HUMAN GENE THERAPY 10:1533–1544 (June 10, 1999)
Mary Ann Liebert, Inc.

Retroviral Display of Functional Binding Domains Fused to the Amino Terminus of Influenza Hemagglutinin

THEODORA HATZIIOANNOU,[1,2] EDWIGE DELAHAYE,[1,2] FRANSISCO MARTIN,[3] STEPHEN J. RUSSELL,[4] and FRANÇOIS-LOÏC COSSET[1,2]

ABSTRACT

We have previously shown that retroviral vector particles derived from Moloney murine leukemia virus (Mo-MuLV) can efficiently incorporate influenza hemagglutinin (HA) glycoproteins from fowl plague virus (FPV), thus conferring a broad tropism to the vectors. To modify its host range, we have engineered the FPV HA to display four different polypeptides on its N terminus: the epidermal growth factor, an anti-human MHC class I molecules scFv (single-chain antibody), an anti-melanoma antigen scFv, and an IgG Fc-binding polypeptide. All recombinant HA glycoproteins were correctly expressed and processed, and efficiently incorporated into Mo-MuLV retroviral particles, indicating that amino-terminal insertion of large polypeptides did not alter the conformation of HA chimeras. Virions carrying the different chimeras bound specifically to cells expressing the targeted cell surface molecules of each ligand. In addition, all virion types were infectious but exhibited various degrees of specificity regarding the use of the targeted cell surface molecule versus the wild-type FPV HA receptor for cell entry and infection. For some ligands tested, infectivity was significantly increased on cells that express the targeted receptor, compared with cells that express only the wild-type HA receptor. Furthermore, some polypeptides could abolish infectivity via the wild-type FPV HA receptor. Our data therefore indicate that it is possible to engineer the HA envelope glycoprotein by fusing ligands to its amino-terminal end without affecting its fusion activity.

OVERVIEW SUMMARY

Retargeting gene delivery to specific cell populations is necessary for gene therapy to be applied *in vivo*. Although retroviral envelope glycoproteins can be readily modified to redirect virion binding to specific cell surface molecules, their practical utility is generally limited by the poor fusogenicity of the chimeras produced. In contrast, the ability of the influenza virus hemagglutinin (HA) glycoprotein to fuse in low-pH endosomes, associated with the high turnover rate of most cell surface molecules, provides the possibility of an alternative glycoprotein as a basis for targeting strategies. We have previously shown that HA can efficiently pseudotype retroviral vectors. In this article, we explore the possibility of modifying the host range of HA and of using HA-derived chimeric glycoproteins to target retroviral vectors to specific cell surface receptors.

INTRODUCTION

THE TROPISM OF RETROVIRUSES and of retroviral vectors is primarily determined by the host-range properties of their envelope glycoproteins. Attachment to the target cells is mediated by the amino-terminal surface subunit, SU, of the retroviral envelope glycoprotein (Hunter and Swanstrom, 1990). After receptor binding, conformational rearrangements are thought to occur that activate the transmembrane envelope subunit, TM, which mediates membrane fusion (Hunter and Swanstrom, 1990). In the case of retroviral vectors based on murine

[1]Laboratoire de Vectorologie Rétrovirale et Thérapie Génique, Unité de Virologie Humaine, INSERM U412, Ecole Normale Supérieure de Lyon, 69364 Lyon Cedex 07, France.
[2]Centre de Génétique Moléculaire et Cellulaire, CNRS UMR5534, Université Claude-Bernard Lyon-1, 69622 Villeurbanne, France.
[3]Department of Immunology, Windeyer Institute for Medical Science, University College London, London, UK.
[4]Molecular Medicine Program, Guggenheim 18, Mayo Clinic, Rochester, MN.

leukemia viruses (MuLVs), those carrying the Moloney MuLV ecotropic envelope glycoproteins infect only rodent cells whereas those carrying the amphotropic MuLV envelope glycoproteins infect most mammalian cell types including human cells. Retroviruses can also incorporate nonretroviral surface glycoproteins from other membrane-enveloped viruses, resulting in the formation of infectious pseudotypes. For example, envelope glycoproteins such as glycoprotein G from vesicular stomatitis virus (Burns *et al*., 1993) and hemagglutinin (HA) from either influenza virus A/Jap/305/57 (Dong *et al*., 1992) or fowl plague virus (FPV) (Hatziioannou *et al*., 1998), can efficiently pseudotype retroviral particles. The host range of retroviral vectors can thus be extended to a wider variety of cell types.

However, for several gene transfer applications a specific targeting of the vector is required. A number of modified retroviral envelope glycoproteins have been generated that are capable of specifically binding to predetermined cell surface molecules (Cosset and Russell, 1996). In most cases, however, the titers of retroviruses carrying the envelope chimeras remain low (Cosset *et al*., 1995a; Marin *et al*., 1996; Konishi *et al*., 1998). In fact, it appears that the ability of the chimeric envelopes to mediate fusion as a consequence of binding to the targeted receptor is usually dramatically impaired. Hence the low retargeted titers can be explained by our inability to understand fully the retroviral fusion process and thus our inability to engineer the fusogenicity of retargeted retroviral envelopes. Indeed, the modification of retroviral envelope glycoproteins by insertion of ligands is likely to severely compromise the fusion activation mechanism. On the other hand, in contrast to retroviral envelopes, the fusion mechanism of influenza HA glycoprotein has been extensively studied and does not appear to depend primarily on binding to the HA receptor. The HA protein consists of two subunits: the amino-terminal HA1 subunit, including the receptor-binding domain, and the HA2 subunit, including the fusion peptide and the membrane-anchoring region. For influenza virus, virion binding is followed by receptor-mediated endocytosis. The acid pH of the endosomes induces conformational changes in both HA subunits, thus resulting in activation of the HA2 subunit, which mediates membrane fusion (White, 1992). Thus the physiological signals that trigger the membrane fusion properties of HA are much less specific than those of retroviral envelope glycoproteins.

Since MuLV retroviral particles can be efficiently pseudotyped with the HA glycoprotein from FPV (Hatziioannou *et al*., 1998), we sought to determine whether it was possible to engineer HA so as to target the pseudotyped viruses to specific cell surface molecules. Although the insertion of peptides with limited size and/or secondary structure into specific loops exposed at the surface of the MuLV retroviral envelope glycoprotein has been a feasible approach to redirect the host range of MuLV retroviral vectors (Valsesia-Wittmann *et al*., 1994), larger modifications, such as the insertion of more structured/longer peptides or the replacement of envelope domains by foreign polypeptide domains, have resulted in MuLV-derived envelope chimeras that were either unstable or not correctly processed and that failed to be efficiently incorporated into retroviral particles (Cosset and Russell, 1996). In contrast, the addition of autonomously folded polypeptides as amino-terminal extensions of the MuLV SU glycoprotein has generally resulted in envelope chimeras able to redirect the binding of retroviruses onto which they were incorporated (Cosset and Russell, 1996). Since the amino-terminal peptide containing the first 10 amino acids of the influenza virus HA1 is not committed in a particular protein structure (Wilson *et al*., 1981) and since its length and sequence are variable between the different influenza virus strains (Nobusawa *et al*., 1991), we sought to display different polypeptides with binding properties by fusing these ligand at the amino terminus of FPV HA as a strategy to modify the tropism of the HA glycoprotein. The four ligands tested here were as follows: (1) the human epidermal growth factor (EGF), (2) an scFv (single-chain variable fragment antibody) directed against human MHC class I molecules, (3) an scFv directed against the high molecular weight melanoma-associated antigen (HMWMAA) expressed on human melanoma cells, and (4) a polypeptide derived from protein A of *Staphylococcus aureus* and able to bind to the Fc fragment of various immunoglobulin types. All four chimeric HA proteins were correctly expressed and processed in cells that produce MuLV Gag–Pol proteins and efficiently incorporated into retroviral particles. Furthermore, all chimeras mediated specific binding to the receptor corresponding to each polypeptide. Finally, the virions pseudotyped with the different chimeric HA envelopes were infectious and showed different levels of infection specificity.

MATERIALS AND METHODS

Cell lines

The TELCeB6 cell line (Cosset *et al*., 1995b) was derived by clonal selection of TE671 human rhabdomyosarcoma cells transfected with both a plasmid expressing Moloney murine leukemia virus (Mo-MuLV) Gag and Pol proteins and an nlsLacZ retroviral vector. TELCeB6 cells produce noninfectious viral core particles, carrying an nlsLacZ reporter retroviral vector.

A431 (ATCC CRL1555) and TE671 (ATCC CRL8805) human cells were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (GIBCO-BRL, Gaithersburg, MD). CHO (Chinese hamster ovary) cells (ATCC CCL-61) were grown in DMEM (Life Technologies) supplemented with 10% fetal bovine serum and proline (Life Technologies). NIH 3T3 mouse fibroblasts were grown in DMEM (Life Technologies) supplemented with 10% newborn bovine serum (Life Technologies).

Plasmid construction

The highly fusogenic H7/Kp Rostock fowl plague virus hemagglutinin (HA) (kindly provided by W. Garten, Marburg University, Marburg, Germany) was expressed from the hCMV-HA expression vector (Hatziioannou *et al*., 1998), using the human cytomegalovirus early promoter and rabbit β-globin intron sequences. The N-terminal end of FPV HA was modified (Fig. 1) by polymerase chain reaction (PCR)-mediated mutagenesis using the FPV HA gene as template and primers UpH7Not (5′-GAA CGC GCG GCC GCA ATC GAG GGA AGG CAA GAC CTT CCA GGA AAT GAC AAC AGC GAC AAA ATT TGT

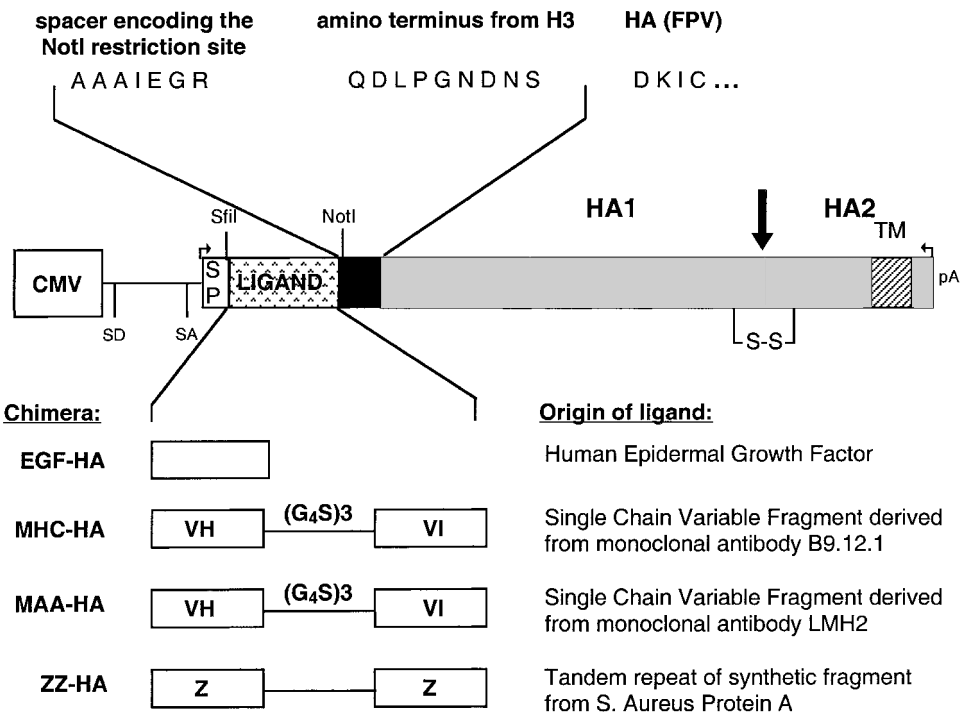

FIG. 1. Schematic diagrams of HA chimeras. The chimeric envelopes are presented diagrammatica lly. The positions of some functional regions are indicated. The site of fusion between the ligands and the HA glycoprotein is shown in detail. HA1, N-terminal subunit of HA; HA2, C-terminal subunit of HA containing the transmembrane-spanning domain (TM). FPV-HA- and HA-derived chimeric glycoproteins were expressed using the human cytomegalovirus (CMV) early promoter. pA, Polyadenylation sequence; SD and SA, donor and acceptor splice sites.

CTT GGA CAT CAT GCT GTA TCA AAT GGC ACC AAA G-3′) and LowH7Xba: (5′-CCC CGG GTA ACA AAC ATC ATT TCC TTC TCG-3′), containing an *Not*I site and an *Xba*I site, respectively. The PCR-amplified DNA fragment was cloned into the phCMV-HA backbone using the *Not*I site located upstream of the CMV promoter and the *Xba*I site inside the HA gene. Both the CMV promoter and the rabbit β-globin intron sequences were reintroduced into the resulting plasmid as an *Not*I–*Xba*I fragment together with an *Xba*I–*Not*I fragment, encoding the MuLV envelope glycoprotein signal peptide followed by EGF (derived from the FBEMOSALF plasmid) (Cosset *et al.*, 1995a). The *Not*I site upstream of the CMV promoter was then removed by filling in and self-ligation, resulting in the pHCMV-EGF-HA expression plasmid. A series of plasmids expressing other HA chimeras were derived from hCMV-EGF-HA by replacing the *Sfi*I–*Not*I fragment encompassing the EGF DNA fragment with sequences (available on request) encoding the other ligands cloned as *Sfi*I–*Not*I fragments (Fig. 1): (1) a DNA fragment encoding an scFv derived from B9.12.1 anti-human MHC class I antigen mouse monoclonal antibody (Marin *et al.*, 1995), for the construct expressing the MHC-HA chimera; (2) a DNA fragment encoding an scFv derived from LMH2 anti-high molecular weight melanoma-associate d antigen (HMW-MAA) (Kupsch *et al.*, 1995), for the construct expressing the MAA-HA chimera; and (3) a DNA fragment encoding an Fc-binding polypeptide derived from domain B of *Staphylococcus Aureus* protein A (Nilsson *et al.*, 1987), for the construct expressing the ZZ-HA chimera.

Cell transfection and virus production

Envelope glycoprotein expression plasmids were transfected by calcium phosphate precipitation into TELCeB6 cells as previously described (Cosset *et al.*, 1995a). Transfected cells were grown for 24–48 hr and virus-containing supernatants were collected after an overnight production from freshly confluent *env*-transfected TELCeB6 cells in regular medium.

Antibodies

The following antibodies were used:

Anti-CA (Quality Biotech, Camden, NJ): A goat antiserum raised against the Rauseher leukemia virus p30 capsid protein (CA), used diluted 1:10,000 for Western blots

Anti-HA (kindly provided by W. Garten): A rabbit antiserum raised against H7/Kp Rostock fowl plague virus hemagglutinin, used at 1:500 dilution for Western blots and at 1:100 dilution for fluorescence-activated cell sorting (FACS) analysis

Anti-EGF receptor (Upstate Biotechnology, Waltham, MA): A mouse monoclonal antibody against human EGF receptor, used at 1:100 dilution for FACS analysis

Anti-HLA ABC (Immunotech, Marseille, France): A mouse monoclonal antibody directed against a monomorphic determinant of human class I HLA molecules (HLA-A,B,C), used at 1:100 dilution for FACS analysis

LMH2 (kindly provided by J. Kupsch, Wellcome Research Lab-

oratoire, Beckeham, UK): A mouse monoclonal antibody recognizing the high molecular weight melanoma-associated antigen (HMWMAA), used at 1:100 dilution for FACS analysis

Immunoblots

Virus producer cells were lysed in a 20 m*M* Tris-HCl buffer (pH 7.5) containing 1% Triton X-100, 0.05% sodium dodecyl sulfate (SDS), sodium deoxycholate (5 mg/ml), 150 m*M* NaCl, and 1 m*M* phenylmethylsulfonyl fluoride (PMSF). 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

Supernatants of virus producer cells were centrifuged for 10 min at 1000 × *g* and then filtered to remove cell debris. Virus samples were obtained by ultracentrifugation of the clarified viral supernatants (5 ml) in an SW41 Beckman (Fullerton, CA) rotor (150,000 × *g*, 1 hr, 4°C). Pellets were suspended in 50 μl of phosphate-buffered saline (PBS) and frozen at −70°C. Samples (30 μg for cell lysates, or 20 μl for purified viruses) were mixed 5:1 (v/v) in a 375 m*M* Tris-HCl (pH 6.8) buffer containing 6% SDS, 30% 2-mercaptoethanol, 10% glycerol, and 0.06% bromophenol blue, boiled for 2 min, then run on 12% SDS-acrylamide gels. After protein transfer onto nitrocellulose filters, immunostaining was performed in TBS (Tris base saline, pH 7.4) with 5% milk powder and 0.1% Tween. The blots were probed with anti-HA antibodies and developed using horseradish peroxidase (HRP)-conjugated IgG (immunoglobulins G) raised against rabbit antibodies (Dako, Carpinteria, CA) and an enhanced chemiluminescence kit (Amersham Life Science, Arlington Heights, IL).

Binding assays

Target cells were washed in PBS and detached by a 10-min incubation at 37°C with 0.02% EDTA tetrasodium (Versene) in PBS. Cells were washed in PBA (PBS with 2% fetal calf serum [FCS] and 0.1% sodium azide). Cells (5×10^5) were incubated with 2.5 ml of viral supernatant for 45 min at 4°C in the presence of Polybrene (4 μg/ml). Cells were then washed twice with pba and were incubated with the anti-HA serum for 45 min at 4°C. Cells were washed twice with PBA and incubated with anti-rabbit IgG antibodies conjugated with fluorescein isothiocyanate (FITC) (Dako) for 45 min at 4°C. Five minutes before the two final washes in PBA, cells were counterstained with propidium iodide (20 μg/ml). Fluorescence of living cells was analyzed with a fluorescence-activated cell sorter (FACSCalibur; Beckton Dickinson, San Jose, CA).

Downregulation of the EGF receptor was performed by incubating A431 cells with 10^{-6} *M* recombinant human EGF (R&D Systems, Minneapolis, MN) for 30 min at 37°C prior to addition of the viral supernatant. Binding assays were then performed as described above.

Competition of MAA-HA virion binding was performed by preincubating TE671 cells with the LMH2 monoclonal antibody for 30 min at 4°C, prior to addition of the viral supernatant. Binding assays were subsequently performed as described above. For the binding of the ZZ-HA chimeric glycoproteins, target cells were incubated with anti-EGF receptor antibodies for 30 min at 4°C before the viral supernatant was added. Binding assays were subsequently performed as described above except that purified mouse IgG (Sigma, St. Louis, MO) was added during the last incubation (used at a final concentration of 10 μg/ml) to inhibit the cross-reaction between the anti-EGF receptor antibody and the anti-rabbit immunoglobulin–FITC conjugate.

Cell surface staining

Cells were washed in PBS and detached by a 10-min incubation at 37°C with 0.02% Versene in PBS. Cells were washed twice in PBA. Cells were then incubated with the anti-EGF receptor or with the anti-HLA or anti-HMWMAA antibodies for 45 min at 4°C. Cells were washed twice in PBA and incubated with an anti-mouse immunoglobulin–FITC conjugated (Dako), 45 min at 4°C. Five minutes before the two final washes in PBA, cells were counterstained with propidium iodide (20 μg/ml). Fluorescence of living cells was analyzed with a fluorescence-activated cell sorter (FACSCalibur; Beckton Dickinson).

Infection assays

Target cells were seeded in 24-well plates at a density of 5×10^4 cells per well. Viral supernatants (5 ml) were centrifuged in an SW41 Beckman rotor at 150,000 × *g* for 1 hr at 4°C. Pellets were suspended in 50 μl of PBS and used for infection assays, at different dilutions, and supplemented with Polybrene (4 μg/ml). Cells were incubated for 3–5 hr at 37°C. Viral supernatant was then removed and cells were incubated in regular medium for 48 hr. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining and viral titer determination were performed as previously described (Cosset *et al.*, 1995b); results are presented as LacZ infectious units (IU) per milliliter.

Infection assays using antibody-coated cells and virions carrying ZZ-HA chimeras

Target cells were detached with Versene, 0.02% in PBS. Cells (6×10^4) were incubated with, or without, anti-EGF receptor antibodies for 30 min at 4°C. Viral supernatant (300 μl), containing Polybrene (4 μg/ml), was added and cells were incubated for 1 hr at 4°C. The supernatant was then removed and cells were washed twice with ice-cold PBS complemented with 2% FCS. The cells were then seeded in 24-well plates in regular medium, incubated for 48 hr, and stained with X-Gal as described above.

RESULTS

Construction of chimeric hemagglutinins

Four different chimeric HA glycoproteins were generated by inserting, at position +1 at the amino terminus of the FPV HA envelope glycoprotein, one of the following ligands (Fig. 1): (1) the human epidermal growth factor (EGF, 53 amino acids) for the EGF-HA-expressing construct; (2) an scFv miniantibody (244 amino acids) recognizing human MHC class I molecules for the MHC-HA construct; (3) an scFv miniantibody (246 amino acids) recognizing the high molecular weight melanoma-associated antigen (HMWMAA) for the MAA-HA construct;

or (4) the IgG-binding domain (126 amino acids) derived from *Staphylococcus aureus* protein A (a synthetic domain based on domain B of protein A) inserted as a dimer for the ZZ-HA construct.

In all chimeric constructs, the same transcription signals [CMV promoter and SV40 poly(A) sequence] as well as the signal peptide of Mo-MuLV envelope of glycoprotein were used. Amino acid alignment of the HA glycoproteins from different influenza subtypes reveals that after cleavage of the signal peptide the remaining amino termini differ in length (Nobusawa *et al.*, 1991). The HA1 subunit from influenza subtype H3 has an amino-terminal "tail" nine amino acids (aa) longer than that of other influenza virus types such as FPV. We have used a 7-aa peptide (encoded by the *Not*I site) in association with the 9-aa tail derived from H3 HA to constitute a 16-amino acid spacer between the added ligand and the amino terminus of the HA glycoprotein of FPV. This was done so that the ligands would be more distant from the HA backbone domains and would thus facilitate proper folding and function of either protein domain.

Expression and virion incorporation

Wild-type HA or HA chimeras were transiently expressed in TELCeB6 cells, which provide MuLV retroviral core particles and a *lacZ* retroviral vector. Lysates of the transfected cells were analyzed by immunoblotting, using a polyclonal serum against HA (Fig. 2). The FPV HA glycoprotein is synthesized as a precursor (HA_0), which is subsequently cleaved by ubiquitously expressed cellular proteases into two subunits, HA1 and HA2, which remain disulfide linked (Klenk and Garten, 1994). The additional binding domain of the chimeric HA glycoproteins should remain associated with the HA1 subunit after cleavage of HA_0. As expected, in the case of the wild-type HA or EGF-HA proteins the envelope precursors could be detected at 90 and 110 kDa, respectively. Similarly, the precursors of MHC-HA, MAA-HA, and ZZ-HA chimeras could be detected at 140 kDa, consistent with the size differences of their respective ligands. All envelope precursors were cleaved and the resulting HA1 processed subunits were detected at 65 kDa for the wild-type HA, at 75 kDa for the EGF-HA chimera, and at about 85 kDa for the other chimeric HA subunits (Fig. 2A). For most HA chimeras, both HA_0 and HA1 proteins were expressed at levels comparable to those of wild-type HA, with the exception of the MAA-HA chimera, which was expressed to a lower level. Concerning only the EGF-HA chimeric envelope glycoprotein, in addition to the expected band corresponding to the chimeric HA1, a faint band that migrated to the same point as wild-type HA1 subunit could be detected in virion pellets and probably resulted from cleavage between the EGF domain and the HA1 subunit. Similar unexpected cleavage products have been observed in EGF-fusion retroviral envelope chimeras and have been attributed to the C-terminal end amino acid sequence of EGF that acts as a cleavage site for a cell protease (Peng *et al.*, 1998).

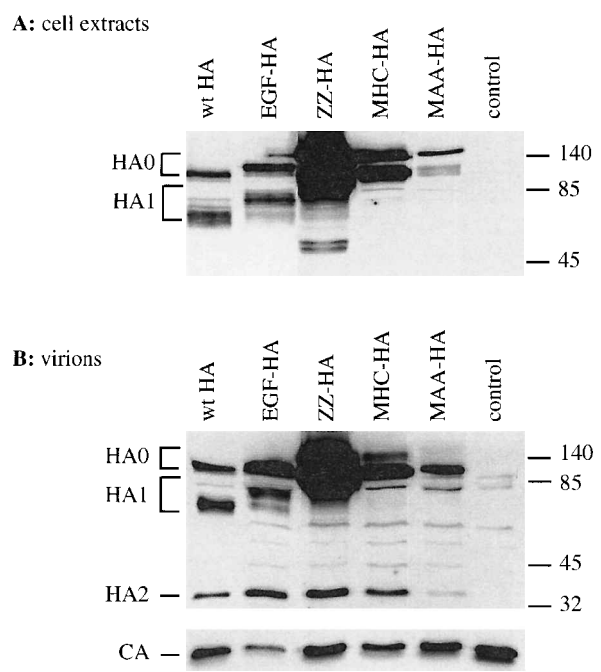

FIG. 2. Detection of envelope glycoproteins. Immunoblots of lysates of nontransfected (control) TELCeB6 cells, or of TELCeB6 cells transfected with the wild-type HA (wt) or with the indicated chimeric HA expression constructs **(A),** and immunoblots of pellets of viral particles produced from the transfected cells **(B).** Blots were stained with an anti-HA serum. The positions of the HA precursors (HA_0) and of the processed HA1 and HA2 subunits are indicated. The bottom part of the immunoblot of viral pellets was stained with anti-p30 antibodies to reveal the retroviral capsid p30-CA protein.

To determine whether the chimeric HA glycoproteins were incorporated into retroviral particles, the supernatant from TELCeB6 cells transfected with the HA chimeras were ultracentrifuged to sediment the viral particles, and the viral pellets were analyzed by immunoblotting with the anti-HA serum. The chimeric HA envelope glycoproteins were readily detected in the virion samples at the expected sizes and at levels comparable to that of wild-type HA (Fig. 2B). Three specific bands could be detected in most samples and corresponded to the HA precursors, to the HA1 subunits, and to the HA2 subunit that migrated to the 33-kDa point for all chimeras. As expected from its weak expression in cell lysates, the protein precursor of the MAA-HA chimera could not be detected in virion pellets and the intensity of its HA2 band was faint. In contrast, the intensities of the protein precursor and of the HA1 subunit for the ZZ-HA chimera were strong compared with the other HA chimeras (Fig. 2). This was due to the ability of the displayed ZZ IgG-binding domain to bind directly to the Fc domain of the conjugate antibody (data not shown).

Binding of viral particles to the corresponding receptors

The binding of virions bearing each of the chimeric HA envelopes was verified on cells expressing the targeted receptors. Target cells were incubated at 4°C with the viral supernatant and binding of the HA chimeras was measured by FACS analysis using anti-HA antibodies. At this temperature binding of HA to sialic acid was not (or was hardly) detectable for all of the target cell types analyzed (Figs. 3A, 4A, and 5A).

Binding of the MHC-HA chimera was carried out on A431

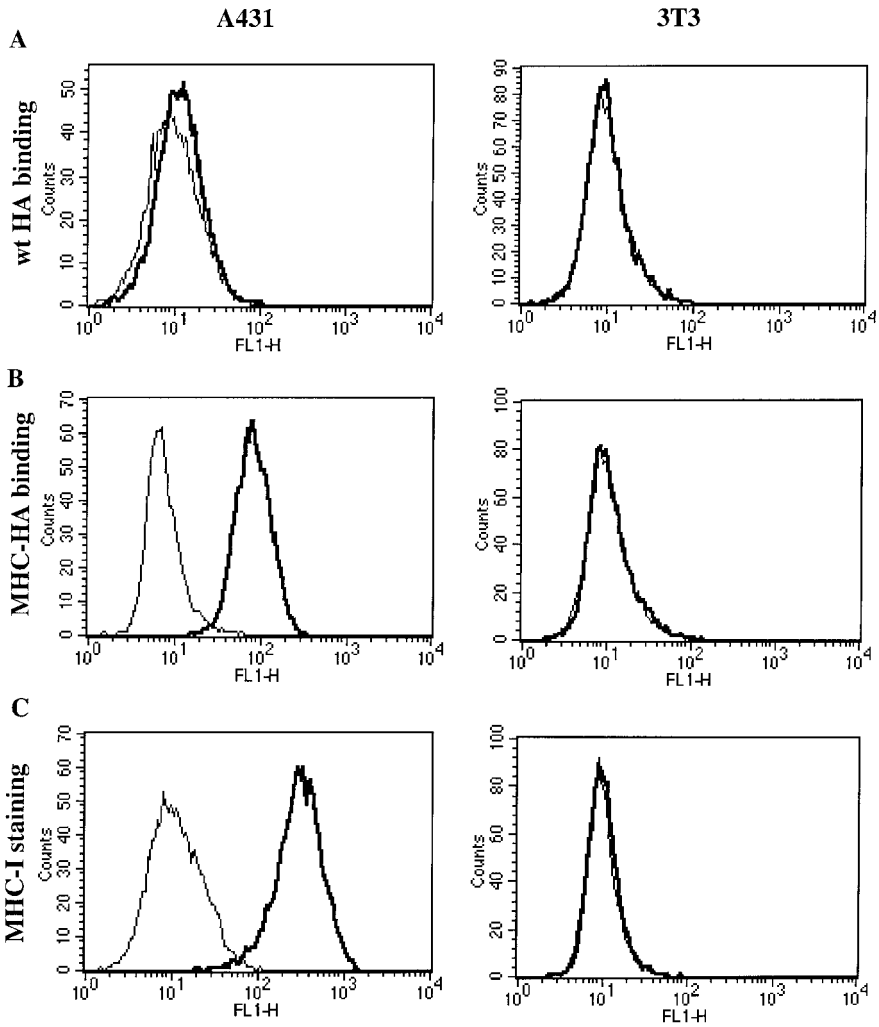

FIG. 3. Binding assays of MHC-HA-bearing virions. The indicated cells were incubated with supernatant containing virions generated with wild-type HA glycoproteins (thick line) **(A)** or incubated with supernatants containing virions carrying MHC-HA glycoproteins (thick line) **(B).** The background of fluorescence was provided by incubating the cells with supernatant containing retroviral particles devoid of envelope glycoproteins (thin line). The indicated cells were stained with an anti-MHC class I monoclonal antibody (thick line) or with the conjugate antibody only (thin line) **(C).**

human cells, which express high amounts of the MHC class I molecules compared with mouse NIH 3T3 cells, which stained negatively for human MHC class I antigens (Fig. 3C). Virions carrying the MHC-HA chimeras could bind efficiently to A431 cells but not to 3T3 cells (Fig. 3B), suggesting that binding of virions generated with MHC-HA glycoproteins was specifically due to interaction with the human class I molecules.

Binding of the EGF-HA-bearing retroviruses was carried out on A431 cells, expressing high numbers of EGF receptors (Fig. 4C). Virions carrying the EGF-HA chimera (Fig. 4B), but not virions loaded with wild-type HA glycoproteins (Fig. 4A), could bind efficiently to the target cells. Moreover, EGF-HA-carrying virions could not bind to 3T3 or CHO cells, which express few or no EGF receptors, respectively (data not shown). Furthermore, EGF-HA binding to A431 cells could be competed by using soluble recombinant human EGF (rEGF). Indeed, preincubation of the A431 target cells with rEGF, at 37°C for 30 min, resulted in downregulation of the EGF receptors from the cell surface (Fig. 4C) and dramatically reduced the binding of virions carrying EGF-HA chimeras to A431 cells (Fig. 4B). As a control, preincubation with rEGF had no effect on the cell surface expression of MHC class I molecules (Fig. 4C) and, as expected, did not decrease the binding of virions generated with MHC-HA chimeric envelope glycoproteins (Fig. 4B). In addition, binding assays were performed with pelleted viral particles obtained by ultracentrifugatio n of supernatants of EGF-HA virus producer cells. Binding of the viral particles was then revealed, as described previously (Hatziioannou *et al.*, 1998), by using an anti-EGF neutralizing monoclonal antibody and the results indicated that the EGF-HA chimera could mediate binding of the virions to A431 cells (data not shown). Altogether these data demonstrated that virions carrying EGF-HA chimeric glycoproteins could interact specifically with the targeted EGF receptors.

Binding of MAA-HA was performed on TE671 cells, which express high densities of HMWMAA antigens (Fig. 5C). Mouse 3T3 cells, which stained negatively for HMWMAA antigens (Fig. 5C), were used as controls. Wild-type HA mediated no or little binding at 4°C to either cell type (Figs. 3A and 5A) and, as expected, the MAA-HA chimera mediated no binding to mouse 3T3 cells (Fig. 5B). In contrast, virions generated with MAA-HA chimeric glycoproteins could bind strongly to HMW-MAA-positive TE671 cells (Fig. 5B). This binding could be competed by preincubation of the target cells with the parental anti-HMWMAA monoclonal antibody (Fig. 5B), thus demonstrating the specificity of interaction between HMWMAA antigens and virions carrying MAA-HA proteins.

The ZZ IgG-binding domain derived from *Staphylococcus aureus* protein A (Nilsson *et al*., 1987) has a strong affinity for the Fc region of various mammalian IgGs, particularly those from mouse (Harlow and Lane, 1988). Thus, as a step toward obtaining a versatile retargeted vector, we displayed the ZZ IgG-binding domain on HA proteins. In theory, coating of either the target cell surface or the ZZ-displaying viral particles with a predetermined antibody should allow a specific interaction between the chimeric viral particle and the desired cell surface molecule. To test the feasibility of this strategy, we used a mouse monoclonal antibody directed against the EGF receptor. A431 EGF receptor-positive cells were preincubated with anti-EGF receptor antibodies and subsequently incubated with the ZZ-HA-bearing virions. While viruses carrying the ZZ-HA chimeras could hardly bind to uncoated A431 cells, they bound easily to cells coated with the anti-EGF receptor antibody (Fig. 6) with an efficiency similar to that of viral particles carrying EGF-HA chimeric envelopes (Fig. 4B).

Collectively, these results demonstrated that the display of different ligands on the amino terminus of HA protein enables a specific retargeted binding of the chimeric virions to the expected cell surface molecules.

Infectivity of chimeric HA pseudotyped virions

Although the addition of ligands at the amino-terminal end of the HA protein allows the specific recognition of the targeted

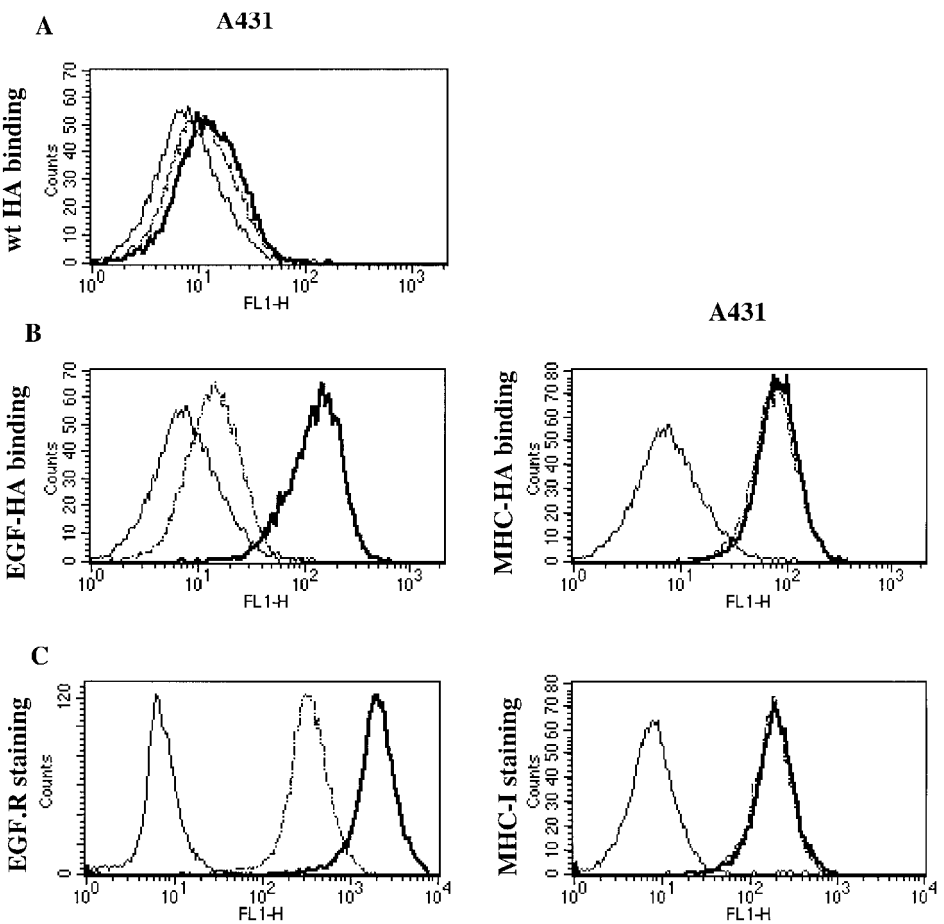

FIG. 4. Binding assays of EGF-HA-bearing virions. Binding of viruses carrying wild-type HA (wt-HA) to A431 cells (thick line) **(A).** Binding of viruses carrying EGF-HA or MHC-HA, as indicated, to A431 cells untreated (thick line) or preincubated with rEGF (broken line) **(B).** The background fluorescence was provided by incubating the A431 cells with retroviruses devoid of envelope glycoproteins (thin line). A431 cells, untreated (thick line) or preincubated with rEGF (broken line), were stained with an anti-EGF receptor or with anti-MHC class I antibodies, as indicated. The background of fluorescence (thin line) was provided by incubating the cells with conjugate antibodies only **(C).**

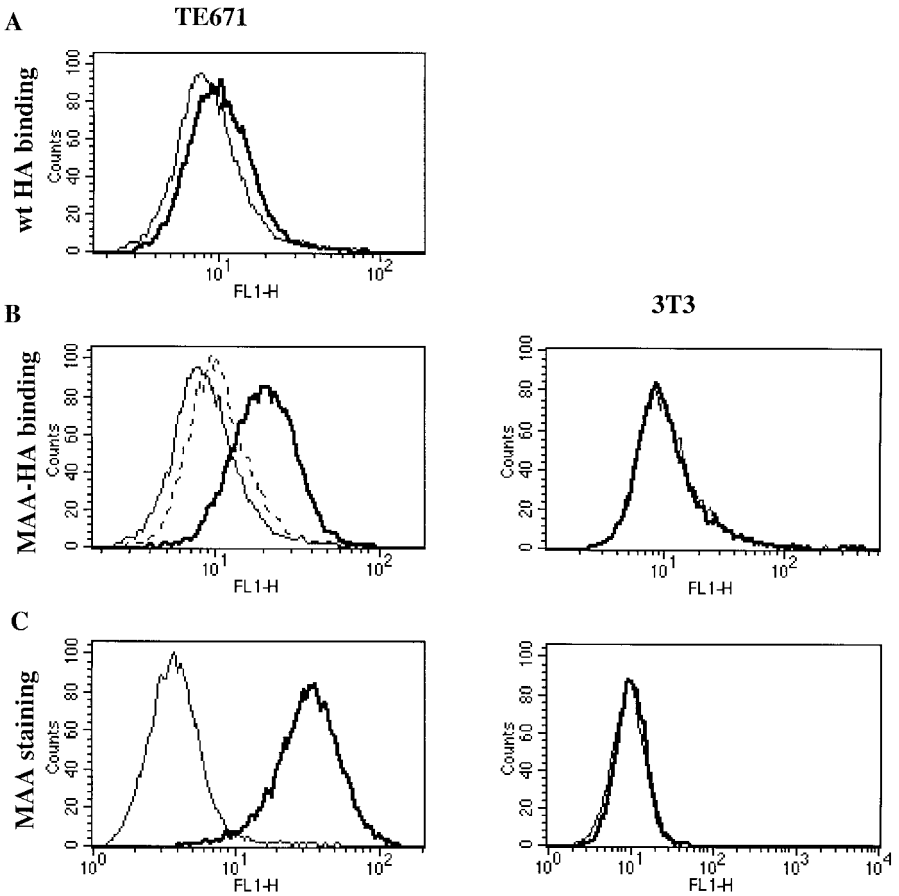

FIG. 5. Binding assays of MAA-HA-bearing virions. Binding of viruses carrying wild-type HA (wt-HA) to TE671 cells (thick line) **(A).** Binding of viruses carrying MAA-HA chimeras to the indicated target cells preincubated (broken line), or not (thick line), with anti-HMWMAA monoclonal antibody **(B).** The background fluorescence was provided by incubating the cells with retroviruses devoid of envelope glycoproteins. The indicated cells were stained with an anti-HMWMAA monoclonal antibody (thick line) or with the conjugate antibody only (thin line) **(C).**

cell surface molecules, the modification leaves the sialic acid-binding site intact. Despite the low or undetectable binding of wild-type HA and of HA chimeras to the natural HA receptor at 4°C (Figs. 3A, 4A, and 5A), binding of wild-type HA is detectable (although weak) at temperatures permissive for infection assays, i.e., 37°C (data not shown). Since the natural receptor of FPV HA is expressed on a wide range of cell types, both this receptor and the receptor recognized by the displayed ligand may contribute to the infectivity of the chimeric retroviruses.

Infection assays performed at 37°C on a variety of cell types indicated that retroviral vectors carrying the EGF-HA, MHC-HA (Fig. 7B), and ZZ-HA (data not shown) chimeric HA glycoproteins were highly infectious with titers similar to those of retroviruses carrying wild-type HA glycoproteins (Fig. 7A), in the range of 10^4–10^5 IU/ml. In contrast, as detailed below, the titers of vectors carrying the MAA-HA chimera were significantly reduced (Fig. 7B). Thus the addition of most ligands on the amino terminus of HA did not seem to affect the fusion activity of the chimeric HA glycoprotein.

Next, to determine whether binding of the displayed ligand to its receptor played a role during infection, we performed infection assays on cells that expressed the targeted receptor and compared the results with those of cells that did not express the targeted cell surface molecule. For each HA chimera, the choice of either cell type was dictated both by the results of binding assays (Figs. 3 to 6) and by their equivalent susceptibility to infection with wild-type HA-carrying retroviruses that use the same receptor for all tested cell lines (Fig. 7A). To indicate clearly the specific differences for a given HA chimeric envelope to allow infection of targeted cells versus control cells (Fig. 7B), the small differences in permissivity among the different cell types used were normalized by using the wild-type HA pseudotype as controls.

Retroviral vectors carrying EGF-HA chimeras were highly infectious on EGF receptor-positive A431 target cells as well as on EGF receptor-negative CHO cells, with titers higher than 10^4 IU/ml (Fig. 7B). Titers were enhanced by approximately twofold on A431 cells compared with CHO cells (Fig. 7B), thus suggesting that binding to EGF receptors could slightly increase the infectivity of the retroviruses but that infection mainly occurred via interaction with the natural FPV HA receptor. These results therefore indicated that the sialic acid-binding sites on the EGF-HA chimera were readily accessi-

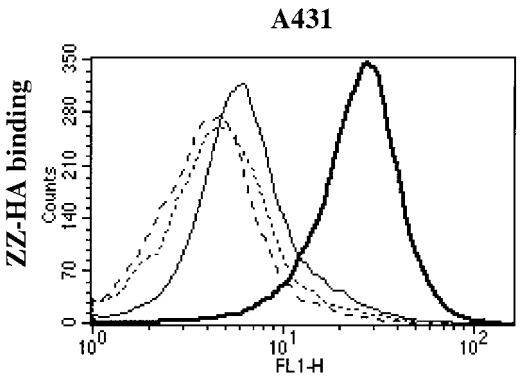

FIG. 6. Binding assays of ZZ-HA chimeras. Binding of viruses carrying ZZ-HA chimeras on uncoated A431 cells (thin line) or on A431 cells coated with anti-EGF receptor antibodies (thick line). The background of fluorescence was provided by incubating retroviruses devoid of envelope glycoproteins with A431 cells (dotted line) or with A431 cells coated with anti-EGF receptor antibodies (dashed line). To minimize the cross-reaction with anti-EGF receptor mouse monoclonal antibodies, excess amounts of mouse IgGs were added during incubation with the conjugate antibodies.

ble and that the displayed EGF did not abrogate the wild-type HA tropism.

Retroviral vectors carrying MHC-HA chimeras were also highly infectious on human MHC-I antigen-positive A431 target cells as well as on MHC-I antigen-negative mouse 3T3 cells (Fig. 7B), confirming the availability of the wild-type HA receptor-binding site for this chimera. However, titers on the target A431 cells were enhanced by sixfold compared with those on 3T3 nontarget cells (Fig. 7B). These data suggested that the displayed anti-MHC-1 scFv could contribute, at least to some extent, to a selective retargeted infection.

A431 cells coated with anti-EGF receptor antibodies were used as target cells for retroviruses carrying ZZ-HA chimeras able to bind the Fc domain of various IgG subclasses. To avoid internalization of cell surface-bound antibodies, infection assays were carried out at 4°C, thus resulting in lower titers relative to infection assays performed at 37°C (data not shown). Compared with uncoated A431 cells used as nontarget cells, a reproducible increase in infectivity, by fourfold, was detected on the anti-EGF receptor-coated A431 cells (Fig. 7B). In contrast, infectivity of retroviruses carrying wild-type HA glycoproteins was slightly reduced on anti-EGF receptor-coated A431 cells (9×10^2 IU/ml) compared with intact A431 cells (1.7×10^3 IU/ml). In addition, no increase in titers of viruses carrying ZZ-HA chimeras was detected on EGF receptor-negative CHO cells (data not shown). These results suggested that, similar to retroviruses loaded with MHC-HA or EGF-HA chimeras, the ZZ displayed domain could optimize the infectivity of retroviral vectors.

In contrast to the three former chimeras, retroviruses generated with MAA-HA glycoproteins could hardly infect HMW-MAA-negative nontarget 3T3 cells (Fig. 7B), although the lat-

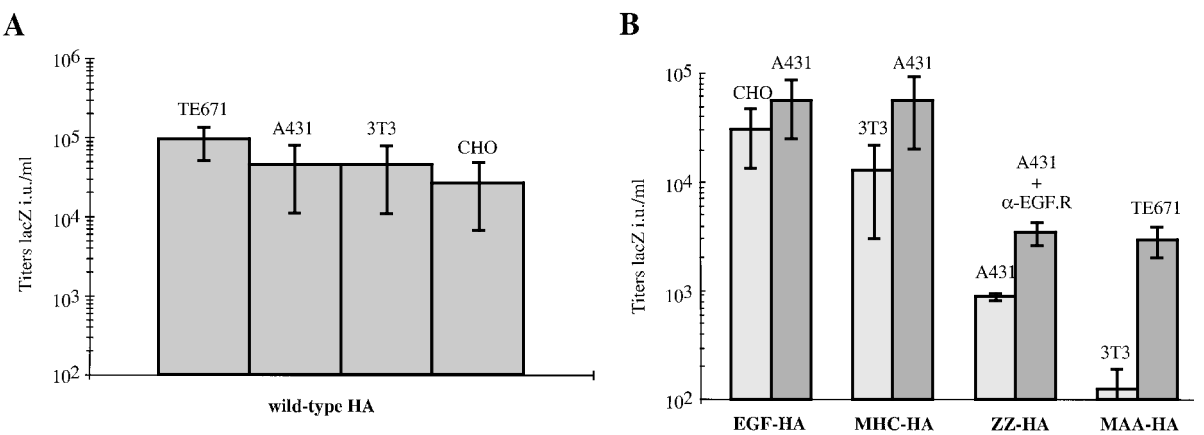

FIG. 7. Infection assays with retroviruses carrying the chimeric HA glycoproteins. Infectivity of retroviruses pseudotyped with wild-type HA glycoproteins **(A).** The cells used for infection assays with retroviruses carrying wild-type HA are indicated. Infectivity of retroviruses pseudotyped with chimeric HA glycoproteins **(B).** For each HA chimera, the infectious titers on the nontarget cells, which express only the wild-type HA receptor (gray columns), and on the target cells, which express both the HA receptor and the targeted receptor cells (hatched columns), are indicated. For each HA chimera, titers on the nontarget cells were normalized to take into account the differences of permissivity between the target cells and the nontarget cells, by using the formula:

$$(\text{normalized titer})_{NT} = (\text{titer})_{NT} \times (\text{HA titer})_T/(\text{HA titer})_{NT}$$

where $(\text{titer})_{NT}$ is the titer of the retrovirus carrying a chimeric HA protein on nontarget cells, $(\text{HA titer})_T$ is the titer of the retrovirus carrying a wild-type HA protein on target cells, and $(\text{HA titer})_{NT}$ is the titer of the retrovirus carrying a wild-type HA protein on nontarget cells. Results of three independent experiments are shown. For infections with the ZZ-HA pseudotyped virions, the target cells were A431 cells coated with the anti-EGF.R antibody whereas the nontarget cells were intact A431 cells. In these experiments infections were carried for 1 hr at 4°C to eliminate endocytosis of the cell surface-bound antibody.

ter cells were fully susceptible to infection with retroviral vectors carrying wild-type HA glycoproteins (titers of 4×10^4 IU/ml; Fig. 7B). Interestingly, HMWMAA-positive TE671 cells used as target cells could be infected at significant titers, 3×10^3 IU/ml on average, by retroviral vectors loaded with the MAA-HA chimeras. This difference in infection efficiency could be due to the ability of the displayed anti-HMWMAA scFv to mask the sialic acid-binding site on the HA backbone, which may prevent infection on 3T3 nontarget cells and allow efficient infection of TE671 target cells via interaction with HMWMAA. However, it is also possible that the lower density of MAA-HA chimera on the retroviral particles (Fig. 2B) becomes a limiting factor and reveals the differential ability of the HA backbone to infect these two cell lines.

Altogether, these data indicated the variable influence of the amino-terminal displayed domain on HA interaction with sialic acid-bearing receptors and suggested that retargeted retrovirus-mediated gene transfer using HA chimeras is feasible, depending on the success of a general strategy aimed to ablate the wild-type HA host range.

DISCUSSION

Strategies of retroviral vector targeting by modification of their envelope glycoproteins fall into two broad classes.

In the first strategy, referred to as "targeting by host range extension," the tropism of the vector is expanded. In most cases, this is achieved by incorporating a new receptor-binding domain into the viral envelope (generally the ecotropic MuLV envelope), so as to allow it to infect nonpermissive cells, such as human cells that lack the natural viral receptor. This strategy relies on the assumption that binding of the added ligand to the targeted cell surface molecule will activate the fusion mechanism of the chimeric glycoprotein. Although for MuLV-derived retroviral vectors such a strategy has worked with limited success in some cases (Cosset *et al*., 1995a; Marin *et al*., 1996; Konishi *et al*., 1998; Martin *et al*., 1998), it was found that most of the molecules targeted failed to allow entry of the virus, often because the fusogenicity of such envelope chimeras was severely compromised (Cosset and Russell, 1996). It is thus likely that this approach will not prove useful for most gene transfer applications owing to the low titers of retargeted retroviruses, unless mutations are found that couple the fusion activity and the retargeted binding properties of the chimeric envelopes. Indeed, it seems possible that the optimization of the interdomain spacing between the displayed ligand and the backbone envelope (Valsesia-Wittmann *et al*., 1996), the coincorporation of nonretroviral fusion glycoproteins (Hatziioannou *et al*., 1998), and the discovery of mutations in the backbone envelope that can facilitate the conversion of the envelope glycoprotein to its fusogenic conformation (Lavillette *et al*., 1998) will improve retargeted envelope glycoproteins that rely on host-range extension.

In the second class of targeting strategies, referred to as "targeting by host-range restriction," the target cells are fully permissive to the unmodified envelope glycoprotein (generally the amphotropic envelope) and the targeting modifications serve primarily to restrict infection of nontarget cells (Cosset and Russell, 1996). An advantage of targeting by host-range restriction is that, because the natural virus entry pathway is exploited, fusion activation of the chimeric envelope is more readily achieved. Since, by itself, the amino-terminal insertion of polypeptides does not generally prevent the ability of the envelope glycoprotein to interact with its retroviral receptor and to proceed to virus–cell membrane fusion, the display of ligands on amphotropic envelopes has resulted, in some cases, in a selective targeting (Martin *et al*., 1998; Nguyen *et al*., 1999). In these chimeras the displayed ligand presumably "tethers" the chimeric virus to the cell surface and allows a preferential infection of cells that express both the targeted cell surface molecule and the amphotropic retroviral receptor, compared with cells that express only the latter receptor. To obtain a highly specific retargeted gene delivery, more sophisticated strategies have aimed at reversibly masking the amphotropic binding and/or fusion functions so as to recruit the amphotropic receptor only when the chimera has bound to the targeted receptor, and to use it as an auxiliary molecule able to promote membrane fusion (Morling *et al*., 1997; Peng *et al*., 1997, 1998; Valsesia-Wittmann *et al*., 1997). Masking of the retroviral envelope can be achieved using trimerizing cleavable spacers that, when inserted between the displayed ligand and the amphotropic envelope, are able to mask its properties by "capping" the envelope complex and then remove the ligand cap by proteolytic digestion of the interdomain spacer (Morling *et al*., 1997). Although the addition of an interdomain spacer introduces an additional degree of specificity to the targeting glycoprotein, the usefulness of this strategy requires that the target cells express three specific molecules to activate the chimeric envelope: (1) the targeted cell surface molecule, (2) the membrane-expressed protease, and (3) the retroviral receptor.

To achieve membrane fusion, both classes of targeting strategies require the activation of the fusion functions of the retroviral glycoprotein backbone, whether such activation is triggered by the interaction of the displayed ligand with its receptor (in the case of targeting by host-range extension) or by interaction with the retroviral receptor itself subsequent to redirected binding (for targeting by host-range restriction). It should be noted that the signals that trigger the fusion activation of the retroviral glycoproteins are promiscuous, in that they essentially reside in intrinsic properties of the retroviral receptor (Weiss and Tailor, 1995). Indeed, virion binding to the retroviral receptor itself is sufficient to induce essential changes in the conformation of the retroviral envelope complex (Gilbert *et al*., 1995; Sullivan *et al*., 1998) that will ultimately lead to exposure of the fusion peptide located at the amino terminus of the TM subunit. In contrast, the signals that trigger the fusion activation of the influenza virus HA glycoproteins are much less specific since HA proteins can use different sialic acid-harboring glycoproteins or glycolipids as receptors (Wiley and Skehel, 1987) and since either partial denaturation or increases in temperature (Carr *et al*., 1997) as well as acid pH, the physiological trigger, can activate HA proteins independent of their binding to receptors (Nobusawa and Nakajima, 1987). Therefore, it is expected that, in contrast to retroviral envelope glycoproteins, the insertion of ligands on the HA protein is less likely to interfere with its fusion trigger.

The goal of the present study was to investigate whether the

display of ligands of varying length and nature would modify the binding and fusion properties of the HA glycoprotein and whether retroviral vectors would be able to incorporate such retargeted HA chimeras. Similar to retroviral envelope chimeras, we found that HA chimeras could be efficiently expressed, were correctly matured and processed, were stably incorporated on retroviral particles, and could specifically target the binding of these virions. However, in sharp contrast to retroviral envelope chimeras, none of the targeted receptors abolished infectivity of retroviral vectors loaded with the HA chimeras on receptor-positive cells. Indeed, an essential feature of several categories of targeted receptors, such as tyrosine kinase receptors, is that they induce sequestration of bound viral particles, thus resulting in abrogation of their infectivity (Cosset *et al*., 1995a; Fielding *et al*., 1998). For example, amphotropic retroviral envelope glycoproteins that display EGF cannot infect human cells that express both EGF receptors and amphotropic receptors (Cosset *et al*., 1995a; Nilson *et al*., 1996), presumably because EGF receptors can sequester and/or traffick bound retroviruses to cell compartments that are not compatible with interaction with the amphotropic receptors (Cosset and Russell, 1996). Conversely, we found that retroviruses generated with EGF-HA chimeras were infections on A431 cells and had titers similar to those of retroviral particles carrying wild-type HA proteins. The absence of receptor sequestration for retroviruses coated with chimeric HA glycoproteins is therefore promising for the development of targeting strategies since it is anticipated that most retroviral chimeric glycoproteins fused to cytokines (Cosset *et al*., 1995a; Fielding *et al*., 1998), or even to some single-chain antibodies (A. Weber, personal communication), will lead to virus sequestration.

Although some polypeptides displayed on HA might significantly restrict the interaction of the chimeric HA with its wild-type receptor (as, e.g., the anti-HMWMAA single-chain antibody), it is clear that the development of targeting strategies that use chimeric HA proteins requires a general method to impede binding to the wild-type HA receptor. It is important to point out, first, that the fusion activation of HA by acid pH does not necessarily require its binding to sialic acid-harboring receptors (Nobusawa and Nakajima, 1987) and, second, that many types of receptor candidates for targeting can recycle from the cell surface and traffick into low-pH endosomes (Schwartz, 1995; Mukherjee *et al*., 1997). Therefore it is likely that the fusogenicity of HA chimeric glycoproteins bound to various targeted cell surface molecules will be activated during endocytosis and trafficking inside the cell, even if the HA chimeras are not attached to the wild-type HA receptors. We are currently exploring the possibility of inactivating the receptor-binding site of FPV HA by point mutation of amino acids critical for interaction with sialic acid. This will render the targeting strategy reported here applicable to a large number of ligands.

ACKNOWLEDGMENTS

We are grateful to W. Garten, J. Kupsch, and M. Piechaczyk for their generous gifts of antibodies and/or scFvs.

This work was supported by the Agence Nationale pour la Recherche contre le SIDA (ANRS), the Association pour la Recherche contre le Cancer (ARC), the Association Française de Lutte contre la Mucoviscidose (AFLM), the Centre National de la Recherche Scientifique (CNRS), the Mutuelle Générale de l'Enseignement National (MGEN), and the Institut National de la Santé et de la Recherche Médicale (INSERM). T.H. was supported by a fellowship from the European Community and by the ARC.

REFERENCES

BURNS, J. C., FRIEDMANN, T., DRIEVER, W., BURRASCANO, M., and YEE, J. K. (1993). Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: Concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. Proc. Natl. Acad. Sci. U.S.A. **90,** 8033–8037.

CARR, C. M., CHAUNDHRY, C., and KIM, P. S. (1997). Influenza hemagglutinin is spring-loaded by a metastable native conformation. Proc. Natl. Acad. Sci. U.S.A. **23,** 14306–14313.

COSSET, F.-L., and RUSSELL, S. J. (1996). Targeting retrovirus entry. Gene Ther. **3,** 946–956.

COSSET, F.-L., MORLING, F. J. TAKEUCHI, Y., WEISS, R. A., COLLINS, M. K. L., and RUSSELL, S. J. (1995a). Retroviral retargeting by envelopes expressing an N-terminal binding domain. J. Virol. **69,** 6314–6322.

COSSET, F.-L., TAKEUCHI, Y., BATTINI, J. L., WEISS, R. A., and COLLINS, M. K. L. (1995b). High titer packaging cells producing recombinant retroviruses resistant to human serum. J. Virol. **69,** 7430–7436.

DONG, J., ROTH, M. G., and HUNTER, E. (1992). A chimeric avian retrovirus containing the influenza virus hemagglutinin gene has an expanded host range. J. Virol. **66,** 7374–7382.

FIELDING, A. K., MAURICE, M., MORLING, F. J., COSSET, F.-L., and RUSSELL, S. J. (1998). Inverse targeting of retroviral vectors: Selective gene transfer in a mixed population of hematopoietic and nonhematopoietic cells. Blood **91,** 1802–1809.

GILBERT, J. M., HERNANDEZ, L. D., BALLIET, J. W., BATES, P., and WHITE, J. M. (1995). Receptor-induced conformational changes in the subgroup A avian leukosis and sarcoma virus envelope glycoprotein. J. Virol. **69,** 7410–7415.

HARLOW, E., and LANE, D. (1988). *Antibodies: A Laboratory Manual*, 1st Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

HATZIIOANNOU, T., VALSESIA-WITTMANN, S., RUSSELL, S., and COSSET, F.-L. (1998). Incorporation of fowl plague virus hemagglutinin into murine leukemia virus particles and analysis of the infectivity of the pseudotyped retroviruses. J. Virol. **72,** 5313–5317.

HUNTER, E., and SWANSTROM, R. (1990). Retrovirus envelope glycoproteins. Curr. Top. Microbiol. Immunol. **157,** 187–253.

KLENK, H. D., and GARTEN, W. (1994). Host cell proteases controlling virus pathogenicity. Trends Microbiol. **2,** 39–43.

KONISHI, H., OCHIYA, T., CHESTER, K. A., BEGENT, R. H., MUTO, T., SUGIMURA, T., and TERADA, M. (1998). Targeting strategy for gene delivery to carcinoembryonic antigen-producing cancer cells by retrovirus displaying a single-chain variable fragment antibody. Hum. Gene Ther. **9,** 235–248.

KUPSCH, J. M., TIDMAN, N., BISHOP, J. A., McKAY, I., LEIGH, I., and CROWE, J. S. (1995). Generation and selection of monoclonal antibodies, single-chain Fv and antibody fusion phage specific for human melanoma-associated antigens. Melanoma Res. **5,** 403–411.

LAVILLETTE, D., MAURICE, M., ROCHE, C., RUSSELL, S. J., SITBON, M., and COSSET, F.-L. (1998). A proline-rich motif downstream of the receptor binding domain modulates conformation and fusogenicity of murine retroviral envelopes. J. Virol. **72,** 9955–9965.

MARIN, M., BROCKLY, F., NOEL, D., ETIENNE-JULAN, M., BIARD-PIECHACZYK, M., HUA, T. D., GU, Z. J., and PIECHACZYK, M. (1995). Cloning and expression of a single-chain antibody fragment specific for monomorphic determinant of class I molecules of the human major histocompatibility complex. Hybridoma **14,** 443–451.

MARIN, M., NOËL, D., VALSESIA-WITTMANN, S., BROCKLY, F., ETIENNE-JULAN, M., RUSSELL, S. J., COSSET, F.-L., and PIECHACZYK, M. (1996). Targeted infection of human cells via MHC class I molecules by MoMuLV-derived viruses displaying single-chain antibody fragment–envelope fusion proteins. J. Virol. **70,** 2957–2962.

MARTIN, F., KUPSCH, J., TAKEUCHI, Y., RUSSELL, S., COSSET, F.-L., and COLLINS, M. (1998). Retroviral vector targeting to melanoma cells by single-chain antibody incorporation in envelope. Hum. Gene Ther. **9,** 737–746.

MORLING, F. J., PENG, K.-W., COSSET, F.-L., and RUSSELL, S. J. (1997). Masking of retroviral envelope functions by oligomerizing peptide adaptors. Virology **234,** 51–61.

MUKHERJEE, S., GHOSH, R. N., and MAXFIELD, F. R. (1997). Endocytosis Physiol. Rev. **77,** 759–803.

NGUYEN, T., PAGES, J.-C., FARGE, D., BRIAND, P., and WEBER, A. (1999). Amphotropic retroviral vectors displaying hepatocyte growth factor–envelope fusion proteins improve transduction efficiency of primary hepatocytes. Hum. Gene Ther. **9,** 2469–2479.

NILSON, B. H. K., MORLING, F. J., COSSET, F.-L., and RUSSELL, S. J. (1996). Targeting of retroviral vectors through protease–substrate interactions. Gene Ther. **3,** 280–286.

NILSSON, B., MOKS, T., JANSSON, B., ABRAHMSEN, L., ELMBLAD, A., HOLMGREN, E., HENRICHSON, C., JONES, T. A., and UHLEN, M. (1987). A synthetic IgG-binding domain based on staphylococcal protein A. Protein Eng. **1,** 107–113.

NOBUSAWA, E., and NAKAJIMA, K. (1987). Amino acid substitution at position 226 of the hemagglutinin molecule of influenza (H1N1) virus affects receptor binding activity but not fusion activity. Virology **167,** 8–14.

NOBUSAWA, E., AOYAMA, T., KATO, H., SUZUKI, Y., TATENO, Y., and NAKAJIMA, K. (1991). Comparison of complete amino acid sequences and receptor-binding properties among 13 serotypes of hemagglutinins of influenza A viruses. Virology **182,** 475–485.

PENG, K. W., MORLING, F. J., COSSET, F.-L., MURPHY, G., and RUSSELL, S. J. (1997). A gene delivery system activatable by disease-associated matrix metalloproteinases. Hum. Gene Ther. **8,** 729–738.

PENG, K. W., MORLING, F. J., COSSET, F.-L., and RUSSELL, S. J. (1998). Retroviral gene delivery system activatable by plasmin. Tumor Targeting **3,** 112–120.

SCHWARTZ, A. L. (1995). Receptor cell biology: Receptor-mediated endocytosis. Pediatr. Res. **38,** 835–843.

SULLIVAN, N., SUN, Y., SATTENTAU, Q., THALI, M., WU, D., DENISOVA, G., GERSHONI, J., ROBINSON, J., MOORE, J., and SODROSKI, J. (1998). CD4-induced conformational changes in the human immunodeficiency virus type 1 gp120 glycoprotein: Consequences for virus entry and neutralization. J. Virol. **72,** 4694–4703.

VALSESIA-WITTMANN, S., DRYNDA, A., DELEAGE, G., AUMAILLEY, M., HEARD, J.-M., DANOS, O., VERDIER, G., and COSSET, F.-L. (1994). Modifications in the binding domain of avian retrovirus envelope protein to redirect the host range of retroviral vectors. J. Virol. **68,** 4609–4619.

VALSESIA-WITTMANN, S., MORLING, F. J., NILSON, B. H. K., TAKEUCHI, Y., RUSSELL, S. J., and COSSET, F.-L. (1996). Improvement of retroviral retargeting by using amino acid spacers between an additional binding domain and the N terminus of Moloney murine leukemia virus SU. J. Virol. **70,** 2059–2064.

VALSESIA-WITTMANN, S., MORLING, F. J., HATZIIOANNOU, T., RUSSELL, S. J., and COSSET, F.-L. (1997). Receptor co-operation in retrovirus entry: Recruitment of an auxillary entry mechanism after retargeted binding. EMBO J. **16,** 1214–1223.

WEISS, R. A., and TAILOR, C. S. (1995). Retrovirus receptors. Cell **82,** 531–533.

WHITE, J. M. (1992). Membrane fusion. Science **258,** 917–924.

WILEY, D. C., and SKEHEL, J. J. (1987). The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. Annu. Rev. Biochem. **56,** 365–394.

WILSON, I. A., SKEHEL, J. J., and WILEY, D. C. (1981). Structure of the hemagglutinin membrane glycoprotein of influenza virus at 3A resolution. Nature (London) **289,** 366–378.

Address reprint requests to:
Dr. François-Loïc Cosset
LVRTG, Unité de Virologie Humaine
INSERM U412
Ecole Normale Supérieure de Lyon
46 Allée d'Italie
69364 Lyon Cedex 07, France

E-mail: Francois-Loic.Cosset@ens-lyon.fr

Received for publication November 9, 1998; accepted after revision March 30, 1999.