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Retroviral Retargeting by Envelopes Expressing an N-Terminal Binding Domain

FRANÇOIS-LOÏC COSSET,[1,2]* FRANCES J. MORLING,[3] YASUHIRO TAKEUCHI,[1] ROBIN A. WEISS,[1] MARY K. L. COLLINS,[1] AND STEPHEN J. RUSSELL[3]

Institute of Cancer Research: Chester Beatty Laboratories, SW3 6JB London,[1] and Cambridge Centre for Protein Engineering, CB2 2QH Cambridge,[3] United Kingdom, and Centre de Génétique Moléculaire et Cellulaire, Centre National de la Recherche Scientifique UMR 106, Université Claude Bernard Lyon-I, 69622 Villeurbanne Cedex, France[2]

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We have engineered ecotropic Moloney murine leukemia virus-derived envelopes targeted to cell surface molecules expressed on human cells by the N-terminal insertion of polypeptides able to bind either Ram-1 phosphate transporter (the first 208 amino acids of amphotropic murine leukemia virus surface protein) or epidermal growth factor receptor (EGFR) (the 53 amino acids of EGF). Both envelopes were correctly processed and incorporated into viral particles. Virions carrying these envelopes could specifically bind the new cell surface receptors. Virions targeted to Ram-1 could infect human cells, although the efficiency was reduced compared with that of virions carrying wild-type amphotropic murine leukemia virus envelopes. The infectivity of virions targeted to EGFR was blocked at a postbinding step, and our results suggest that EGFR-bound virions were rapidly trafficked to lysosomes. These data suggest that retroviruses require specific properties of cell surface molecules to allow the release of viral cores into the correct cell compartment.

Retroviral infection is initiated by viral recognition of a variety of cell surface molecules (47). Retroviral envelope glycoproteins, expressed on the surface of virions, are responsible for the attachment of viral particles to these cell surface receptors and for the subsequent fusion between the viral and cell membranes. The CD4 antigen, the receptor for human immunodeficiency virus, was the first retroviral receptor to be identified (14, 21). Its expression is mainly restricted to helper T lymphocytes and macrophages. More recently, genes encoding the receptors for three mammalian type C retroviruses, Moloney murine leukemia virus (MoMLV) (1), gibbon ape leukemia virus (30), and amphotropic murine leukemia virus (MLV-A) (26, 45) have been cloned. The three receptor molecules belong to the family of permeases and are phosphate transporters in the cases of gibbon ape leukemia virus and MLV-A (20) and a cationic amino acid transporter in the case of MoMLV (46). They are widely expressed on most cell types, and sequence analysis suggests that they possess multiple membrane-spanning domains.

It is of interest that groups of mammalian retroviruses share common receptors (47). For example, all primate lentiviruses use CD4 as their main receptors and bind to the same portion of the molecule (35). A number of type D retroviruses and the type C retroviruses RD114 and baboon endogenous virus, with envelopes related to those of type D retroviruses, share a receptor on human cells (40). All receptors cloned to date for type C mammalian retroviruses belong to a family of permease molecules (27, 47). Furthermore, the gibbon ape leukemia virus receptor is also used by feline leukemia virus subgroup B (43), which interacts with the same region of the molecule but slightly differs in its receptor amino acid sequence requirement (41), and by some other murine type C viruses (27). In this case, there is limited homology between the envelope sequences of gibbon ape leukemia virus and feline leukemia virus subgroup B (41), although they share common structural features together with other type C retrovirus envelope glycoproteins (4, 32). Although different groups of retroviruses use quite different receptors (47), such observations suggest that only particular types of cell surface molecules can serve as retroviral receptors and that a property, or properties, such as topology or route of internalization, of certain molecules may be required for events after virus binding, like fusion and uncoating.

To test this hypothesis, we have introduced modifications in the envelope of ecotropic MLV (MLV-E) to redirect virions to two different receptors on human cells, Ram-1 (the phosphate transporter used by MLV-A to initiate infection) and epidermal growth factor receptor (EGFR). While both receptors allow specific virion binding, only Ram-1 permits viral infection. These observations demonstrate that it is feasible to engineer retargeted recombinant retroviruses. Such vectors will be of value for targeted gene delivery in various gene therapy applications. It is also apparent that only some cell surface molecules will be suitable for targeting with retroviruses displaying similar types of chimeric envelopes.

MATERIALS AND METHODS

Cell lines. The TELCeB6 cell line (13) was derived from the TELac2 line (42) after transfection and clonal selection of cells containing a plasmid expressing MoMLV Gag and Pol proteins. TELCeB6 cells produce noninfectious viral core particles, carrying an nlsLacZ reporter retroviral vector.

A431 (ATCC CRL1555), TE671 (ATCC CRL8805), and HT1080 (ATCC CCL121) cells were grown in Dulbecco's modified Eagle medium (DMEM) (Gibco-BRL) supplemented with 10% fetal bovine serum (Gibco-BRL). K422 cells (15) were kindly provided by A. Karpas (Medical Research Council, Cambridge, United Kingdom) and were grown in RPMI 1640 (Gibco-BRL) supplemented with 10% fetal bovine serum (Gibco-BRL). NR6 murine fibroblasts lacking detectable EGFRs (37), NR6-C′973 (an NR6 subclone obtained after transfection of a plasmid expressing the C′973 mutant human EGFR [hEGFR]) and NR6-hEGFR (an NR6 subclone obtained after transfection of a plasmid expressing the wild-type hEGFR) cells were kindly provided by G. Gill (La Jolla, Calif.). psi2 cells (22) and GP+EAM12 cells (23) were derived from NIH 3T3 cells and express MoMLV and MLV-A envelopes, respectively, which block the corresponding receptors (Rec-1 and Ram-1) by interference. NIH 3T3 and NIH

* Corresponding author. Mailing address: CGMC, CNRS UMR106, UCB Lyon-I, 43 bd. du 11 Novembre 1918, 69622 Villeurbanne Cedex, France. Phone: 33 72 44 81 90. Fax: 33 72 44 05 55. Electronic mail address: cosset@univ-lyon1.fr.

3T3-derived cell lines were grown in DMEM (Gibco-BRL) supplemented with 10% new-born bovine serum (Gibco-BRL).

Chimeric envelopes. DNA fragments encoding polypeptides binding either EGFR or Ram-1 were generated by PCR with oligonucleotides containing restriction sites. They were introduced at the N terminus of MLV surface protein (SU) gp70 in which the *Sfi*I and *Not*I restriction sites have been engineered at codon 6 (33). (For a schematic diagram of the various env genes used in this report, see Fig. 1.) Briefly, a PCR-derived DNA fragment encoding the 53 amino acids of hEGF (5) was generated with a cDNA template (ATCC 59957) and two primers, i.e., OUEGF (5′-ATGCTCAGAGGGGTCAGTACGGCCCAGCCG GCCATGGCCAATAGTGACTCTGAATGTCCC), with an *Sfi*I restriction site, and OLEGF (5′-ACCTGAAGTGGTGGGAACTGCGCGCGGCCGCATGTG GGGGTCCAGACTCC), with a *Not*I site, and were cloned after digestion with *Sfi*I and *Not*I in either MoMLV SU for the EMO chimeric envelope or 4070A SU for the EA envelope. The EMOA chimeric envelope was generated by engineering a *Bam*HI site by PCR-mediated mutagenesis of the 4070A env gene at a position corresponding to the *Bam*HI site of the MoMLV env gene at position 6538 (38). The 5′ half of the EMO env gene up to *Bam*HI was then fused with the 3′ half of the 4070A env gene. For the AMO envelope construct, a *Not*I site was engineered at the end of the receptor binding domain in the 4070A envelope (3), at nucleotide 750 (31) with a PCR fragment generated from *Xho*I (at nucleotide 594) to nucleotide 750 before the proline-rich hinge with the two oligonucleotides 805FC (5′-TCCAATTCCTTCCAAGGGGC) before *Xho*I and 806FC (5′-ACCCCCACATGCGGCCGCTCCCACATTAAGGACCTGCCG) with a *Not*I restriction site. The chimeric envelope was assembled by cloning both the PCR *Xho*I-*Not*I fragment and the *Not*I-*Cla*I fragment from the EMO env gene (encoding most of the MoMLV SU and p15E transmembrane proteins [TM]) between *Xho*I and *Cla*I sites of the 4070A MLV env gene.

All envelope constructs were expressed as *Bgl*II-*Cla*I fragments (corresponding to positions 5408 and 7676 in MoMLV), cloned between *Bam*HI and *Cla*I sites of the FBMOSALF expression vector (13), in which a phleomycin-selectable marker (17) fused to the phosphoglycerate kinase gene polyadenylation sequence was introduced downstream to the C57 MLV long terminal repeat of FB3 (4).

Production of viruses. Envelope expression plasmids were transfected by calcium phosphate precipitation (34) into TELCeB6 cells. Transfected cells were selected with phleomycin (50 μg/ml) and pools of phleomycin-resistant clones were used to harvest viruses from confluent cells after overnight incubation in DMEM and fetal bovine serum (10%). These supernatants were used for ultracentrifugation (to provide Western immunoblot virus samples), for binding assays, and for infection assays. Viruses (in 100 μl of producer cell supernatant) were also purified by gel filtration on 2-ml columns (Bio-Rad) of S-1000 Sephacryl (Pharmacia). Fractions were obtained by elution with phosphate-buffered saline (PBS) at 4°C.

Immunoblots. Virus producer cells were lysed in a 20 mM Tris-HCl buffer (pH 7.5) containing 1% Triton X-100, 0.05% sodium dodecyl sulfate (SDS), 5 mg of sodium deoxycholate per ml, 150 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride. Lysates were incubated for 10 min at 4°C and were centrifuged for 10 min at 10,000 × *g* to pellet the nuclei. Supernatants were then frozen at −70°C until further analysis. Virus samples were obtained by ultracentrifugation of viral supernatants (10 ml) in an SW41 Beckman Rotor (30,000 rpm, 1 h, 4°C). Pellets were suspended in 100 μl of PBS and frozen at −70°C. Samples (30 μg for cell lysates, or 10 μl for purified viruses) were mixed 5:1 (vol/vol) in a 375 mM Tris-HCl (pH 6.8) buffer containing 6% SDS, 30% β-mercaptoethanol, 10% glycerol, and 0.06% bromophenol blue, were boiled for 3 min and then were run on 10% polyacrylamide (SDS) gels. After protein transfer onto nitrocellulose filters, immunostaining was performed in Tris base saline, pH 7.4, with 5% milk powder and 0.1% Tween 20. Antibodies (Quality Biotech Inc., Camden, N.J.) were goat antisera raised against either Rausher leukemia virus (RLV) gp70 SU or RLV p30 capsid protein (CA) and were diluted 1/1,000 and 1/10,000, respectively. Blots were developed with horseradish peroxidase-conjugated rabbit anti-goat immunoglobulin antibodies (DAKO Ltd., United Kingdom) and an enhanced chemiluminescence kit (Amersham Life Science).

Binding assays. Target cells were washed in PBS and detached by a 10-min incubation at 37°C with 0.02% EDTA in PBS. Cells were washed in PBA (PBS with 2% fetal calf serum and 0.1% sodium azide). Cells (10^6) were incubated with viruses for 30 min at 4°C. Cells were then washed with PBA and incubated in PBA containing Rausher leukemia virus gp70 immune serum (1/200) for 30 min at 4°C. Cells were washed twice with PBA and incubated with rabbit anti-goat immunoglobulin fluorescein isothiocyanate-conjugated antibodies (DAKO). At 5 min before the two final washes in PBA, cells were stained with 20 μg of propidium iodide per ml. Fluorescence of living cells was analyzed with a fluorescence-activated cell sorter (FACS) (FACScan; Beckton Dickinson). For hEGFR staining, 10^6 cells in 100 μl of PBA were incubated with 10 μl of anti-EGFR antibodies (M886; DAKO) for 30 min at 4°C.

Infection assays. Target cells were seeded in 24-well plates at a density of 3×10^4 cells per well. Viral supernatant dilutions containing 4 μg of Polybrene per ml were added, and cells were incubated for 3 to 5 h at 37°C. Viral supernatant was then removed, and the cells were incubated in regular medium for 24 to 48 h. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining was performed as previously described (42). Viral titers were estimated as previously reported (12), in *lacZ* CFU per milliliter.

To block EGFRs, target cells were incubated 30 min at 37°C in a medium containing 10^{-6} M recombinant EGF (rEGF) (236-EG; R&D Systems). The cells were then washed, and infections were carried out as previously described. To block lysosomal acidification, 100 μM chloroquine phosphate (Sigma-Aldrich Co., Ltd.) was added to the medium. At 6 h after infection, the cells were washed and incubated in regular medium.

Internalization assays. A431 cells were seeded in six-well plates at a density of 10^6 cells per well and were incubated overnight. Viruses (5 ml) were plated onto the cells and incubated for 45 min at 37°C. The cells were then trypsinized (10 min at 37°C) and were washed three times in PBS. The cells were lysed as indicated above, and SU and CA contents were analyzed by immunoblotting as described above.

RESULTS

Construction of mutant envelopes. Two series of modified envelopes that would bind either a retroviral receptor (Ram-1) (26, 45) or a heterologous receptor (EGFR) were generated. The Ram-1 targeting envelope, AMO, was constructed by inserting a Ram-1-binding polypeptide, provided by the 208 first amino acids of the MLV-A SU (3), close to the N terminus of the MoMLV envelope (codon 6). The sequence coding for EGF was inserted in the MLV env gene in a position corresponding to amino acid 6 in the SU of MoMLV (Fig. 1). This position of insertion was previously shown to allow the functional display of single-chain antibodies at the surface of virions (33). The EGF domain was separated from the wild-type receptor binding domain in the envelope by a small linker containing three alanines. In the chimera EMO, EGF was inserted in the MoMLV envelope, whereas the chimera EA had an EGF insertion in the MLV-A envelope at position 5. The mutant EMOA was a chimeric envelope between EMO and EA, containing the EMO envelope N terminal to the proline-rich region (site *Bam*HI) and the MLV-A fusion domain.

Envelopes, including the control envelopes from MLV-E (MO envelopes) and MLV-A (A envelopes) were transfected into TELCeB6 cells which express MLV Gag-Pol core particles and an nlslacZ retroviral vector (13).

Expression and incorporation of envelopes into virions. Lysates of TELCeB6 cells were analyzed for envelope expression with antibodies against RLV SU (Fig. 2). For all chimeric envelopes, both a precursor and a processed SU product were detected at ratios similar to those for wild-type envelopes, suggesting that the mutants were correctly expressed and processed. Cell surface expression of mutant envelopes was examined by FACS analysis of producer cells, with antibodies against the SU or a monoclonal anti-hEGF antibody. All transfected cells were stained with the anti-SU antibodies, and cells expressing the EGF fusion envelopes were stained with anti-EGF monoclonal antibodies (data not shown).

To demonstrate the incorporation of the chimeric envelope glycoproteins into retroviral particles, supernatants of the various TELCeB6-transfected cell lines were ultracentrifuged to pellet viral particles. Pellets were then analyzed on immunoblots for their Gag (p30 CA) and envelope protein contents (Fig. 2). Viral SU could be detected for all mutants at similar Env-to-Gag ratios compared with the wild-type envelope. Only in the case of mutant EMOA was significantly less envelope found in the viral pellet. As expected, no SU was found in pellets when either of the envelopes was transfected in TElac2 cells, which do not express Gag and Pol, thus demonstrating that the SU found in the pellets of env-transfected TELCeB6 cells was associated with Gag-Pol viral particles.

These data demonstrated that insertion of large polypeptides at the N terminus of the MLV SU did not impair expression, processing, and viral incorporation of the mutant envelopes.

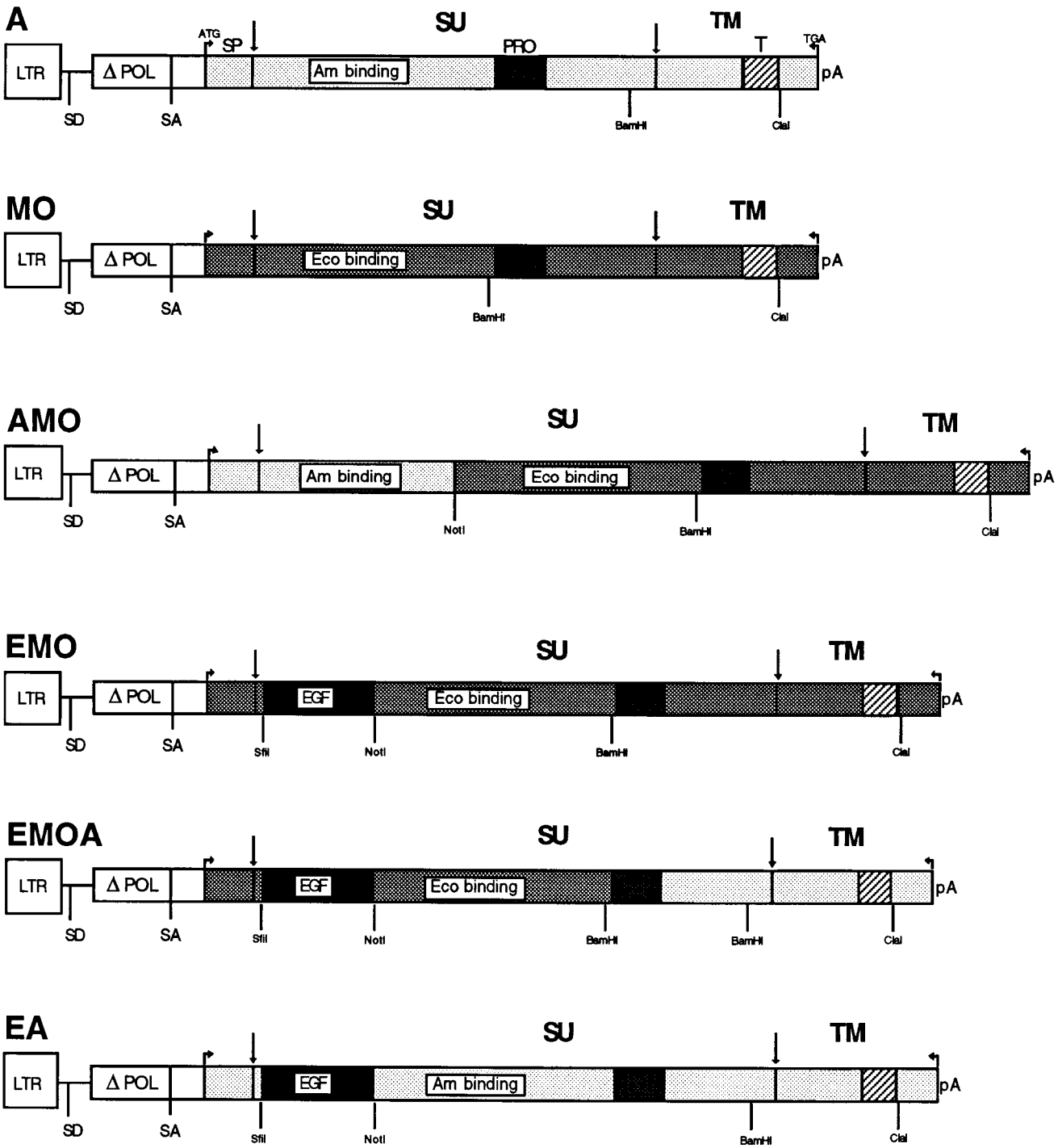

FIG. 1. Schematic diagrams of envelope chimeras. The positions of some functional regions are indicated. All env genes were expressed with the same promoter (LTR) and polyadenylation sequence (pA) from a subgenomic mRNA with retroviral splice donor (SD) and acceptor (SA) sites with an identical 190-nucleotide intron containing the end of pol gene (ΔPOL). The positions of some restriction sites are indicated. Vertical arrows indicate protein cleavage sites. Abbreviations: SP, env signal peptide; PRO, polyproline hinge; T, transmembrane domain; Am binding, MLV-A receptor binding domain; Eco binding, MLV-E receptor binding domain.

Receptor binding of envelopes. Human cell lines expressing different numbers of EGFRs (Fig. 3B) were used for binding assays. Cells were incubated with virus supernatants, and binding of viral envelopes to the target cell surface was analyzed by FACS with antibodies against the RLV SU (Fig. 3 and 4). As expected, no binding was detected for viruses carrying ecotropic envelopes (MO envelopes), whereas amphotropic envelopes (A envelopes) and fusion envelopes with the Ram-1-binding domain (AMO envelopes) could similarily bind to TE671 cells (Fig. 4).

MoMLV-derived EGF fusion envelopes (EMO envelopes) were found to bind to A431 cells (Fig. 3A) overexpressing

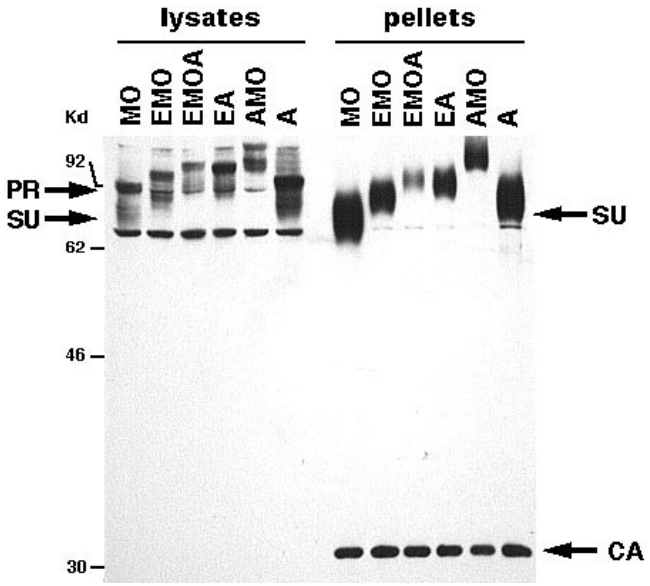

FIG. 2. Detection of envelope SUs. Immunoblots of lysates of TELCeB6 cells (transfected with the envelopes shown in Fig. 1) and of pellets of viral particles produced from these cells are shown. Both blots were stained with an SU antiserum. The immunoblot of pellets was cut at 46 kDa, and the lower part was stained with a p30 antiserum to detect the p30 CA protein. The positions of MO env precursors (PR) and SU are shown.

EGFR (Fig. 3B). Less binding was found on TE671 and HT1080 target cells, which express less EGFR (Fig. 3). No binding could be detected on K422 lymphoma cells with no detectable expression of EGFR (Fig. 3). The two other EGF fusion envelopes (EMOA and EA) bound to A431 cells as well as did EMO envelopes (data not shown). EGFRs on A431 cells were down-regulated by preincubation with rEGF. This treatment did not affect the binding of amphotropic envelopes (Fig. 5B) but abolished the binding of EMO envelopes (Fig. 5A).

SU envelope glycoproteins of MLVs are known to be weakly associated with their TM counterparts (18), and a low proportion of SU is retained on virions. Therefore, it is likely that the results of the binding assays shown in Fig. 3 and 4 are due in part to soluble envelope glycoproteins shed from virions. To determine whether viral particles could also bind, the supernatant of producer cells was separated by gel filtration and fractions were analyzed for binding activity on A431 cells (Fig. 6). As expected, very little binding activity was found in the early fractions containing the viral particles, with most of the binding activity occurring in the late fractions containing soluble envelopes. However, when viral particles were produced at 32°C in order to reduce the dissociation between SU and TM, a significant binding activity was also found in the fractions containing the virions (Fig. 6), demonstrating that viral particles could bind EGFR.

Infection with viruses carrying mutant envelopes. TE671 human cells and derivatives chronically infected with either MLV-A (which blocks Ram-1) or RD114 (which binds a cell surface receptor other than Ram-1) were used as target cells for infection. As expected, the nlsLacZ vector pseudotyped with the wild-type amphotropic envelope could infect at a titer of 10^7 *lacZ* CFU/ml. Infection was blocked by 4 log units on MLV-A-infected cells by receptor interference (Table 1). Pseudotypes with AMO envelopes could also infect TE671 cells. However, infection was severely reduced compared with that of the wild type, despite a comparable binding efficiency

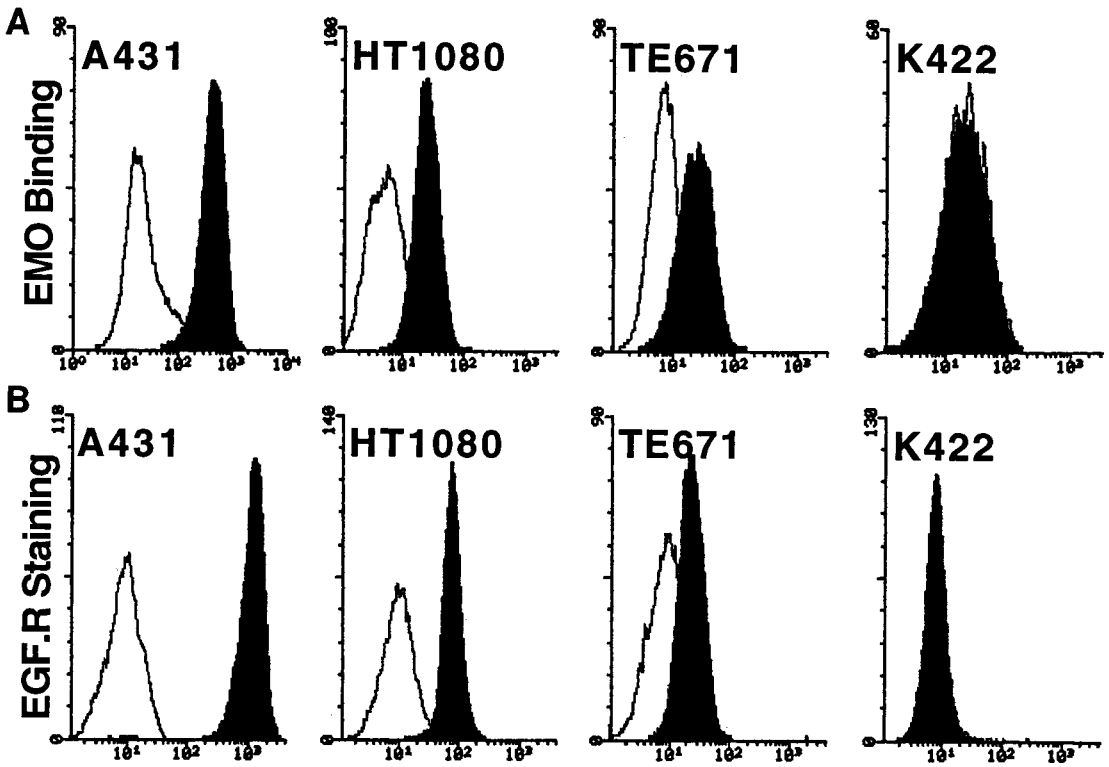

FIG. 3. EGFR binding assays. (A) Binding assays were done with EMO envelopes (black histograms) and MO envelopes (white histograms). (B) Cells were stained with an anti-hEGFR antibody (black histograms) versus no primary antibody (white histograms).

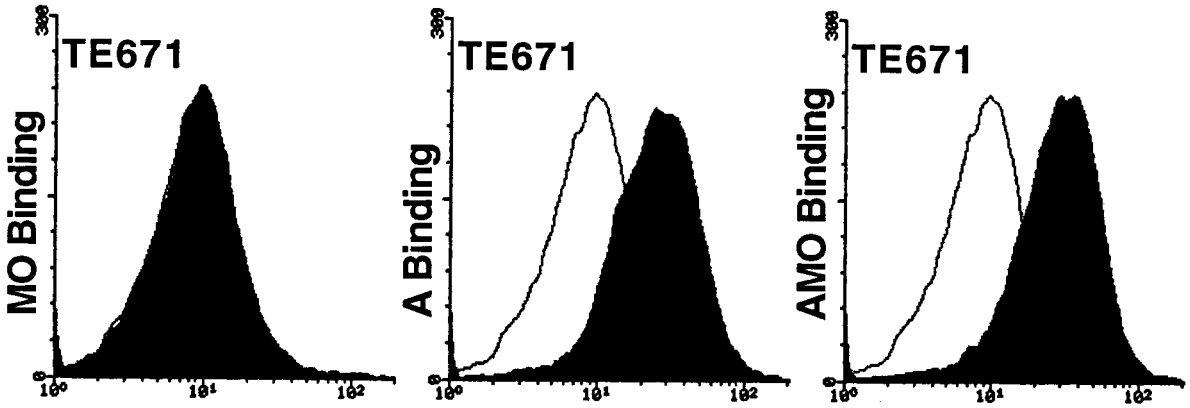

FIG. 4. Ram-1 binding assays. TE671 cells were used as target. The background of fluorescence was provided by incubating the cells with DMEM only (white histograms). Binding assays (black histograms) were made with MO, A, or ANO envelopes. The Env glycoprotein contents of the different samples were normalized by immunoblot.

(Fig. 4). Infection of human cells occurred through the Ram-1 receptor, as shown by interference in MLV-A-infected TE671 cells (Table 1). When wild-type MO ecotropic envelopes were coexpressed along with AMO envelopes to provide a helper for membrane fusion, no better titers could be obtained (Table 1). These data demonstrate that chimeric envelopes with an N-terminal addition of a new binding domain can direct retroviral infection via a new receptor.

Human cells expressing various densities of EGFRs were used for infections with viruses carrying EMO envelopes (Table 2). As expected, viruses with wild-type amphotropic envelopes could infect all types of cells while wild-type ecotropic

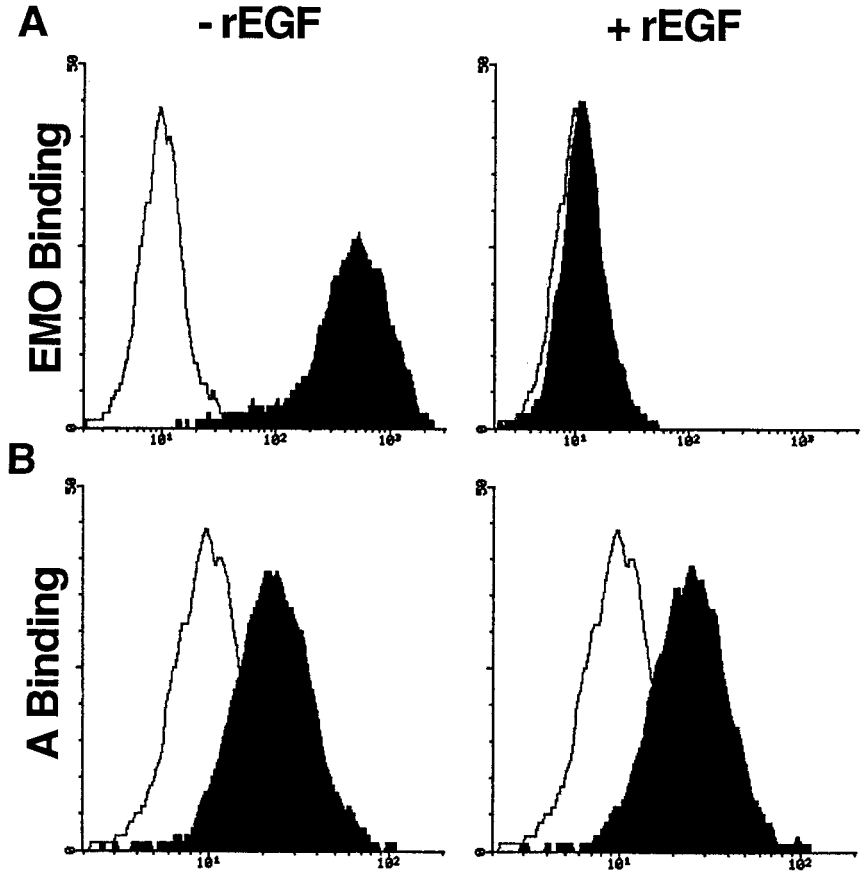

FIG. 5. Specificity of EGFR binding. A431 cells were used as target cells. Cells were untreated (−rEGF) or were treated with rEGF (+rEGF) (10^{-6} M, 30 min, 37°C) prior to binding assays with either EMO (A) or A (B) envelopes (black histograms). The background of fluorescence was provided by incubating the cells with DMEM only (white histograms).

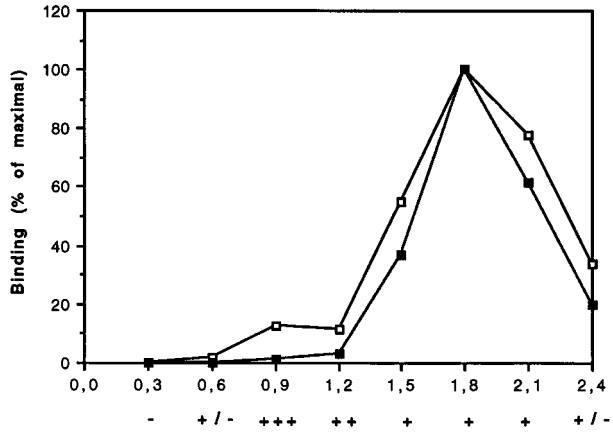

FIG. 6. EGFR binding assays after S-1000 chromatography. Each fraction was analyzed both for its binding activity with A431 cells as targets and for infectivity on NIH 3T3 cells. Amounts of fractions given in milliliters. Levels of infectivity: −, no infectivity; +/−, 1 to 10 *lacZ* CFU; +, 10 to 100 *lacZ* CFU; ++, 100 to 1,000 *lacZ* CFU; +++, >1,000 *lacZ* CFU. Results for 32 (□) and 37°C (■) are shown.

envelopes could not. However, virus pseudotypes carrying EMO envelopes could not infect cells (Table 2), despite their ability to bind (Fig. 3A). Fusogenicity of MLV-E envelopes is reported to be pH dependent and to be triggered by acidification after internalization of virus-receptor complexes into endosomes (25). Conversely, MLV-A can directly fuse at the cell surface at neutral pHs (25), and hybrid ecotropic-amphotropic envelopes with the MLV-A TM behave similarly to wild-type amphotropic envelopes for fusion (29). To overcome a possible postbinding block due to the absence of endocytosis-mediated fusion, the infection of the same cells was also carried out with viruses carrying EMOA envelopes, which should have the pH-independent fusion properties of MLV-A. These EMO and EMOA envelopes were competent for fusion following binding to the ecotropic receptor, as demonstrated by their abilities to allow infection of NIH 3T3 cells (Table 2). However, no infection could be detected on human cells (Table 2). Similarly, viruses with EA envelopes (which should also be pH independent for fusion) were not able to infect human cells expressing EGFR, with the exception of TE671 cells, on which a very low titer (10 *lacZ* CFU/ml) was obtained. EMO or EMOA EGF fusion envelopes were also coexpressed in TELCeB6 producer cells with wild-type MO ecotropic envelopes in order to generate viral particles with mixed envelope oligomers, but no infectious virions were produced (Table 2).

TABLE 1. Infection by virions expressing Ram-1 targeting envelopes

Env[a]	Titer (*lacZ* CFU/ml) for cell line[b]		
	TE	TE-A	TE-RD
A	10^7	10^2	10^7
MO	<1	<1	<1
AMO	10^3	2	10^3
AMO/MO[c]	10^3	ND[d]	ND

[a] Envelope expressed on *lacZ* virions.
[b] Abbreviations for cell lines: TE, TE671; TE-A, MLV-A-infected TE671; TE-RD, RD114-infected TE671.
[c] Chimeric envelopes were coexpressed with MO ecotropic wild-type envelopes.
[d] ND, not determined.

TABLE 2. Infection by virions expressing EGFR targeting envelopes

Env[a]	Titer (*lacZ* CFU/ml) for cell line[b]						
	A431	HT1080	TE671	K422	3T3	3T3-E	3T3-A
A	10^7	10^7	10^7	10^5	10^7	10^7	10^2
MO	<1	<1	<1	<1	10^7	<1	10^7
EMO	<1	<1	<1	<1	10^5	<1	10^5
EMO/MO[c]	<1	<1	<1	<1	10^7	<1	10^7
EMOA	<1	<1	<1	<1	10^3	<1	10^3
EMOA/MO[c]	<1	<1	<1	<1	10^7	<1	10^7
EA	<1	<1	10^1	10^4	10^6	ND[d]	10^1

[a] Envelope expressed on *lacZ* virions.
[b] Abbreviations for cell lines: 3T3, NIH 3T3; 3T3-E, psi2; 3T3-A, GP+EAM12.
[c] Chimeric envelopes were coexpressed with MO ecotropic wild-type envelopes.
[d] ND, not determined.

Infection is competitively blocked by EGFR expression. A surprising result was found for viruses with EA envelopes following infection of human cells. Although viruses with such envelopes could easily infect K422 human cells devoid of EGFR, presumably through the Ram-1 receptor (Table 2), they could only weakly infect TE671 cells and could not infect human cells with higher densities of EGFR, like A431 or HT1080 cells. These data suggested that EGFR expression led to a competitive inhibition of viral infection. Therefore, NR6 murine fibroblasts, which lack EGFRs, and derivatives of these fibroblasts which were engineered to express hEGF receptors by transfection with a plasmid encoding hEGFR, were challenged with EMO and EA enveloped viruses (Table 3). The titers of viruses carrying EMO or EA EGF fusion envelopes were reduced by approximately 100- to 200-fold by hEGFR expression. When NR6-hEGFR cells were pretreated with rEGF, which down-regulates EGFR as confirmed by antibody staining (not shown), titers of viruses coated with EGF fusion envelopes were greatly enhanced, reaching the range of titers obtained on parental NR6 cells (Table 3). These data suggested that the interaction of virions with EGFRs could specifically interfere with the postbinding events required to release viral cores in the cytoplasm.

After binding to its receptor, EGF induces receptor dimerization and signal transduction, followed by ligand-receptor internalization and routing to lysosomes, in which EGF-EGFR complexes are degraded (7). To see whether viruses were following a similar degradative pathway after their binding to EGFR, we examined internalization of viral particles. A431 cells were incubated 45 min at 37°C with viruses coated with the various envelopes. Cells were then washed and trypsinized

TABLE 3. Inhibition of infection by EGFR

Env[a]	Titer (*lacZ* CFU/ml) (% infection) for cell line[b]			
	NR6	NR6-C′973	NR6-hEGFR[c]	
			−rEGF	+rEGF
MO	1.4×10^5 (100)	5.6×10^5 (400)	5×10^5 (357)	5×10^5 (357)
EMO	4.9×10^4 (100)	1.4×10^4 (28.6)	1.4×10^3 (2.9)	10^5 (200)
A	7×10^4 (100)	3.7×10^5 (529)	1.7×10^5 (243)	2×10^5 (286)
EA	2.1×10^5 (100)	9×10^4 (42.9)	3×10^3 (1.4)	5×10^5 (238)

[a] Envelope expressed on *lacZ* virions.
[b] Percentage of infection with the same viruses relative to parental NR6 cells.
[c] Cells were (+) or were not (−) preincubated with 10^{-6} M rEGF for 30 min at 37°C.

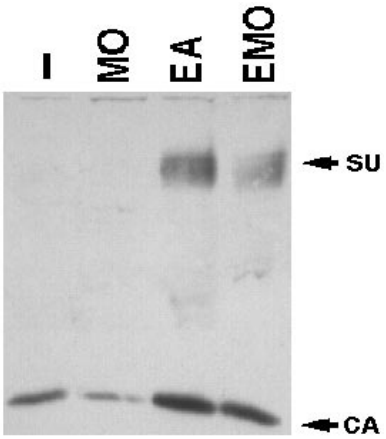

FIG. 7. Internalization assays. A431 cells were used as targets. Lysates of cells incubated with viruses carrying the different envelopes were analyzed by Western blot. Filters were stained with SU antiserum for proteins with atomic masses of >46 kDa or with CA antiserum for proteins with lower molecular masses. −, bald viruses from nontransfected TELCeB6 cells.

to remove cell surface-bound virions. Lysates of cells were then analyzed by immunoblotting with antibodies against either CA or SU (Fig. 7). A low level of CA was detected when viruses without envelopes or with ecotropic MO envelopes were plated on the cells, although these latter viral preparations were not infectious on these cells as judged by X-Gal staining (Table 2). By comparison, viral core proteins could be easily detected in A431 cells incubated with viruses with EMO or EA envelopes, demonstrating that virions were more readily internalized in cells following interaction with EGFR. These results also directly confirmed that the EMO-carrying virions and not only the soluble EMO envelopes were able to bind cells via EGFR.

Two further experiments supported the idea that ligand-mediated internalization was involved in the inactivation of virus infectivity following EGFR binding. Firstly, NR6 cells expressing the C′973 hEGFR mutant (8) lacking the determinants for ligand-induced internalization, which are located in the cytoplasmic tail (7), showed a much-reduced ability to inactivate viruses expressing EMO or EA envelopes (Table 3). Secondly, when EMO- or EA-carrying viral particles were used to infect A431 cells treated with the inhibitor of lysosomal degradation, chloroquine, a significant increase of infectivity (by approximately 100-fold) was obtained (Table 4). This effect was specific to EGFR, as EGFR-negative cells such as K422 cells did not respond similarly (Table 4).

Our results suggest that the interaction of virions with EGFR does not lead to a productive infection because viral particles are routed to a cell compartment which does not allow the postbinding events required for retroviral infection. Viral particles bound to EGFR, like EGF, may be routed to a late endosome and destroyed by lysosomal enzymes. However, it seems likely that other infection-inactivating factors were involved, as viruses with EMO envelopes were inactivated by binding to NR6-C′973 EGFR (Table 3) and were unable to infect TE671 cells engineered to express a similar EGFR mutant, C′958 (data not shown).

TABLE 4. Effects of chloroquine on infection

Env[a]	Titer (*lacZ* CFU/ml) for cell line[b]							
	NIH 3T3		A431		TE671		K422	
	−	+	−	+	−	+	−	+
MO	10^6	5×10^5	<1	6	<1	1	<1	<1
EMO	10^5	5×10^4	1	225	<1	46	<1	<1
A	10^6	5×10^5	10^6	5×10^5	10^6	10^4	10^4	5×10^2
EA	10^5	5×10^4	2	156	59	29	10^3	10^2

[a] Envelope expressed on *lacZ* virions.
[b] Cells were untreated (−) or treated with chloroquine (+).

DISCUSSION

Several strategies can be used to engineer retroviral envelopes with altered host range. Small modifications of the retroviral SU can be introduced by substitutions with linear peptides displaying binding properties. Some such minimally modified retroviral envelopes have been shown to be processed and incorporated into virions, and redirection of tropism at very low efficiency has been achieved (44). Larger modifications of the retroviral envelope, such as replacing the receptor binding domain, have also been analyzed. Replacement with an antibody (9, 10) or the cytokine erythropoietin (19) has been reported to retarget viral infection. In a previous approach to modify retroviral host range, we replaced the receptor binding domain of the MoMLV envelope with EGF or erythropoietin (13a). Such chimeric envelopes were secreted in the supernatant of transfected cells, but they were not correctly processed or expressed on the cell surface or incorporated on viral particles. Consequently, no infectious viruses were detected even when wild-type MoMLV envelope glycoproteins were coexpressed with the recombinant envelopes. The contrast between these data and those recently reported by Kasahara et al. (19) remains unexplained, although the different cells used to generate retroviral particles may account for the discrepancies in the results.

We have recently reported evidence that single-chain recombinant antibodies inserted at the N terminus of MoMLV SU enabled the generation of retroviruses capable of binding to antigen (33). We report here that identical N-terminal insertion of two ligands, either for Ram-1 phosphate transporter, or for EGFR, resulted in virions expressing correctly processed envelopes, which bound to the expected receptor. We have additional evidence (12a) that chimeric envelopes fused to a variety of single-chain antibodies and polypeptide ligands are correctly folded and displayed on virions, allowing a variety of specific targeting of infection.

AMO viruses targeted to Ram-1 were able to specifically infect cells, though with a low efficiency. Because similar binding levels were achieved with the wild-type Ram-1-binding envelopes (MLV-A) and the Ram-1-targeted chimeric envelopes (AMO), such a low level of infection is likely to be due to an inefficient fusion between viral and cell membranes after AMO binding to Ram-1. The fusogenicity of viral envelopes is thought to be triggered by conformational changes in the envelope glycoprotein (47). Like the envelopes of most retroviruses, MLV-A envelopes are known to induce virus fusion directly at the cell surface. Conversely, MLV-E envelopes have been reported to be dependent on acidic pHs for fusion and to require virus internalization (2, 25). A recent report has mapped the sequence responsible for the pH dependence of MLV-E to a region of Env C terminal to the polyproline hinge (29). Swapping this particular region between MoMLV and MLV-A envelopes resulted in MoMLV/MLV-A hybrid envelopes which had ecotropic host ranges and were pH independent for fusion (29). However, the same modifications introduced in AMO envelopes did not result in increased titers

(data not shown). Therefore the low level of infectivity of AMO envelopes was not due to incompatibility between their fusion domains and the Ram-1 receptor. It is therefore more likely that the low level of infectivity was due to the inability of chimeric envelopes to optimally transduce a signal to trigger fusion after they had bound.

For orthomyxoviruses like influenza, the binding of the hemagglutinin to the sialic acid residues on the receptor induces endocytosis of the virus-receptor complex (24) and a lowering of pH in the endosome leads to structural changes in the hemagglutinin protein which trigger fusion (6, 39). For the majority of retroviral Env glycoproteins, conformational changes which lead to fusion must be triggered by events other than acidification, such as receptor binding (28, 36). For example, the interaction of human immunodeficiency virus gp120 SU with CD4 results in dissociation between SU and TM and in increased exposure of V3, a gp120 loop involved in fusion (11, 36). The insertion of new binding domains in an ectopic location on the envelope is likely to interfere with such a conformational trigger. It is nevertheless interesting to note that EMO envelopes were fusion competent and fully infectious when the wild-type MO binding domain was used to infect NIH 3T3 cells (Table 2). This suggests that an N-terminal ligand insertion does not necessarily inhibit fusion triggered by the MO binding domain.

Our results suggest that not all cell surface molecules can act as retroviral receptors for such targeted envelopes. The infectivities of viral particles targeted to EGFRs were specifically inhibited after EGFR binding. Virion-EGFR complexes were internalized, and lysosomal enzymes might contribute to the inactivation of viruses, as the use of lysosomotropic agents such as chloroquine rescued a low level of infectivity (Table 4). However, EGFR mutants which do not undergo ligand-activated endocytosis were also able to inhibit virus infectivity, though less efficiently (Table 3), and did not permit viral infection (data not shown). This suggests that the routing of particles with EMO or EA envelopes to lysosomes might be a consequence of binding to EGFR rather than the primary cause of viral inactivation. Therefore, some property of initial EGFR interaction probably leads to virus inactivation. In a recent report, Etienne-Julan et al. (16) have linked MoMLV-derived recombinant retroviruses to EGFRs on A431 cells using anti-SU monoclonal antibodies and various EGFR binding components. Such complexes were found to allow infection (16). Interestingly, the efficiency of infection was higher when the bridge was made with anti-EGFR antibodies rather than with biotinylated EGF. This demonstrates that EGFR can function as a retroviral receptor under certain experimental conditions and suggests that the nature of the bridge between envelope and cell is critical for postbinding steps leading to infection.

A number of *in vivo* gene therapy protocols require the development of novel retroviral vectors able to specifically recognize target cells. Our data will be helpful in the design of such targeting vectors with genetic modifications in the envelope glycoprotein and in the selection of cell surface molecules suitable for targeting. Our data also suggest a novel approach to targeting, whereby the host range of a promiscuous retroviral vector can be selectively restricted by displaying a ligand on its surface.

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