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Retroviral Vector Particles Displaying the Antigen-Binding Site of an Antibody Enable Cell-Type-Specific Gene Transfer

TE-HUA TEARINA CHU AND RALPH DORNBURG*

Department of Molecular Genetics and Microbiology, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, Piscataway, New Jersey 08854

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Retroviral vectors are the most efficient tool for stably introducing genes into vertebrate cells. However, their use is limited by the host range of the retrovirus from which they are derived. To alter the host range, we recently constructed retrovirus vector particles, derived from spleen necrosis virus, that display a single-chain antigen-binding site of an antibody (scA) on the viral surface (T.-H. T. Chu, I. Martinez, W. Sheay, and R. Dornburg, Gene Ther. 1:292–299, 1994). Using a hapten (2,4-dinitrophenol) model system, we showed that such particles are competent for infection. In this study, we repeated our experiments using an scA directed against a cell surface protein expressed on various human carcinoma cell lines. We found that such scA-displaying particles can efficiently infect human cells that express the corresponding antigen. Particles with wild-type spleen necrosis virus envelope are minimally infectious on such cells. The addition of the original monoclonal antibody to the viral vector particle solution prior to infection inhibited infection. This competition assay showed that the infection is mediated by the antibody moiety and, therefore, is antibody specific. These data indicate that retroviral vectors with antibody-envelope fusion proteins may be a valuable tool for selectively introducing genes into any target cell.

Retroviral vectors are the most efficient tool for stably transducing genes into vertebrate cells. They are widely used to study various aspects of retroviral replication and cell differentiation, to make transgenic animals, and recently, to introduce therapeutic genes into humans (5, 8, 12, 16, 21, 22). Retroviral vectors have been mainly obtained from murine leukemia virus (MLV), from avian leukosis virus, and from the reticuloendotheliosis virus strain A (REV-A) and its close relative spleen necrosis virus (SNV) (7, 16, 21).

Although these vector systems have been proven to be very suitable for a large variety of gene transfer applications, they also have some limitations. For example, the in vivo application of retroviral vectors is restricted by the host range of the virus from which the vector particle has been derived: some retroviral vectors infect only various tissues of one species (e.g., those derived from ecotropic MLV retroviruses). Other retroviral vectors have a very broad host range and infect various cell types of different species, including human tissues (e.g., those derived from amphotropic MLV retroviruses) (16). Thus, it is not possible to use such vectors to selectively target genes to distinct cell types in vivo.

The host range of the virus is determined by the interaction of the viral envelope (Env) glycoprotein with a specific receptor on the cell surface (9). To alter the host range, we have modified the envelope protein of SNV. In one set of experiments, we substituted amino-terminal parts of the SU peptide of Env for a single-chain antigen-binding protein (scA) (3). scAs are encoded by a single gene which codes for the variable domains of both the heavy and the light chain of an antibody connected by a region coding for a peptide bridge (1, 23). To test whether such particles are competent for infection, we recently established a model system with an antibody directed against the hapten 2,4-dinitrophenol (DNP). We found that such particles are competent for infection (3). While this work was in progress, Russel et al. developed a similar hapten system with MLV-derived vector particles and obtained similar results (18).

In this report, we describe experiments performed with retroviral particles that display the single-chain antibody against a cell surface protein expressed on various human carcinoma and sarcoma cells. We found that such particles are competent for infection as well. Infectivity was blocked in competition assays with the original monoclonal antibody from which the scA was derived.

Experimental design. The construction of retroviral particles displaying an scA was achieved by fusing an scA coding gene to 3′ portions of the envelope gene. This fusion replaced parts or the complete SU coding region (Fig. 1). In this study we used the single-chain antibody B6.2 (4) (Enzon). The monoclonal antibody B6.2 was obtained by immunizing mice with a membrane-enriched fraction of a human breast tumor metastasized to the liver. The corresponding antigen was later found to be also expressed on human colon carcinoma cells (4).

The original gene coding for the B6.2 scA was designed for expression in *Escherichia coli*. It contains an *ompA* leader sequence at the 5′ end and stop codons at the 3′ end. These signals were eliminated by PCR technology. The PCR product which contained the complete scA coding region without a leader sequence was cloned (19) into the eucaryotic gene expression vector pTC13 to give plasmid pTC23 (Fig. 1). The universal gene expression vector for peptide secretion, pTC13, was constructed in our laboratory recently. A detailed description of its construction is described elsewhere (2a). Briefly, pTC13 contains the promoter and enhancer of MLV followed by the adenovirus tripartite leader sequence for further enhancement of gene expression (20). Translation starts at the ATG codon of the SNV *env* gene hydrophobic leader sequence, used for transport through the endoplasmic reticulum. Downstream of this sequence are two unique cloning sites for insertion (blunt-end ligation) of DNA fragments, i.e., PCR

* Corresponding author. Mailing address: UMDNJ-Robert Wood Johnson Medical School, Dept. of Microbiology, 675 Hoes Ln., Piscataway, NJ 08854.

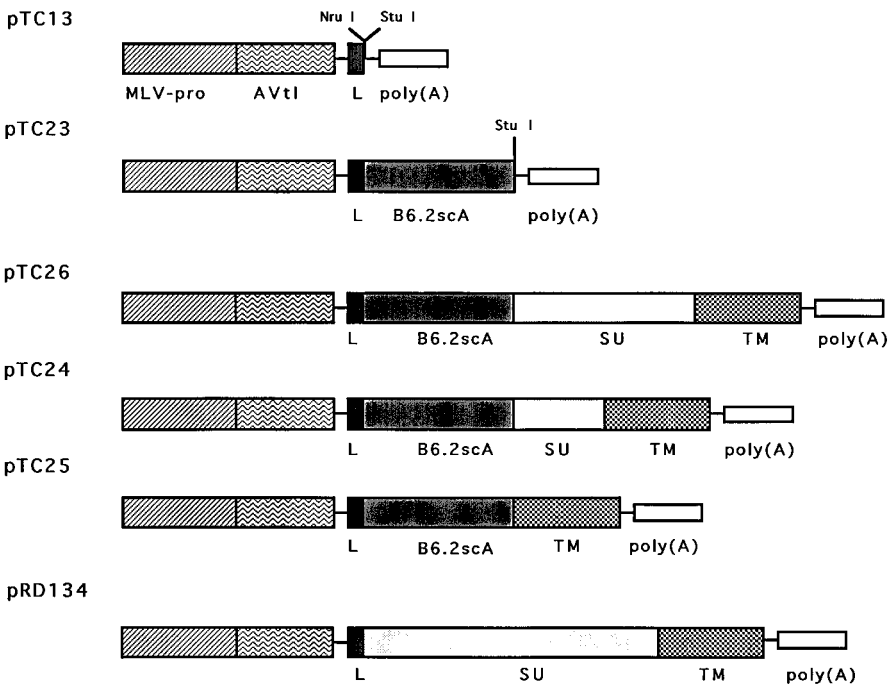

FIG. 1. Plasmid constructs for the expression of chimeric retroviral envelope proteins. All plasmids contain the MLV promoter (MLV-pro), the adenovirus tripartite leader sequence (AVtl), and the poly(A) addition site of simian virus 40 (SV40-ter). Plasmid sequences (derived from pUC19) that abut the vector sequences are identical in all constructs and are not shown. pTC13 is a universal eucaryotic gene expression vector for peptide secretion (2a). It contains the SNV leader sequence for transport through the endoplasmic reticulum and then two unique cloning sites and stop codons in all three reading frames. pTC23 contains a PCR product, the B6.2 *scA* gene inserted into the *Nru*I site of pTC13. In pTC26, pTC24, and pTC25, various amino-terminal parts of the envelope gene have been fused to the B6.2 gene of pTC23. pRD134 has the wild-type envelope of SNV. L, leader sequence; TM, transmembrane protein; SU, surface protein.

products. The vector also contains stop codons in all reading frames and the polyadenylation signal sequence of simian virus 40.

The *scA* gene in pTC23 was fused to various carboxy-terminal parts of the envelope gene. In plasmids pTC26 and pTC24, about one-third or two-thirds of the SU peptide coding regions have been replaced with the *scA* gene, respectively. In plasmid pTC25, the complete SU region has been replaced with the B6.2 *scA* gene (Fig. 1). Since all chimeric envelope genes were inserted into the same expression vector, efficiency of transcription is approximately the same for all constructs.

Expression of chimeric antibody-Env proteins. To test whether the chimeric envelope proteins are transported to the cell surface, D17 cells were cotransfected with pSV2neo, a plasmid expressing the G418 resistance gene (10), and pTC24 or pRD134. After G418 selection, cells were subjected to fluorescence-activated cell sorter (FACS) analysis with a rabbit polyclonal antiserum against the SNV envelope. Antibodies of this serum also bind to domains of TM (6). The secondary antibody was a fluorescein-labelled goat anti-rabbit immunoglobulin G. We found that the chimeric envelope was present at the cell surface at approximately the same density as the wild-type envelope. These data indicate that the SNV hydrophobic leader works as a valid endoplasmic reticulum transport signal sequence when juxtaposed to the coding region of another peptide (these data are described in detail in the article describing the pTC13 plasmid [2a]). The finding that the chimeric envelope is correctly processed coincides with our data obtained with vector particles displaying an anti-DNP scA (3). Constructs expressing the anti-DNP scA-Env fusion proteins were identical to the B6.2 scA-Env expression plasmids except for the antibody-coding region.

Infectivity studies. To test for infectivity, the constructs expressing the chimeric fusion proteins were transfected into the SNV-derived helper cell lines DSgp13 and DSH-CXL. These cell lines, derived from D17 cells (a dog osteosarcoma cell line, obtained from the American Type Culture Collection), were made with highly efficient *gag-pol* and *env* expression vectors (termed pRD136 and pRD134, respectively) constructed in our laboratory (14a). Briefly, both vectors are similar to pTC26. However, they contain the complete *gag-pol* (pRD136) or complete *env* (pRD134) coding region in place of the chimeric envelope (a detailed description of the new helper cell lines will be published elsewhere [14b]). DSgp13 cells have been derived from a single isolated cell clone transfected with the *gag-pol* expression vector only. They do not express wild-type envelope. They also contain the retroviral vector pCXL, which transduces the bacterial β-galactosidase (*lacZ*) gene (15). DSH-cxl cells are complete SNV-based helper cells. They were derived from DSgp13 cells by the transfection of plasmid pRD134 expressing the SNV wild-type envelope. Thus, the levels of expression of *gag-pol* and pCXL in DSH-cxl cells is the same as in DSgp13 cells. All experiments described were performed at least twice with virus harvested from two different transfected cell cultures.

As outlined above, the antigen of the monoclonal antibody B6.2 is expressed on human colon carcinoma cells. Thus, we started our experiment with the human colon carcinoma cell line DLD-1. In a first set of experiments, *scA-env* gene expression vectors pTC24, pTC25, and pTC26 were transfected into the DSgp13 cells, which do not contain a wild-type SNV *env*. Transfection was performed with Lipofectamine reagent purchased from GIBCO. Using this agent and control plasmids, we found that up to 5% of D17 cells transiently express gene

TABLE 1. Infectivity of retroviral vector particles displaying the B6.2 single-chain antigen-binding protein[a]

Construct	Titer (CFU/ml) against cell line:				
	D17	DLD-1	HeLa	HOS	CHO
DSgp13	1	<1	1	ND	ND
DSgp13 + pTC26	4×10^1	<1	1×10^1	ND	ND
DSgp13 + pTC24	2×10^1	<1	0.5×10^1	ND	ND
DSgp13 + pTC25	4.5×10^1	<1	2×10^1	ND	ND
DSH-cxl	1×10^6	2.5×10^1	3.5×10^1	3×10^1	<1
DSH-cxl + pTC26	4×10^5	1.3×10^2	9×10^2	5×10^2	<1
DSH-cxl + pTC24	1.5×10^5	2.4×10^2	1.3×10^3	4×10^2	<1
DSH-cxl + pTC25	3.4×10^5	3.5×10^2	9.4×10^2	1.4×10^2	<1

[a] DSgp13 cells are SNV helper cells that express *gag-pol* only and contain the retroviral vector pCXL (transducing the bacterial *lacZ* gene). DSH-cxl cells were derived from DSgp13 cells by the transfection of the SNV-*env* expression plasmid pRD134. These cell lines were transfected with plasmid DNAs (pTC24, pTC25, and pTC26) to express chimeric B6.2-scA-Env proteins. Two days after transfection, virus was harvested and fresh target cells were infected. Virus titers are given in CFU/milliliter of supernatant tissue culture medium. ND, not done. The experiments were repeated three times, and similar data were obtained.

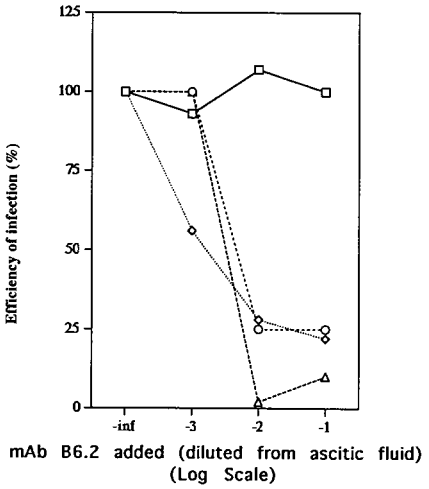

FIG. 2. Antibody competition assay. Increasing amounts of B6.2 monoclonal antibody (mAb) were added to tissue culture supernatant medium harvested from helper cells that produce retroviral vector particles displaying B6.2-scA-envelope fusion proteins. DLD-1 cells were infected, and the number of infected cells was determined. Symbols: □, infectivity on D17 cells with TC25; ○, ◇, and △, infectivity on DLD-1 cells with TC26, TC24, and TC25, respectively; -inf, infectivity without the addition of monoclonal antibody B6.2. The experiment has been reproduced, and similar data were obtained.

products of transfected DNAs. Other transfection protocols were less efficient (data not shown). Virus was harvested 2 days after transfection and used to infect DLD-1 and D17 cells. Two days after infection, infected cells were assayed for expression of the *lacZ* gene by X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining (15), and the number of infected cell colonies was determined.

Virus harvested from DSgp13 cells (Env-negative virus) was minimally infectious on D17 or DLD-1 cells (Table 1). No infectivity on DLD-1 cells was obtained with particles that display the B6.2 scA. Surprisingly, low levels of infectivity were observed on D17 cells. Similar data had been obtained with virus particles displaying an anti-DNP scA (3). At this point, we do not know whether this low level of infectivity results from a weak interaction (with high avidity) of the scA to a homologous B6.2 receptor or, more likely, whether it is mediated by the remaining portion of the SNV envelope. This latter hypothesis is supported by the finding that truncated envelope proteins of SNV retain a low level of infectivity on D17 cells. For example, virus particles containing the same amount of carboxy-terminal SU sequence as the chimeric constructs or TM alone were still able to infect D17 cells with efficiencies similar to those of TC24, TC25, or TC26 (14b).

In a previous study we tested the infectivities of chimeric envelope proteins of MLV and SNV. We found that particles displaying such chimeric envelopes were not infectious on CHO cells that expressed the ecotropic MLV receptor. However, such particles became infectious after wild-type SNV envelope was added (3). We hypothesized that wild-type envelope can act as a helper for membrane fusion. To test whether wild-type envelope would also have such a helper function in particles displaying the chimeric B6.2 envelope, plasmids pTC24, pTC25, and pTC26 were transfected into DSH-cxl cells, which express the wild-type SNV envelope. Virus harvested from these cells was able to infect DLD-1 cells with titers of up to 3×10^2 (Table 1). We found that there was no remarkable difference in titers among the different scA-Env constructs.

The finding that the addition of wild-type envelope to particles containing a targeting envelope bestows infectivity coincides with our earlier observations with chimeric envelopes of SNV and MLV (3). They suggest (i) that the chimeric Env protein can act as a targeting molecule to bind the virus to the cell surface of the target cell and (ii) that wild-type envelope is needed as a helper for efficient membrane fusion.

Specificity of infection. To test whether binding of the vector virus to DLD-1 cells was mediated by the scA moiety, a series of competition assays was performed. We hypothesized that the addition of increasing amounts of antibody to the virus solution would block the B6.2 antigen, preventing viral infection. We found that the infectivity was partially blocked if the antibody (ascites fluid) was added to the virus solution in a ratio of 1:1,000. Ascites fluid in a dilution of less than 1:100 blocked infectivity at up to 90% for all three constructs (Fig. 2). The addition of the antibody to the virus solution in any concentration did not inhibit infectivity of D17 cells which contain a receptor for the SNV wild-type envelope (Fig. 2). These data further show that the block of infectivity was not mediated by nonspecific inhibitory effects of the antibody and that the binding of the vector virus was mediated by the scA-Env protein.

To further test the infectivities of particles displaying the B6.2 scA, we performed infectivity experiments on HeLa, HOS (a human osteosarcoma cell line), and CHO cells. First, we tested whether the B6.2 antigen was present on these cells by FACS analysis with the monoclonal antibody B6.2 and a fluorescein-labelled anti-mouse immunoglobulin G antibody (Fig. 3). The intensity of fluorescence was compared in cells stained with the second antibody only. We found that HOS cells expressed the B6.2 antigen as well as DLD-1 cells. In the case of HeLa cells, the main shift of fluorescence was not as strong as in the other two human cell lines. However, about 5% of the cells appear to express even higher levels of the B6.2 antigen than HOS or DLD-1 cells (Fig. 3). CHO cells and D17 did not reveal a significant shift of the intensity of fluorescence, indicating that these cells were B6.2 antigen negative.

Virus particles containing the chimeric scA-Env alone infected HeLa cells with a very low efficiency (Table 1). However, both HeLa cells and HOS cells could be infected at significant levels with particles that incorporated wild-type envelope in addition to the chimeric scA-Env protein (Table 1). The level of infectivity was higher than that on DLD-1 cells. In

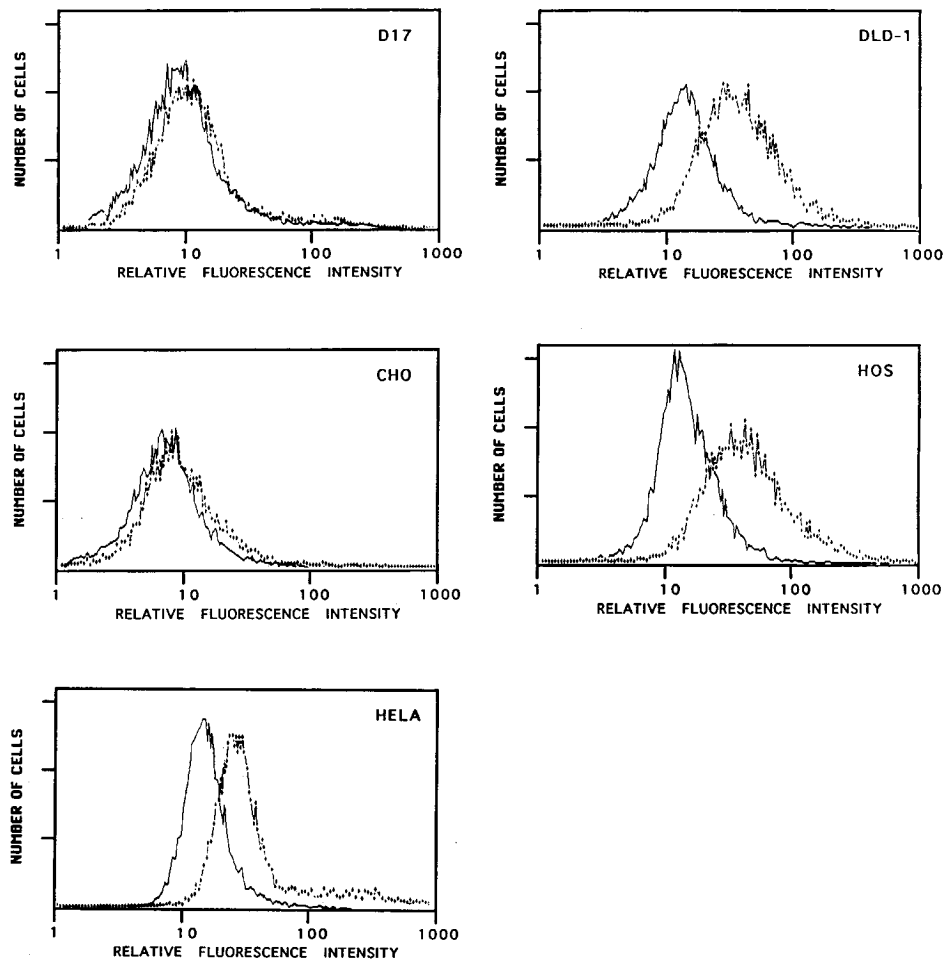

FIG. 3. Distribution of the B6.2 antigen on various cell lines. Indirect immunofluorescence flow cytometry patterns of D17, CHO, HeLa, DLD-1, and Hos cells are shown. The solid lines indicate cells stained with the secondary antibody (fluorescein-labelled goat anti-mouse immunoglobulin G) only. The dashed lines indicate cells preincubated with the monoclonal antibody B6.2 and stained with the secondary antibody.

the case of TC24, HeLa cells were infected with virus titers of more than 10^3 CFU/ml of tissue culture supernatant medium. Particles containing the SNV wild-type envelope alone infected D17 cells with titers of about 10^6 (Table 1). They were also able to infect all three human cell lines. However, the efficiency of infection was significantly lower than that obtained with particles containing scA-Env chimeras. Particles containing wild-type plus chimeric scA-Env proteins had slightly reduced virus titers on D17 cells. This titer reduction may result from displacement of wild-type envelope with the chimeric construct. Earlier, it was reported that HeLa cells could be efficiently infected with REV-A-derived vectors which are closely related to SNV (11, 13, 14). However, the SU peptides of REV-A and SNV differ by 33 amino acids (14), which may account for the difference of infectivity on human cells.

No infectivity on CHO cells which do not contain the B6.2 antigen was observed. Earlier we found that CHO cells expressing the ecotropic MLV receptor could be infected with SNV particles that contained chimeric MLV-SNV envelope proteins with titers of about 10^3 (3). In these studies, the MLV SU was fused to carboxy-terminal parts of the SNV Env, as in pTC24 and pTC25. Thus, the lack of infectivity with particles displaying the B6.2 scA does not result from a postpenetration block of infection on these cells but is the result of a missing receptor on the cell surface. These data further confirm that the infectivity of the scA displaying retroviral particles is not mediated by unspecific adsorption but by specific interactions of the antigen-binding site to its corresponding antigen.

Conclusion. In summary, in this article, we give final proof of the principle that retroviral vector particles that display a single-chain antigen-binding protein directed against a cell surface receptor are competent for infection. We show that the infectivity was mediated by the antibody moiety. We hypothesize that this technique can be used with any scA directed against any cell surface protein. This technology, once optimized for higher virus titers, may enable not only in vivo gene therapy in humans but also the creation of experimental animal systems for various diseases. For example, viral or toxic genes could be targeted to distinct organs to induce the symptoms of specific diseases.

In these studies, virus titers of $>10^3$ CFU/ml of tissue culture supernatant medium were obtained for HeLa cells with virus harvested from transiently transfected cell cultures. Because such cell cultures contain a maximum of 5% transfected cells, higher titers may be obtained from single isolated and stably transfected cell clones. The reason for using mass-transfected cells in this study was to get average virus titers from many transfected cells for each construct and experiment.

Gene expression from stably transfected DNA can vary by as much as 2 orders of magnitude depending on the site of integration. Thus, working with single-cell clones would have required the establishment of hundreds of cell lines. This latter statement is also based on our observation that after cotransfection, often only 10% of isolated antibiotic-resistant D17 cell colonies express the gene of interest (data not shown).

While this work was in progress, it was shown that D17 cells can be infected 30 times more efficiently with particles containing the REV-A matrix protein instead of that of SNV (2). Thus, in future studies, we will test whether this also applies to human cells. Another variable that may lead to better titers is the variation of the ratio of wild-type to scA-Env protein.

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