The block to membrane fusion differs with the site of ligand insertion in modified retroviral envelope proteins

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Efforts to achieve cell type-specific transduction of retroviral vectors for gene therapy have centred on modification of the envelope protein (Env). Typically, addition of a ligand to Env gives binding to the new or target receptor, but little or no infection, and affects the subunit association of the modified Env. We previously discovered two point mutations that increase targeted infection by over 1000-fold when added to an Env modified by N-terminal insertion of the receptor-binding domain from amphotropic murine leukemia virus Env. Here, we asked whether these mutations would similarly increase transduction by Env modified with a clinically relevant ligand, human interleukin-13 (IL-13L). Addition of the point mutations stabilized the weak subunit association observed in some IL-13L-modified Env proteins, but infection via the target IL-13 receptor still did not occur. Fluorescence-based cell–cell fusion assays and studies with a membrane-curving agent revealed that defects in membrane fusion differed with the site of ligand insertion. When IL-13 was inserted into the N terminus of Env, membrane fusion was blocked prior to membrane-lipid mixing, regardless of whether flanking flexible linkers were added. Unexpectedly, insertion of IL-13 in the proline-rich region showed evidence of initiation of fusion and fusion-peptide exposure, but fusion was blocked at a subsequent step prior to fusion-pore formation. Thus, the site of ligand insertion influenced initiation of membrane fusion and its progression. These observations suggest that a novel site for ligand insertion must be identified before clinically useful targeted transduction will be achieved.

INTRODUCTION

The envelope protein (Env) of the gammaretrovirus Moloney murine leukemia virus (MoMLV) has been used most often for the purpose of modifying Env to achieve cell type-specific transduction by retroviral vectors. It has been modified with many different ligands, including growth factors (Kasahara et al., 1994), single-chain antibodies (Benedict et al., 1999; Lorimer & Lavictoire, 2000; Somia et al., 1995; Zhao et al., 1999) and short peptides (Gollan & Green, 2002; Hall et al., 1997).

Retroviral Env proteins consist of a surface protein or SU and a transmembrane anchor subunit (TM), many of which, including those of MoMLV, are covalently associated (Pinter et al., 1997). The N-terminal residues of SU form a receptor-binding domain (RBD) containing the sequences that bind the natural virus receptor. The RBD is followed by the proline-rich region (PRR) and a C-terminal domain (Battini et al., 1995). Functionally, Env is responsible for attachment, through its RBD, to host cells and for fusion of the viral and cellular membranes to give penetration of the virion core into the cell cytoplasm (Hunter, 1997).

The N terminus of SU has been the site used most frequently for insertion of ligands into Env, and insertion into the PRR has also been reported (Lavillette et al., 2001). In most reported cases, ligand insertion into either site did not interfere with incorporation of the modified Env into virions. Importantly, the modified Env proteins mediated specific attachment of virus to cells through the target receptor, but gave poor or no transduction of the target cells (Lavillette et al., 2001; Russell & Cosset, 1999). The modified Env proteins retained their ability to mediate fusion, as they gave efficient entry via the natural MoMLV receptor (Russell & Cosset, 1999). The accumulated findings led investigators to conclude that the modifications imposed
a post-binding block to entry through the target receptor alone (Lavillette et al., 2001).

The nature of the post-binding block was investigated previously by using an Env modified by a CD33-specific single-chain antibody inserted into the N terminus (Zhao et al., 1999). Virions containing this modified Env bound to cells expressing the target CD33 receptor, but these cells were not transduced (Zhao et al., 1999). In further analysis, Zhao et al. (1999) showed that the virus genome did not penetrate into the cell cytoplasm, indicating that virus–cell membrane fusion was blocked.

We showed previously that addition of two point mutations, Gln227 changed to arginine and Asp243 to tyrosine (Q227R and D243Y), to a modified Env increased infection via the target receptor substantially (Zavorotinskaya & Albritton, 2001). In that study, Env was modified by N-terminal insertion of the RBD from amphotropic MLV Env. Whilst this chimeric Env was not a clinically useful one because expression of the target amphotropic receptor is so widespread in vivo, it recapitulated a well-studied model reported previously by Cossett et al. (1995), who had shown it to be defective for infection.

In the current study, we sought to determine whether Q227R and D243Y might be similarly useful in enhancing the infectivity of Env modified with a clinically relevant ligand. We also asked whether the mutations increase infection when the ligand is inserted into the PRR. For this investigation, we constructed a set of modified Env proteins in which the human interleukin-13 (IL-13) sequence was inserted into the N terminus or the PRR of MoMLV Env. MLV vectors pseudotyped with IL-13-modified Env bound IL-13 receptor (IL-13R)-positive human cells, but none gave targeted transduction. Addition of Q227R and D243Y did not increase infection via IL-13R.

Fluorescence-based cell–cell fusion assays and studies with a membrane-curving agent revealed defects in membrane fusion. Importantly, the results showed that the block to fusion differed with the site of ligand insertion. When IL-13 was inserted into the N terminus of Env, membrane fusion was blocked prior to membrane–lipid mixing. However, Env with IL-13 inserted in the PRR showed evidence of outer-leaflet membrane mixing consistent with initiation of fusion and fusion-peptide exposure, but was blocked at a subsequent step prior to fusion-pore formation. We interpret these results as indicating that lack of transduction results primarily from failure of Env–target receptor binding to induce all of the conformational changes in Env that are required for viral and cell membrane fusion. We propose that novel positions for placing the ligand in Env will need to be identified in order to achieve targeted transduction approaching an efficiency that is clinically useful.

METHODS

Cell lines. Human 293 cells were the gift of Dr M. Quinlan (Guthrie Research Institute, Sayre, PA, USA). The human glioblastoma cell line U251 MG was kindly provided by Raj Puri (US Food and Drug Administration, Center for Biologics Evaluation and Research, Rockville, MD, USA).

Generation of 293 cells stably expressing human IL-13Rα2. The coding sequence of IL-13Rα2 was amplified from total RNA from U373 MG cells (ATCC) by using the OneStep RT-PCR protocol (Qiagen) with primers based on the human sequence (GenBank accession no. NM_000640); the amplified RT-PCR product was verified by DNA sequencing and subcloned into pcDNA3 (Invitrogen). 293 cells transfected with this plasmid were selected for growth in medium containing 1 mg G418 ml–1, then live cells immunostained with anti-human IL-13Rα2 antibody B-D13 (Cell Sciences, Inc.) were sorted for high expression by fluorescence-activated cell sorting (FACS) (Coulter).

Construction of retroviral vectors. The gag-pol expression plasmid pcDNA MoMLV was described previously (Zavorotinskaya & Albritton, 1999). Human IL-13 sequences were amplified from pc6h-IL-13 (a gift of Dr Gerard Zurawski, DNAX Research Institute, Palo Alto, CA, USA) and ligated into the NotI site of three altered env constructs: pcDNA-MoMLV-NotI, containing a unique NotI site between codons 6 and 7 of SU; pcDNA-MoMLV-NFex, containing a unique NotI site between codons 6 and 7 flanked by the peptide Ser-Gly-Gly-Gly; and pcDNA-MoMLV-PRR, containing a unique NotI site between codons 264 and 265 flanked by Ser-Gly-Gly-Gly-Gly.

Retroviral vector production, transduction and immunoblotting, and virus binding. Retroviral vector production and titration, Western blotting and virus-binding assays were performed as described previously (Zavorotinskaya & Albritton, 1999). For Western blot analysis, proteins were separated on reducing SDS/8 % polyacrylamide gels and transferred onto nitrocellulose membranes. IL-13R-positive 293-IL13R, MoMLV and IL-13R-negative 293 cells were added and then incubated for an additional 1 h at 4 °C prior to addition of primary and secondary antibodies and flow cytometry.

Cell–cell fusion assay. Cell–cell fusion assays were performed as described elsewhere (Melikyan et al., 2000a; Munoz-Barroso et al., 1998; Zavorotinskaya et al., 2004) with the following modifications. Forty-eight hours after transfection with a plasmid encoding the R- less form (Rein et al., 1994) of each Env, Env-expressing effectors 293 cells were labelled with 20 μM 7-amino-4-chloromethylcoumarin (CMAC; Molecular Probes), a blue-fluorescent, viable cytoplasmic dye. As target cells, 293-IL13R cells were preincubated with 1 μM recombinant IL-13 ligand (Cell Sciences, Inc.) for 30 min at 4 °C, concentrated virions were added and then incubated for an additional 1 h at 4 °C prior to addition of primary and secondary antibodies and flow cytometry.
target cells were mixed and incubated for 1 h as described above. After initial micrographs were taken, cells were treated with 0.4 mM chlorpromazine (CPZ) in BES-buffered medium (pH 7.4) for 1 min. Cells were washed once, fed with fresh medium and incubated at 37 °C for an additional 1 h, then a second set of micrographs was captured. To determine the effect of CPZ on transduction by retroviral vectors, U251 MG cells or 293-IL13R cells were seeded in 24-well plates coated with poly-l-lysine. Twenty-four hours later, 10-fold serial dilutions of retroviral vectors were applied to quadruplicate wells and incubated for 2 h. Cells were washed once to remove unbound vector particles, then exposed to freshly made 0.4 mM CPZ in BES-buffered medium (pH 7.4) for 1 min (Zavorotinskaya et al., 2004), after which cells were washed once, then fed with fresh culture medium. Forty-eight hours later, cells were fixed and stained for LacZ transduction.

**Spinoculation.** Transduction of target cells by centrifugal inoculation (spinoculation) was performed as described elsewhere (O’Doherty et al., 2000). Briefly, 4 × 10⁴ U251, 293, 293-IL13R or NIH 3T3 cells were seeded into quadruplicate wells on two 24-well plates and cultured for 24 h. Each retroviral vector (250 µl) was applied to cells on both plates. One plate was centrifuged at 1200 g at 25 °C for 2 h, whereas the other plate was left on the laboratory bench (1 g) for 2 h. Cells were fed with fresh medium, incubated for an additional 48 h, then fixed and stained with X-Gal for transduction.

**RESULTS**

**IL-13-modified Env exhibited a phenotype typical of modified Env: binding but not transduction of cells expressing the target IL-13 receptor**

These studies were originated to determine whether the addition of Q227R and D243Y to an Env modified by a clinically relevant ligand would result in reasonable levels of transduction via the target receptor for the ligand. For this purpose, we chose human IL-13 as a ligand. In addition to its role in inflammatory and immune responses, IL-13 has been implicated in tumour progression because various human tumours, including glioblastomas, express relatively high levels of IL-13R on their cell surface (Debinski et al., 1999). Thus, if addition of Q227R and D243Y proved effective at increasing infection of chimeric Env directed at the IL-13R as the target receptor, then targeted transduction of tumour cells might be possible.

Three chimeric Env proteins were constructed (Fig. 1a): IL-13N, with IL-13 inserted between Ser6 and Pro7 of the MoMLV SU (amino acid numbering from the N terminus of mature SU); IL-13NFlex, which differed from IL-13N by a Ser-Gly-Gly-Gly flexible linker flanking the ligand; and IL-13PRR, with IL-13 inserted between Gly264 and Thr265 in the PRR flanked by the flexible Ser-Gly-Gly-Gly linker. Flexible peptide linkers have been used to prevent steric hindrance in single-chain antibodies, as well as in N-terminally modified retroviral Env (Valsesia-Wittmann et al., 1996).

Chimeric Env proteins modified by a ligand in either of these two sites typically retain use of the natural MoMLV receptor for entry, but fail to give infection via the target receptor (Benedict et al., 1999; Waehler et al., 2007; Weimin Wu et al., 1998). In murine NIH 3T3 cells that express the natural MoMLV receptor, IL-13PRR Env gave similar levels of transduction to control MoMLV, whilst the IL-13N and IL-13NFlex vectors showed 100- and 50-fold less infection, respectively (Fig. 1b). None of the IL-13-modified Env proteins infected the IL-13Rα-positive human glioblastoma cell lines U251 MG (Fig. 1b) or U373 MG (Bernard et al., 2001) (data not shown). The lack of transduction via IL-13Rα mapped to the modified Env and target IL-13R, as MLV containing amphotropic MLV Env transduced the glioblastoma cells (Fig. 1b). We generated 293-IL13R, a human 293 cell line that stably expressed IL-13Rα at over 3-fold higher surface levels than U251 and U373 MG glioblastoma cells, as judged by the difference in mean fluorescence intensity from flow-cytometry analysis of live cells (see Supplementary Fig. S1, Fig. 1).

**Fig. 1.** IL-13-modified Env proteins do not give transduction of IL-13R-positive cells. (a) Schematic diagrams of IL-13-modified Env. For simplicity, only the SU subunit of Env is illustrated. The sites of insertion are indicated by ▼. The positions of flexible Ser-Gly₄ linker peptides flanking IL-13 in the IL-13NFlex and IL-13PRR Env are shown by black bars. RBD, Receptor-binding domain; PRR, proline-rich region. (b) Vectors transduced mouse NIH 3T3 cells expressing endogenous MLV receptor, but not a human glioblastoma line, U251 MG, expressing endogenous IL-13R. LacZ transducing units (TU) ml⁻¹ were calculated from the end-point dilutions. Values shown are the mean ± SD from three independent experiments. AmphoMLV, MLV coated with amphotropic 4070A Env.
available in JGV Online). The chimeric Env also failed to
give transduction of 293-IL13R cells (data not shown).

Vector binding to IL-13Rx2 was assessed by using a FACS-based
virus-binding assay. Vectors containing IL-13-modified Env showed comparable binding to each other
(Fig. 2, solid lines), whereas control MoMLV did not bind
to cells expressing IL-13Rx2 (Fig. 2, solid line). The binding
was specific to IL-13R, as preincubation of the target cells
with 1 µM recombinant IL-13 gave a 12-fold reduction in
the mean fluorescence intensity (Fig. 2, dotted lines). By
inference, this finding is evidence that the lack of
transduction resulted from a block in a post-binding step,
similar to reports for other chimeric Env proteins.

**Insertion of the ligand in the PRR does not
weaken subunit association of the modified Env**

We and others have reported previously that the asso-
ciation of N-terminally modified SU with the TM subunit
is weaker than in the wild-type Env (Kizhatil et al.,
2001; Valsesia-Wittmann et al., 1996; Zavorotinskaya &
Albritton, 2001), but association of PRR-modified subunits
was shown previously to be comparable to the wild-type
levels (Weimin Wu et al., 1998). Comparable numbers of
particles were produced, as judged by the level of capsid
protein in each vector stock by using Western blot analysis
(Fig. 3a, left panel). The modified Env showed a larger
molecular mass of 100 kDa, reflecting the addition of
114 aa and four new N-linked glycosylation sites in the IL-
13 ligand sequences. IL-13N and IL-13NFlex SU were
consistently less abundant on vectors, whereas IL-13PRR
Env incorporation was comparable to that of MoMLV. The
lower amount of N-terminally modified SU on virions was
not due to lower expression, as approximately equal
amounts of SU and precursor Env were present in equal
masses of producer-cell lysate (Fig. 3b).

These results suggested that less N-terminally modified SU
was recovered in virus pellets because the association
between these SU and their TM subunit was relatively
weak, whereas insertion of IL-13 in the PRR did not
influence subunit association. If so, then application of
mechanical shear by ultracentrifugation through a sucrose
cushion should increase dissociation. Indeed, almost no
virion-associated IL-13N and IL-13NFlex SU was observed
in virus centrifuged through a sucrose cushion, whereas the
amounts of IL-13PRR and MoMLV SU were similar
(Fig. 3a, middle panel). Further, when vectors were
concentrated under relatively gentle conditions by using
low-speed centrifugation on Centricon devices, the
amounts of virion-associated SU were comparable for all
vectors (Fig. 3a, right panel).

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**Fig. 2.** IL-13-modified Env give binding to the target receptor IL-
13Rx2. 293-IL13R cells were incubated with vectors and bound
virus was detected by incubation with anti-SU antiserum followed
by flow cytometry. Grey histogram, background fluorescence
measured by incubation of 293-IL13R cells with antiserum in
the absence of virus; solid lines, fluorescence histograms in the
presence of the indicated virus; dotted lines, histograms in the
presence of virus and using cells incubated with 1 µM rIL-13 prior
to and during incubation with vectors.

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**Fig. 3.** Western blot analysis of modified Env. (a) Western blot
analysis of vector particles purified by direct ultracentrifugation
direct pelleting), purified by ultracentrifugation through a sucrose
cushion or concentrated by using a Centricon device, as described
in Methods. (b) Equal masses of producer-cell lysates were loaded
on each lane. (c) Addition of point mutations stabilized the subunit
association of IL-13N and IL-13NFlex. Q227R/D243Y, Env
containing Glu227-to-Arg plus Asp243-to-Tyr changes. SU, Env
surface subunit; CA, capsid protein. Numbers on the left indicate
relative molecular masses in kDa.
Two point mutations stabilize subunit association, but do not give transduction via the target IL-13R

We showed previously that Q227R and D243Y gave a 1500-fold increase in transduction by a modified Env called ampho-eco (Zavorotinskaya & Albritton, 2001) and stabilized the subunit association of this chimera, as well as that of a non-targeting chimera, GFP-SU (Kizhatil et al., 2001). The ampho-eco chimera contains the RBD of amphotropic 4070A MLV (Cosset et al., 1995) and GFP-SU contains green fluorescent protein inserted between Ser6 and Pro7 of MoMLV Env.

We asked whether Q227R and D243Y give a similar increase in infection when added to the IL-13-modified Env proteins, particularly IL-13N and IL-13NFLEX, which are similar in design to the ampho-eco Env. Unlike in ampho-eco Env, adding the two mutations did not result in transduction of IL-13R-positive cells by IL-13N, IL-13NFLEX or IL-13PRR Env (data not shown), although they stabilized the association of the N-terminally modified SU with vector particles (Fig. 3c). However, this stabilization did not correlate with an increase in infection of NIH 3T3 cells (see Supplementary Fig. S2, available in JGV Online).

IL-13PRR Env initiates membrane fusion, but is blocked at a subsequent step, whereas N-terminal insertions of IL-13 block fusion completely

As the point mutations had not increased transduction, we sought to identify the post-binding step that is blocked. For this purpose, we used a fluorescence-based cell–cell fusion assay in which Env-expressing effector cells are labelled with a blue-fluorescent cytoplasmic dye, CMAC, and receptor-expressing target cells are doubly labelled with a green-fluorescent cytoplasmic dye, calcineAM, and a red-fluorescent lipid-membrane dye, DiI. Complete fusion is observed as transfer of red membrane marker and green cytoplasmic dye from target cells to effectors, plus blue cytoplasmic-dye transfer from effectors to target cells, indicating continuity of the membrane lipids and mixing of the cell contents. In the positive control, about 21% of cell pairs between MoMLV Env effector cells and target cells expressing natural MLV receptor showed both lipid and content mixing, indicative of complete fusion (Table 1; Supplementary Fig. S3, available in JGV Online). This fusion activity was specific to the natural MLV receptor; no dye transfer was seen in mixtures of MoMLV Env effectors and 293-IL13R targets (see Supplementary Fig. S3). Negative-control mixtures of IL-13NFLEX or IL-13PRR Env effectors and natural MLV receptor-positive targets also showed both lipid and content mixing, reflecting retention of their ability to infect via the natural receptor (see Supplementary Fig. S3).

When IL-13N and IL-13NFLEX Env effector cells were mixed with 293-IL13R target cells, none of the cell pairs showed transfer of fluorescent dyes, consistent with an absence of fusion activity (Fig. 4a, b). Unexpectedly, red-fluorescent membrane-dye transfer from target 293-IL13R cells to IL-13PRR Env effector cells was observed in some cell pairs, although none showed cytoplasmic-dye transfer (Fig. 4c). The observation of lipid-dye transfer in the absence of cytoplasmic-dye transfer is consistent with a block midway through fusion, referred to as the hemifusion stage. To quantify the fusion, micrographs of live cells were captured at low magnification and more than 2000 cell pairs per sample were scored for fluorescence-dye transfer. In cell pairs between IL-13PRR Env effector and 293-IL13R target cells, 1.1% showed lipid mixing without cell-content mixing, whereas the percentage of hemifusion in IL-13N or IL-13NFLEX Env effector cells did not exceed the background level in MoMLV Env effector cells (Table 1).

To test further whether IL-13PRR Env can reach hemifusion, whilst the N-terminal insertions are unable to initiate fusion, we examined the effect of the membrane-curving agent CPZ on cell fusion by using the modified Env proteins. CPZ can induce complete cell–cell fusion in hemifusion mutants of influenza virus haemagglutinin (HA) (GIS, containing a glycine-1 to serine change) and MoMLV Env (H8R, containing a histidine-8 to arginine change), but has no effect if fusion has not been initiated (Melikyan et al., 1997; Zavorotinskaya et al., 2004). Live cell pairs that had been captured in micrographs after 1 h incubation in the absence of any drug were then exposed briefly to CPZ and given an additional 1 h incubation at

<table>
<thead>
<tr>
<th>Env protein</th>
<th>Fusion (%)</th>
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<tbody>
<tr>
<td></td>
<td>No CPZ</td>
</tr>
<tr>
<td></td>
<td>Hemifusion</td>
</tr>
<tr>
<td>MoMLV⁺</td>
<td>0.1 0.1</td>
</tr>
<tr>
<td>IL-13N⁺</td>
<td>0.1 0.2</td>
</tr>
<tr>
<td>IL-13NFLEX⁺</td>
<td>0.1 0.0</td>
</tr>
<tr>
<td>IL-13PRR⁺</td>
<td>1.1 0.5</td>
</tr>
<tr>
<td>MoMLV†</td>
<td>0.0 21.3</td>
</tr>
<tr>
<td>H8R MoMLV</td>
<td>3.2 1.2</td>
</tr>
<tr>
<td>Env†</td>
<td></td>
</tr>
<tr>
<td>Influenza HA†</td>
<td>0.0 12.5</td>
</tr>
<tr>
<td>GIS HA†</td>
<td>8.0 1.6</td>
</tr>
</tbody>
</table>

*293-IL13R cells were used as target cells.
†Human 293 cells stably expressing the natural MoMLV and influenza A virus receptors were used as target cells. Numbers are modified from a previous report (Zavorotinskaya et al., 2004) by normalizing the percentage fusion in MoMLV Env effectors mixed with target cells expressing MoMLV receptors.
37 °C, after which a second set of micrographs of live cell pairs was taken. This experimental design provided a comparison before and after CPZ exposure that was not biased by variations in transfection and dye-labelling efficiencies.

Brief exposure to CPZ induced apparently all IL-13PRR Env effectors to complete fusion (Table 1). After CPZ exposure, no hemifused cells were present in the IL-13PRR samples; all cell pairs that showed lipid-dye mixing also had content-dye mixing, indicative of complete fusion. The 4.4-fold increase in IL-13PRR-mediated fusion after exposure to the drug was about half that seen in control H8R MoMLV Env (8.4-fold) and slightly more than that seen in the influenza virus hemifusion mutant G1S HA (2.8-fold). In contrast, CPZ had little effect on the percentage of hemifusion and fusion in IL-13N, IL-13NFlex and wild-type MoMLV Env effectors.

**CPZ also promotes transduction of IL-13R-positive human cells by IL-13PRR vector**

Whilst the fold increase in IL-13PRR Env fusion was comparable to those of the control hemifusion mutants, the actual percentage of hemifused (−CPZ) and fused (+ CPZ) cells was lower, raising the question of how much the hemifusion block actually contributes to the failure of targeted entry. We reasoned that if the hemifusion block is inconsequential to infection, then exposure of virus–cell complexes to CPZ will have a minimal effect, if any, on infection of IL-13R-positive cells. IL-13R-positive cells were incubated for 2 h with virus, then a 1 min pulse of CPZ or a mock pulse was applied, followed by an additional 48 h incubation, after which cells were fixed and stained to detect infection.

The pulse of CPZ increased IL-13PRR virus transduction of U251 MG cells from none to over 400 transduced tumour cells (ml virus)−1 (Fig. 5). On cells expressing three times as many target receptors, e.g. 293-IL13R cells, the drug increased infection from none to 4000 transduced cells (ml virus)−1. These values were over 50-fold and 2000-fold greater than negative-control MoMLV transduction of U251 MG and 293-IL13R cells, respectively, in the presence of CPZ (Fig. 5). In contrast, IL-13N and IL-13NFlex showed no increase over MoMLV infection of U251 MG after the drug pulse, although there was a small increase in infection of the 293-IL13R cells. These results are consistent with IL-13PRR Env having the ability to initiate fusion, but being blocked at the subsequent step of fusion-pore formation, whilst the N-terminal IL-13 insertions had little ability to initiate fusion.
Spinoculation has no significant effect on transduction

CPZ induced a large increase in IL-13PRR infection, but the drug also induced a low level of transduction by control MoMLV. As U251 MG and 293-IL13R cells do not express the natural MoMLV receptor, this background infection probably resulted from receptor-independent attachment (Pizzato et al., 1999) and pleiotropic effects of CPZ (Taylor et al., 2003). In light of the background, we asked whether any of the CPZ-induced IL-13PRR infection resulted from drug-induced membrane curvature, independently of the target IL-13R. To address this question, IL-13R-negative 293 and NIH 3T3 cells were added as host-cell lines, and the number of virions attached to cells at the time of CPZ pulse was increased by using centrifugal inoculation (spinoculation). Spinoculation apparently works by depositing vector particles onto the target-cell surface (O’Doherty et al., 2000).

IL-13R-positive or -negative cells were inoculated with vectors in the presence or absence of centrifugation, then half of each set of samples was pulsed with CPZ and the other half received a mock pulse. The results indicated that >99% of drug-induced IL-13PRR infection was dependent on IL-13R, as judged by the level of infection of receptor-negative 293 cells compared with IL-13R-positive cells when pulsed with CPZ (Table 2). In the absence of CPZ, increasing attachment by using centrifugal inoculation had little effect on IL-13PRR and no apparent effect on IL-13N and IL-13N Flex infection via the target IL-13R, whereas infection by the IL-13PRR virus was more than doubled when spinoculation was followed by a CPZ pulse (Table 2). These results are consistent with previous reports that spinoculation had no significant effect on transduction of receptor-negative cells (Lavillette et al., 2002; Smith et al., 2004).

DISCUSSION

To our knowledge, this is the first report identifying the step in membrane fusion that is inhibited during vector transduction by a modified MoMLV Env and the first to present evidence that the site of ligand insertion influences the functionality of a modified Env. The IL-13 chimeras showed a phenotype similar to those of previously reported modified Env proteins, e.g. robust target-receptor binding that does not result in transduction (reviewed by Lavillette et al., 2001). Unexpectedly, the site of insertion influenced subunit association: insertion in the N-terminal site

Table 2. Effect of centrifugal inoculation (spinoculation) on transduction of IL-13-modified vectors

<table>
<thead>
<tr>
<th>Env target cells</th>
<th>Infected cells (%)</th>
<th>No spinoculation (1 g)</th>
<th>Spinoculation (1200 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT*</td>
<td>IL-13N</td>
<td>IL-13N Flex</td>
</tr>
<tr>
<td>No CPZ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U251 MG</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>293-IL13R</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>293</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>NIH 3T3†</td>
<td>0.68</td>
<td>0.00</td>
<td>0.005</td>
</tr>
<tr>
<td>0.4 mM CPZ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U251 MG</td>
<td>0.20</td>
<td>0.040</td>
<td>0.040</td>
</tr>
<tr>
<td>293-IL13R</td>
<td>0.11</td>
<td>0.050</td>
<td>0.130</td>
</tr>
<tr>
<td>293</td>
<td>0.10</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

*WT, Wild type.
†100-fold-diluted viruses were used.
Weakened association, whereas insertion in the PRR did not appear to affect subunit stability. Thus, the failure of N-terminal insertion chimeras to mediate infection through the target IL-13R might be due partly to dissociation of SU. This possibility is consistent with their reduced ability to infect via the natural receptor on NIH 3T3 cells compared with wild-type Env and IL-13PRR (Fig. 1b). However, as it does not explain why NIH 3T3 infection did not increase when subunits were stabilized by Q227R and D243Y, there are probably additional defects in these chimeras.

It has been suggested that N-terminal insertion of a ligand interferes with the function of the essential histidine residue at position 8 (His8) in Env (Bae et al., 1997). Alternatively, N-terminal insertion may cause subtle misfolding of Env. We favour the latter possibility for the following reasons. First, loss of His8 function results in a block at hemifusion, but IL-13N and IL-13NFlex showed an earlier fusion block. Second, Q227R and D243Y were discovered for their ability to suppress the hemifusion block of His8 mutants, yet they did not increase infection of NIH 3T3 cells by the N-terminal IL-13 chimeras. Lastly, the approximately 10-fold-increased infection of NIH 3T3 cells when a flexible linker was added suggested that steric hindrance of Env folding was involved.

Why, then, did the IL-13-modified Env proteins fail when interacting with IL-13Rs2? As they gave infection of NIH 3T3 cells and cell–cell fusion with target cells expressing the MoMLV receptor, they must possess membrane-fusion function. Although there are exceptions, most retroviral receptors are multiple membrane-spanning proteins (Sommerfelt, 1999). In particular, all known receptors for members of the genus Gammaretrovirus, to which MoMLV belongs, are multiple membrane-spanning proteins. Do multipass membrane proteins possess a common property that is important for membrane fusion? One possibility is that multipass membrane proteins have a more intimate association with the membrane lipids than single-pass membrane proteins and this arrangement may reduce the energy needed to open a fusion pore. Alternatively, Katane et al. (2002) suggested that binding to multipass membrane proteins may be important to position the viral membrane close enough for the extended fusion peptide to enter the host-cell membrane.

Alternatively, an effective retroviral receptor might need to fulfil two functions: a direct function in virus binding and an indirect function in the opening of the fusion pore. If so, then IL-13Rs2 may provide attachment, but lack this essential property. Entry of human immunodeficiency virus (HIV) exemplifies these two receptor functions: they are clearly separated in its receptor and coreceptor, CD4, a single membrane-spanning protein, provides high-affinity initial binding, while the multipass membrane-spanning chemokine coreceptors function in fusion-pore opening. Our results are most consistent with virus–cell membrane fusion involving at least two discrete steps, rather than a single step. Thus, membrane fusion in MoMLV may be analogous to that in HIV, in which conformational changes in Env are triggered first by CD4 and then by a coreceptor, leading to complete fusion. As certain primary HIV-2 and simian immunodeficiency virus strains, as well as some laboratory-adapted HIV-1 isolates, do not require CD4 for infection (Dumonceaux et al., 1998; Edinger et al., 1997; Endres et al., 1996), the most important contributions to membrane fusion are apparently provided by Env and the multipass chemokine coreceptors.

Although our observations cannot rule out the possibility that IL-13Rs2 lacks an important property, the differences between the characteristics of Env proteins with insertions in their N terminus versus their PRR argue strongly that the principal defect in the fusion function of Env is inherent to its interaction with the target IL-13Rs2. The exact correlations between these molecular events and dye transfer in the cell–cell fusion assay have not been established. However, it appears that fusion-peptide insertion gives mixing of membrane lipids, but is not sufficient for six-helix bundle and fusion-pore formation, which are observed as cell–content mixing (Melikyan et al., 1995, 2000a, b; Munoz-Barroso et al., 1998). The N-terminal insertions did not appear to initiate fusion, as only a background level of lipid transfer was observed in the cell–cell fusion assay (Fig. 4). Brief exposure of their cell–cell pairs to the membrane-curving agent CPZ had no significant effect (Table 1). In agreement with this interpretation, CPZ elicited very little infection of IL-13R-positive cells by the N-terminal IL-13 chimeras (Fig. 5).

In contrast, IL-13 insertion into the PRR could give membrane-lipid transfer, although content mixing was not attained (Fig. 4c; Table 1). These observations are consistent with some molecules of IL-13PRR Env changing conformation to expose their fusion peptide. CPZ could resolve the block in hemifusion to achieve complete cell–cell fusion (Table 1). The drug has previously shown a similar ability to resolve the blocks in hemifusion mutants of influenza HA and MoMLV Env (Chernomordik et al., 1998; Zavorotinskaya et al., 2004). Importantly, the IL-13PRR chimera appeared to expose fusion peptide when on virions interacting with the target IL-13R in sufficient numbers for CPZ to elicit substantial infection of U251 MG glioblastoma and 293-IL13R cells (Fig. 5).

If these IL-13PRR Env molecules initiate fusion, then why do they not complete it? One possibility is that they are unable to undergo the additional conformational changes required to form six-helix bundles. Another possibility is that some of these molecules form helical bundles after their fusion peptides are exposed, but there are too few of them to catalyse fusion-pore formation. For example, it has been shown that a minimum number of viral Env trimers are necessary in order to complete membrane fusion and that a suboptimal number induces hemifusion (Blumenthal et al., 1996; Danieli et al., 1996). In this regard, it is noteworthy that IL-13PRR Env gave a lower...
percentage of hemifusion than the previously published H8R (Table 1) and that the infection elicited by CPZ in 293-IL13R cells was 100-fold lower than that elicited by transduction of NIH 3T3 cells (Fig. 5). Considering that CPZ could rescue infection of H8R mutant virus to levels close to those of wild-type MoMLV infection (Zavorotinskaya et al., 2004), our observations are consistent with the possibility that too few IL-13PRR Env molecules undergo conformational changes and form six-helix bundles to form a fusion pore in response to IL-13R binding.

As receptor binding induces the key conformational changes in retroviral Env, we propose that the principal cause for lack of transduction through IL-13Rα2 was that the interaction of the ligand with its receptor did not induce the same spectrum of conformational changes as were induced by the natural MoMLV receptor and, further, that this defect is inherent to the basic design of inserting ligands essentially as separate domains within Env. How can this problem be solved to achieve cell type-specific entry? It is likely that a new design for modifying Env will need to be implemented. We propose that one such novel design is replacement of the sequences that normally bind the natural viral receptor with the ligand sequences. As interaction of the natural receptor with this location on the wild-type Env sets all of the necessary conformational changes in motion, it seems reasonable to expect that swapping this sequence with a heterologous ligand would be an effective new approach to targeted infection.

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