gene therapy clinical trials. Nevertheless, several fundamental properties of retroviral vectors remain to be improved before these vectors will be safe and effective for clinical use (Baum et al., 2006). For example, the safety and effectiveness of retroviral vectors will be greatly enhanced if their cell entry receptor tropism is artificially altered. There have been various attempts to establish strategies for redirecting the entry receptor tropism of ecotropic and amphotropic murine leukemia virus (MLV) vectors by genetically incorporating heterogeneous ligand sequences, such as those of peptides, small proteins and single-chain antibodies, into the viral envelope (Env) proteins (Sandrin et al., 2003).

One potential way to alter entry receptor tropism is a direct targeting strategy, where the ligand directs the binding of the chimeric Env protein to an alternative entry receptor and this binding activates the membrane fusion potential of the chimeric Env protein (Russell & Cosset, 1999). However, a reliable and versatile method for production of high-titre retargeted vectors based on this strategy has not yet been obtained (Sandrin et al., 2003). Major difficulties lie in (i) the cellular transport of nascent chimeric Env precursors to the cell surface and subsequent incorporation into the viral envelope, (ii) coupling between the alternative entry receptor binding of the chimeric Env protein and activation of its membrane fusion function, and (iii) possible structural requirements for the targeted membrane protein to be used as an alternative MLV entry receptor (Benedict et al., 1999; Katane et al., 2002; Wu et al., 2000; Zhao et al., 1999).

We have recently reported the successful direct targeting of the ecotropic (mouse and rat cell-tropic) Moloney (Mo) MLV vector (Katane et al., 2002, 2004). The retargeted vectors (S3 and S3-D84K) contain the Mo-MLV Env protein into which a full-length human stromal cell-
derived factor-1alpha (SDF-1α) sequence (68 aa) was inserted at Pro-79 as a model ligand (Fig. 1a). These vectors transduce human cells (HOS.CXCR4 cells) through the CXC chemokine receptor 4 (CXCR4) and number in parentheses indicate those of the MO protein. Various structural elements of SDF-1α are labelled below the S3-D84K protein. NTD, N-terminal domain.

METHODS

Construction of expression plasmids for the mutant S3-D84K Env proteins. The S3-D84K Env expression plasmid (pT-S3-D84K) (Katane et al., 2004) had inadvertently incorporated a ~110 bp Mo-MLV env sequence between the EcoRI and BamHI sites in the upstream multiple cloning region of pTarget (Promega). This sequence is located 47 bp upstream of the translation initiation ATG codon for the S3-D84K Env protein, thus leaving the chimeric Env reading frame intact. We constructed a new S3-D84K Env plasmid without this extraneous sequence. Briefly, a 0.8 kb BamHI fragment corresponding to the N-terminal portion of the Mo-MLV Env surface unit (SU) was removed from pCE4.1 that encodes the whole Env protein (Katane et al., 2002). The rest of pCE4.1 was ligated with the 1.0 kb BamHI fragment from pKBS-S3-D84K (Katane et al., 2004). This fragment had a full-length SDF-1α sequence inserted at the Pro-79 site and the env codon for Asp-84 had been mutated to Lys. We confirmed that the resulting construct (pT-S3-D84K-2) lacked the extraneous sequence. pT-S3-D84K-2 was used to produce the S3-D84K Env protein. There were no differences between the two S3-D84K Env plasmids with regard to the transduction ability of retroviral vectors pseudotyped by the encoded chimeric Env proteins.

Plasmids encoding mutant S3-D84K Env proteins were constructed by replacing the SDF-1α-encoding SacI cassette of pT-S3-D84K-2 with that encoding the modified SDF-1α. Two SacI cassettes encoding full-length SDF-1α had inadvertently incorporated a ~110 bp Mo-MLV env sequence between the EcoRI and BamHI sites in the upstream multiple cloning region of pTarget (Promega). This sequence is located 47 bp upstream of the translation initiation ATG codon for the S3-D84K Env protein (Katane et al., 2002). However, the S3-D84K vector lacks this ecotropism due to an Asp-84-to-Lys (D84K) mutation (Katane et al., 2004) known to abolish the binding of Mo-MLV Env protein to mCAT1 (MacKrell et al., 1996). As the titres of retargeted transduction by these vectors may be insufficient for practical applications, we investigated how changes in the structure of the SDF-1α ligand affect these titres. We hypothesized that certain modifications of the ligand might improve incorporation of the chimeric Env protein into the envelope and/or increase the efficiency in coupling between the alternative receptor-binding and post-binding fusion events.

In this study, we modified the SDF-1α ligand of the S3-D84K Env protein by truncations from its C terminus or by Cys-to-Ala changes of the disulfide-forming cysteines. Modified chimeric Env proteins were evaluated for their incorporation into the envelope and their binding to CXCR4. Retargeted transduction titres of the vectors displaying the modified chimeric Env proteins in the envelope were measured using two CXCR4-expressing human cell lines.
Oligonucleotides 1F(C9A) and 68R for CA-N, and 1F(2CA) and 68R(C50A) for CA-NC were used as primers. For a SacI cassette for the CA-C Env, pT35SN was used as a PCR template and 1F(C11A) and 68R(C50A) as primers.

Cell lines. The human cell line TELCeB6 expresses Mo-MLV Gag and Pol proteins and an nlsLaC-zoding MLV vector genome RNA (Cosset et al., 1995). HOS.pBABE-puro and HOS.CXCR4, human osteosarcoma HOS cell lines transduced with MLV vectors expressing either the puromycin-resistance gene or human CXCR4 and puromycin-resistance genes (Deng et al., 1996), were obtained through the AIDS Research and Reference Reagent Program, NIH, USA. The human glioma cell line NP-2 (Yamazaki, 1982) and its CD4, CXCR4-expressing derivative, NP-2.CXCR4, were kindly provided by J. Komano (National Institute of Infectious Diseases, Japan). In the latter, CXCR4 is expressed as a fusion protein with a C-terminal green fluorescent protein. Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum.

Western blot analysis of chimeric Env proteins. To analyse the expression of chimeric Env proteins, TELCeB6 cells were transfected with each Env expression plasmid by lipofection using TransIT-LT1 (5 μg per μg DNA; Mirus). Forty-eight hours after transfection, cell lysates were prepared using RIPA buffer by incubation at 4 °C for 30 min and centrifugation at 16 000 g for 10 min to remove cell debris, filtered through a 0.45 μm membrane filter (Millex-HV; Millipore) and used immediately or stored at −80 °C. Wild-type Mo-MLV and amphotropic MLV vectors were similarly produced from TELCeB6 cells after stable transfection of pCE4.1 and pCMV4070A (kindly provided by J. M. Heard, Institut Pasteur, France), respectively.

Cell transduction assay. Recipient cells (1 × 10⁶ HOS.pBABE-puro and HOS.CXCR4 cells, or 2–5 × 10⁵ NP-2 and NP-2.CXCR4 cells) were seeded in six-well tissue culture plates. The next day, cells were exposed to 1 ml retroviral vector solution at 37 °C for 4 h. Appropriately diluted vector solutions were used to transduce cells at low m.o.i. (<0.1 c.f.u. per cell). Polybrene was included at 1 μg ml⁻¹ except in the case of the amphotropic MLV vector, where a concentration of 5 μg ml⁻¹ was used. Fresh medium (2 ml) was added, and the cells were cultured for 44 h prior to staining with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside to detect cells expressing Escherichia coli β-galactosidase. The number of colonies containing stained cells was counted under a microscope. Transduction titres of the vectors (c.f.u. ml⁻¹) were determined from duplicate assays. For the SDF-1α inhibition experiment, NP-2.CXCR4 cells were exposed to 1 ml retroviral vector solution containing an indicated concentration of soluble SDF-1α (S190; Sigma-Aldrich) for 4 h and then the fresh medium (1 ml) containing the same concentration of SDF-1α was added.

Retroviral vector binding to cells. Retroviral vector binding to cells was detected by a modification of the method described previously (Kadan et al., 1992). NP-2.CXCR4 cells (5 × 10⁵) were resuspended in 200 μl PBS containing 0.2% BSA (PBS-BSA). To this, 1 ml freshly prepared retroviral vector solution containing polybrene (1 μg ml⁻¹) was added, and the mixture was incubated on ice for 30 min for binding. In several cases, retroviral vector solutions were concentrated 10-fold by a centrifugal filter device (Centricon Plus-20, 100K NMWL; Millipore) before use. A culture supernatant of the untransfected TELCeB6 cells was used as a background control after centrifugation and filtration (no-Env vector). To terminate binding, cells were washed twice with 2 and 2.5 ml cold PBS-BSA. Cells were then resuspended in 500 μl PBS-BSA, anti-SU (1 μl) was added, and the mixture was incubated on ice for 30 min. Cells were washed with 2 ml PBS-BSA. They were resuspended again in 500 μl PBS-BSA, phycoerythrin (PE)-conjugated donkey F(ab)²-anti-goat IgG (H + L) (Oxford) was added, and the mixture was incubated on ice for 30 min. Cells were washed similarly with PBS-BSA. Stained cells were resuspended in 500 μl PBS containing 1% formaldehyde and analysed on an Epic X flow cytometer (Beckman Coulter). For the SDF-1α inhibition experiment, an indicated concentration of SDF-1α was included in the binding mixtures containing either the S3-D84K vector or the no-Env vector.

RESULTS

C-terminal truncations of the SDF-1α ligand in the S3-D84K Env protein

Since the CXCR4 binding by SDF-1α is thought to occur through the N-terminal portion of SDF-1α (NTD and N-loop, Fig. 1b) (Crump et al., 1997), we first examined the effects of truncations from the C terminus of SDF-1α. By considering the secondary structure of the protein, which includes three β-strands and one α-helix (Crump et al., 1997), a total of seven truncation mutant plasmids were constructed (Fig. 1b). C-terminal truncations were by 3 (CT65), 12 (CT56), 17 (CT51 and CT51M), 33 (CT35M) and 51 (CT17 and CT17M) aa from the 68 aa sequence. The full-length SDF-1α in the chimeric Env protein probably forms two disulfide linkages, between Cys-9 and Cys-34 and between Cys-11 and Cys-50. Several of the imposed C-terminal truncations cause a loss of cysteine residue(s) or might prevent disulfide linkage formation, which leads to the appearance of unlinked cysteine(s) that might inhibit proper folding of the chimeric Env proteins. Therefore, we introduced Cys-to-Gly changes into three of the truncation mutants (CT51M, CT35M and CT17M). We examined how C-terminal truncations affected the amount of chimeric Env protein incorporated into the envelope. By Western blot analysis of TELCeB6 cells transiently transfected with a chimeric Env expression plasmid, we found that the amount of the cellular Env
Mock, virions prepared from the untransfected TELCeB6 cells.

The lower part of the same blot was probed with the anti-capsid protein plasmid, and were analysed using anti-SU (upper panel). The TELCeB6 cells stably transfected with each Env expression plasmid were analysed using the anti-SU proteins. (a) Lysates of TELCeB6 cells transiently transfected with each Env expression plasmids were collected by centrifugation from the culture supernatants (2 ml) of TELCeB6 cells stably transfected with each Env expression plasmid, and were analysed using anti-SU (upper panel). The lower part of the same blot was probed with the anti-capsid protein (anti-CA) antibody to confirm collection of the virions (lower panel). Mock, virions prepared from the untransfected TELCeB6 cells.

![Western blot analysis of expression (a) and virion incorporation (b) of the truncation mutant SDF-1z-chimeric Env proteins.](image)

**Fig. 2.** Western blot analysis of expression (a) and virion incorporation (b) of the truncation mutant SDF-1z-chimeric Env proteins. (a) Lysates of TELCeB6 cells transiently transfected with each Env expression plasmid were analysed using the anti-SU antibody. Mock, transfection without a plasmid. (b) Virions were collected by centrifugation from the culture supernatants (2 ml) of TELCeB6 cells stably transfected with each Env expression plasmid, and were analysed using anti-SU (upper panel). The lower part of the same blot was probed with the anti-capsid protein (anti-CA) antibody to confirm collection of the virions (lower panel). Mock, virions prepared from the untransfected TELCeB6 cells.

**Table 1.** Transduction titres of retroviral vectors containing the mutant S3-D84K Env protein

<table>
<thead>
<tr>
<th>Env†</th>
<th>Transduction titre (c.f.u. ml⁻¹) for the cell line*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HOS.pBABE-puro</td>
</tr>
<tr>
<td>S3-D84K</td>
<td>2.0 × 10⁴</td>
</tr>
<tr>
<td>CT65</td>
<td>&lt;10</td>
</tr>
<tr>
<td>CT56</td>
<td>&lt;10</td>
</tr>
<tr>
<td>CT51</td>
<td>&lt;10</td>
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<tr>
<td>CT51M</td>
<td>&lt;10</td>
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<tr>
<td>CT35M</td>
<td>&lt;10</td>
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<tr>
<td>CT17</td>
<td>&lt;10</td>
</tr>
<tr>
<td>CT17M</td>
<td>&lt;10</td>
</tr>
<tr>
<td>CA-N</td>
<td>&lt;10</td>
</tr>
<tr>
<td>CA-C</td>
<td>&lt;10</td>
</tr>
<tr>
<td>CA-NC</td>
<td>&lt;10</td>
</tr>
<tr>
<td>AM‡</td>
<td>8.0 × 10⁵</td>
</tr>
</tbody>
</table>

*Each value shown is an average of the duplicate assays.
†Env protein that pseudotyped the nlsLacZ MLV vector.
‡Amphotropic MLV.

The precursor was similar among the Mo-MLV Env (MO), S3-D84K Env and the truncation mutant Envs (Fig. 2a). In contrast, the amount of the processed form of chimeric Env protein produced by the stably transfected TELCeB6 cell pool and incorporated into the envelope was highly variable (Fig. 2b). Similar to our previous results (Katane et al., 2004), the amount of S3-D84K SU protein per virion was very low compared with the MO SU protein. In mutants with a C-terminal truncation but without a Cys-to-Gly change, the amount of virion SU protein either did not increase much relative to the S3-D84K Env (CT65 and CT17) or decreased severely (CT56 and CT31). Cys-to-Gly changes were effective in enhancing incorporation of the chimeric Env protein into the envelope, as seen with CT51M, CT35M and CT17M. Of the seven truncation mutants, two (CT35M and CT17M) showed a significantly increased amount of chimeric Env protein incorporated into the envelope as compared with the S3-D84K Env.

We then measured retargetted transduction by nlsLacZ retroviral vectors displaying each mutant chimeric Env protein in the envelope. Two CXCR4-expressing human cell lines (HOS.CXCR4 and NP-2.CXCR4) were used as recipients of transduction. Flow cytometric analysis revealed that NP-2.CXCR4 cells expressed 2.4–2.9 times as much cell surface CXCR4 per cell as HOS.CXCR4 cells (Supplementary Fig. S1, available in JGV Online). Table 1 summarizes transduction titres of the vectors towards these cell lines. As references, those of the S3-D84K Env and the amphotropic MLV (AM) Env vector are also shown. Similar to our previous report (Katane et al., 2004), the S3-D84K Env vector showed a titre of ~10⁴ c.f.u. ml⁻¹ in HOS.CXCR4 cells. Remarkably, in NP-2.CXCR4 cells the S3-D84K vector exhibited a more than 10-fold higher titre (almost 10⁶ c.f.u. ml⁻¹) than in HOS.CXCR4 cells. Of the seven truncation mutant vectors, only the CT65 vector transduced both cell lines, but the titres were significantly lower than those of the S3-D84K vector. The other six mutant vectors did not transduce HOS.CXCR4 cells at all, and two of them (CT51M and CT35M) showed a marginal titre in NP-2.CXCR4 cells. Transduction of the NP-2.CXCR4 cell line by the SDF-1α-chimeric Env retroviral vectors was apparently dependent on the expression of CXCR4, as suggested by the result that the NP-2 cell line could be efficiently transduced by the AM vector but not at all by the SDF-1α-chimeric Env vectors (Table 1). More direct evidence showing that the SDF-1α-chimeric Env vectors transduce NP-2.CXCR4 cells through CXCR4 was obtained from the soluble SDF-1α inhibition experiment. As shown in Fig. 3, soluble SDF-1α inhibited transduction of NP-2.CXCR4 cells by the S3-D84K vector specifically and dose-dependently.

**Cys-to-Ala changes of the SDF-1α ligand in the S3-D84K Env protein**

As described above, Cys-to-Gly changes in the truncated SDF-1α ligand increased virion incorporation of the chimeric Env protein. We next examined whether alanine substitution
for disulfide-forming cysteines of the SDF-1α ligand in the absence of truncation improved virion incorporation of the chimeric Env protein and transduction titre of the vector. We constructed three alanine mutant plasmids, as shown in Fig. 1(b). CA-N and CA-C had their N- and C-terminal disulfide-forming cysteines replaced by alanines, respectively, and CA-NC had all four cysteines replaced. All alanine mutant chimeric Env proteins showed a level of incorporation into the viral envelope similar to the S3-D84K Env protein (Fig. 4). The CA-N vector exhibited transduction titres similar to those of the S3-D84K vector, whereas the other alanine mutant vectors were not able to transduce HOS.CXCR4 cells and had only marginal titres in the NP-2.CXCR4 cell line (Table 1).

CXCR4 binding by retroviral vectors containing the mutant SDF-1α-chimeric Env protein

As described above, eight of the ten mutant chimeric Env proteins, CT65, CT51M, CT35M, CT17, CT17M, CA-N, CA-C and CA-NC, were incorporated into the viral envelope with an efficiency that was similar to or greater than the parental S3-D84K Env protein. Only the CT65 and CA-N Envs were found to retain the ability to mediate the retargeted transduction of the vector. To identify the steps in the Env function defective in the other six mutant proteins, we performed flow cytometric virus vector-to-cell binding assays using NP-2.CXCR4 cells.

When the parental S3-D84K vector was used in this assay, a twofold increase in mean fluorescence intensity (MFI) over the no-Env vector control was observed between two histograms (Fig. 5b). In a separate experiment, the presence of soluble SDF-1α (100–200 nM) in the virus binding mixtures significantly suppressed this increase in MFI (Fig. 5a). We observed no increase in MFI by the S3-D84K vector in the NP-2 cell line (data not shown). These results suggested that this increase in MFI represents specific binding of the virus vector to CXCR4 on the cell surface. As only the CT65 and CA-N vectors exhibited CXCR4 binding (Fig. 5b), the inability of the six mutant chimeric Env proteins to mediate the retargeted transduction was mainly due to a loss of the CXCR4-binding activity.

DISCUSSION

Sequence modifications of the SDF-1α ligand in the S3-D84K chimeric Env protein examined in this study were found to affect variably the virion incorporation of the chimeric Env protein, to abolish readily its CXCR4-binding activity, and to be unable to improve the retargeted transduction titres in two CXCR4-expressing human cell lines. This study, however, should be useful for understanding the nature of the retargeted retroviral vectors and for constructing such vectors with different ligands.

The retargeted transduction titre of the parental S3-D84K vector in NP-2.CXCR4 cells was close to $10^6$ c.f.u. ml$^{-1}$ (Table 1), which was nearly the same as that of the AM vector in these cells. Several lines of evidence (Figs 3, 5a) confirmed that the S3-D84K vector transduces NP-2.CXCR4 cells through CXCR4. A significantly higher transduction titre
positive correlation between the entry receptor density on the target cell and the transduction efficiency has been previously reported for another retroviral vector (Kurre et al., 2001). As NP-2.CXCR4 cells are artificially engineered cells and may be an exceptional case, it remains necessary to develop a strategy for improving the transduction efficiency of the S3-D84K vector.

A ligand will not be able to bind to the receptor unless the native conformation of its receptor-binding site is maintained. Even if the ligand sequence modification does not involve a change in the amino acid sequence of the receptor-binding site, it may cause a conformational change in that site. Many of the ligand modifications examined in this study must have induced a conformational change in the receptor-binding site of SDF-1α. This idea maybe useful for explaining the CXCR4-binding abilities of the CA-N and CA-C mutants. The CA-N mutant retained, while the CA-C mutant nearly lost, the abilities to transduce CXCR4-expressing cells and bind to CXCR4 (Table 1 and Fig. 5b). The CA-C mutant lacked the disulfide bond between Cys-11 and Cys-50 (Fig. 1b). This may have led to a conformational rearrangement of the N-loop region containing the RFFESH motif (residues 12–17). This motif has been proposed to be an initial receptor-binding site of SDF-1α (Crump et al., 1997). Although the CA-N mutant lacked the disulfide bond between Cys-9 and Cys-34 (Fig. 1b), maintenance of the Cys-11/Cys-50 linkage may have allowed the N-loop region to assume its native conformation.

Following sequence modification, a small protein ligand flanked covalently by heterologous protein sequences at both its N and C termini may undergo a conformational rearrangement more readily than the one with free termini. Consequently, a full-length sequence that folds into a distinct domain within the ligand-chimeric Env protein is preferable as a targeting ligand. Related discussion by Bahrami et al. (2007) has appeared elsewhere.

Eight of the ten mutant chimeric Env proteins were assayed for their abilities to bind to CXCR4. As a result, we found a good correlation between the ability to bind to CXCR4 (Fig. 5b) and the ability to mediate the retargeted transduction (Table 1). This suggests that the post-binding fusion events are invariably induced in the chimeric Env protein upon binding of the ligand moiety to CXCR4, if the ligand is inserted at Pro-79. For construction of the functional retargeted Env protein, usefulness of the Pro-79 site as a ligand insertion site should be emphasized.

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