A TVA–Single-Chain Antibody Fusion Protein Mediates Specific Targeting of a Subgroup A Avian Leukosis Virus Vector to Cells Expressing a Tumor-Specific Form of Epidermal Growth Factor Receptor

SOPHIE SNITKOVSKY, THOMAS M. J. NIEDERMANN, BOB S. CARTER, RICHARD C. MULLIGAN, and JOHN A. T. YOUNG

Committee on Virology, Department of Microbiology and Molecular Genetics, Howard Hughes Medical Institute at the Children’s Hospital, and Harvard Institute of Human Genetics, Harvard Medical School, Boston, Massachusetts 02115, and Department of Oncology, McArdle Laboratory for Cancer Research, University of Wisconsin at Madison, Madison, Wisconsin 53706

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We have previously described an approach that employs retroviral receptor-ligand bridge proteins to target retroviral vectors to specific cell types. To determine whether targeted retroviral entry can also be achieved using a retroviral receptor–single-chain antibody bridge protein, the TVA-MR1 fusion protein was generated. TVA-MR1 is comprised of the extracellular domain of the TVA receptor for subgroup A avian leukosis viruses (ALV-A), fused to the MR1 single-chain antibody that binds specifically to EGFRvIII, a tumor-specific form of the epidermal growth factor receptor. We show that TVA-MR1 binds specifically to a murine version of EGFRvIII and promotes ALV-A entry selectively into cells that express this cell surface marker. These studies demonstrate that it is possible to target retroviral vectors to specific cell types through the use of a retroviral receptor–single-chain antibody fusion protein.

Several different strategies have been developed for targeting infection of specific cell types by retroviral vectors. The most common approach has employed recombinant viral envelope (Env) proteins that contain either cell type-specific ligands or single-chain antibodies that bind to specific cell surface molecules (1, 4, 7–12, 15, 18–20, 24–28, 31–35, 38, 39, 41, 42, 44, 46, 48, 51–54, 57). This approach requires that the specific alterations made to Env do not affect the biosynthesis, virion assembly, or fusogenic function of the viral glycoprotein (12, 54, 57).

An alternative approach for targeting retroviral entry that employs soluble retroviral receptor-ligand bridge proteins was recently developed (5, 47). These bridge proteins are bifunctional reagents: the ligand moiety binds to specific cell surface receptors and the retroviral receptor moiety binds to Env, activating viral entry. This technique does not require making alterations to the viral glycoprotein but instead relies on “wild-type” Env-receptor interactions to target viral entry.

To demonstrate the feasibility of this approach, the TVA-EGF and TVB-EGF fusion proteins were generated. These bridge proteins contained human epidermal growth factor (EGF) fused to the extracellular domains of either the TVA receptor for subgroup A avian leukosis viruses (ALV-A) or the TVB receptor for ALV-B and ALV-D, respectively. Each of these bridge proteins promoted specific retroviral entry into cells that express the EGF receptor (EGFR) when bound to cell surfaces before viral challenge (5, 47). Furthermore, murine leukemia virus (MLV) pseudotypes bearing ALV-B Env (EnvB) and preloaded with TVB-EGF were targeted specifically to cells that express EGF (5). These data demonstrated that retroviral vectors could be targeted to specific cell types by binding retroviral receptor-ligand bridge proteins to virions or to cell surfaces before viral challenge.

To extend the utility of this approach, we have now asked whether targeted retroviral entry into cells can also be achieved using TVA-MR1, a bridge protein that contains the extracellular domain of TVA fused to the MR1 single-chain antibody. This antibody binds specifically to the extracellular region of EGFRvIII, a variant form of the EGF receptor that is expressed at the surface of human tumor cells, including those derived from lung and breast carcinomas and glioblastomas (14, 17, 30, 37, 40, 49, 55, 56). EGFRvIII lacks a substantial portion of the extracellular domain of the wild-type receptor as a consequence of a deletion or rearrangement that commonly occurs when the EGFR gene is amplified during tumor biosynthesis. These alterations result in constitutive, ligand-independent activation of EGFRvIII (22), which, in turn, confers a transformed phenotype upon various cell lines (22, 40).

The MR1 antibody binds to a novel polypeptide sequence that is formed at the site of the deletion or rearrangement that gives rise to EGFRvIII (29). The combination of the tumor-restricted expression of EGFRvIII and the binding specificity of the MR1 antibody makes this an attractive model system to test whether retroviral receptor–single-chain antibody bridge proteins can mediate targeted viral entry into cells. In this report, we demonstrate that TVA-MR1 can support efficient and specific ALV entry into mammalian cells that have been engineered to express a murine form of EGFRvIII.

Materials and Methods

Viruses and Immunoadhesins. The SUA-rIgG immunoadhesin was described elsewhere and is comprised of the surface protein of the Schmidt-Ruppin A strain of Roux sarcoma virus fused to a rabbit Fc chain (56). ALV-A-specific vectors encoding the enhanced green fluorescent protein (EGFP; Clontech) were generated by transfection of DF1 cells (45) with the RCASBP(A)-EGFP plasmid (provided by Mark Federspiel and Matt van Brocklin). The transfected...
cells were propagated until 100% of the population expressed EGFP as determined by fluorescence microscopy. Cells were then seeded in 1,700-cm² roller bottles, and vireons were harvested every 12 h in 20 ml of medium that was equal to 10% of the original volume of TNE buffer (5).

Replication-defective MLV vectors encoding a synthetic transmembrane form of TVA (TVA[E])3 (or a murine form of EgfrvIII) were generated. The MLV vector pMMp.TVAM was generated by first preparing a DNA fragment that encoding TVA[E]3 by PCR amplification using pMD1 plasmid template DNA (A. Wenzke and J. A. T. Young, unpublished data) and the following two primers: 5'-GGACTGACGCTACGAGTATAGTCTCTCATCAGG-3' and 5'-GGATCTCCAGGAGGTTAAGGTCGAGTTAAGGGGTTTGAAGTACC-3'. The resultant DNA fragment was digested with NcoI and BglII restriction enzymes and was subcloned into the NcoI and BglII restriction sites of the pMD.MP.EGFP plasmid (K. Bradley and J. A. T. Young, unpublished data), generating the pMMp.TVAM plasmid. The MLV vector pSFG.EGFRvIII contains a gene encoding a murine form of EgfrvIII (R. Carter and R. C. Mulligan, unpublished data) located between the NcoI and BamHI restriction enzyme sites of the pSFG vector.

Stocks of MLV vectors pseudotyped with VSV-G protein were prepared from human 293T cells by using a transient transfection system essentially as described previously (5) with 5 µg of the pMD.G plasmid encoding VSV-G, 15 µg of the pMD.old.gagpol plasmid encoding MLV Gag and Pol, and 15 µg of either plasmid pSFG.EGFRvIII or plasmid pMMp.TVAM. Virens were harvested 3 d after transfection and 18 h after supernatants containing pSFG.EGFRvIII (VSV-G) and pMMp.TVAM (VSV-G) viruses were separately pooled and filtered through a 0.45-µm-pore-size filter and then stored at −80°C.

Cell lines. Human 293T cells were propagated in Dulbecco modified Eagle medium containing 5% fetal bovine serum. 293T-EGFR/III cells were generated by transducing 293T cells with MLV-EGFR/III(VSV-G). Approximately 72 h after viral challenge, the transduced cell population (10⁷ cells) was incubated for 30 min at 4°C with 5 ml of extracellular supernatant containing TVA-MR1 and then for 30 min at 4°C with fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit antibody (DAKO Corp.) diluted 1:100 in medium. The cells were washed with ice-cold medium, and those expressing the EGFRvIII receptor were isolated by flow cytometric sorting after binding SUA-rIgG to the surface EGFRvIII protein. Transfected human 293T cells expressing a TVA-MR1 fusion protein, plasmid pSS11 (Fig. 1B, compare lanes 1 and 2), or plasmid pSFG.EGFRvIII were similarly incubated with 500 µl of extracellular supernatant containing TVA-MR1. 293T-EGFR/III cells were incubated with 500 µl of extracellular supernatant containing TVA-MR1 for 1 h at 4°C with a final 100 nM concentration of either the MR1 epitope-containing peptide (LEEKNGYVYTDH1) or a scrambled control peptide (TKELEGVDNJKHTI). These cells were then washed again and resuspended in 500 µl of ice-cold PBS containing 1% formaldehyde, and the bound TVA-MR1 fusion proteins were detected by flow cytometry using a Coulter Epics XL flow cytometer. Dead cells that had taken up propidium iodide were excluded from this analysis by electronic gating. The viral titer was measured by first calculating the multiplicity of infection (MOI) observed from the input virus: MOI = −ln(1 − (number of EGFP fluorescent cells/total number of cells analyzed)). The actual titers (per microliter of 100-fold-concentrated virus) were calculated by using the following equation (MOI = ln[1/v, where v was determined from the MOI = 10⁻⁵ experiment to be 10⁻⁵, because that was the case of 293T-EGFRvIII cells). The number of fluorescent cells seen in the absence of virus was subtracted from each of the calculations.

Production of the TVA-MR1 protein. A gene that encodes the recombinant TVA-MR1 bridge protein was constructed in plasmid pSS11 (described in Materials and Methods). TVA-MR1 is comprised of the extracellular portion of the TVA receptor (2) fused to the N terminus of the MR1 single-chain antibody, which recognizes a unique sequence in the extracellular domain of EgfrvIII (29) (Fig. 1A). A proline-rich polypeptide linker that was described previously (47) was inserted between each of the two domains of TVA-MR1 (Fig. 1A) in order to maximize the probability that each domain would fold and function independently. TVA-MR1 was produced as a secreted protein, approximately 54 kDa in size, in the extracellular supernatants taken from human 293T cells transfected with plasmid pSS11 (Fig. 1B, compare lanes 1 and 2).

TVA-MR1 specifically binds to cells expressing an EGFRvIII protein. To determine whether the Env-binding and antigen-binding domains of TVA-MR1 can each function independently, we investigated whether this bridge protein can bind both to the ALV-A surface envelope (ALV-A SU) and to a cell surface EgfrvIII protein. Transfected human 293T cells expressing a murine form of EgfrvIII (293T-EGFRvIII cells)
were incubated with increasing amounts of an extracellular supernatant containing TVA-MR1. For control purposes, the parental 293T cells were also incubated with TVA-MR1-containing supernatant. The bridge proteins that were bound to these cells were then detected by flow cytometry after the cells had been incubated with SUA-rIgG, an immunoadhesin composed of the ALV-A surface envelope fused to a rabbit Fc chain (58), and a horseradish peroxidase-conjugated secondary antibody. TVA-MR1 bound specifically and in a dose-dependent manner to 293T-EGFRvIII cells (Fig. 2A), indicating that this bridge protein can bind simultaneously to both ALV-A SU and to cell surface EGFRvIII.

To obtain direct evidence that TVA-MR1 binds to the EGFRvIII protein, we examined whether the interaction of this bridge protein with 293T-EGFRvIII cells could be blocked by a synthetic peptide that contains the target epitope for the MR1 antibody (29). Indeed, this peptide blocked the binding of TVA-MR1 to these cells (Fig. 2B). By contrast, a control peptide (with the same amino acids scrambled in a different order) did not affect TVA-MR1 binding to these cells (Fig. 2B). To rule out the possibility that the MR1 epitope-containing peptide interfered nonspecifically with ALV-A SU–TVA interactions, this peptide was also tested for its effect on the binding of SUA-rIgG to a stably transfected line of human 293T cells (designated as 293T-TVAsyn) that express a transmembrane form of the TVA receptor. The MR1 epitope-containing peptide did not block the binding of SUA-rIgG to these cells (Fig. 2C), confirming that this peptide specifically blocks the interaction between TVA-MR1 and the EGFRvIII protein.

**TVA-MR1 mediates specific ALV-A entry into 293T-EGFRvIII cells.** To determine whether TVA-MR1 can facilitate ALV-A infection of 293T-EGFRvIII cells, these cells and, for control purposes, 293T cells, were incubated with this bridge protein and then challenged with an ALV-A vector [RCASBP(A)-EGFP], encoding EGFP. The addition of TVA-MR1 led to a significant enhancement of ALV-A entry into 293T-EGFRvIII cells (Table 1) (Fig. 3, compare panels B and C). Indeed, TVA-MR1 acted in a dose-dependent manner to increase the susceptibility of these cells to ALV-A infection (data not shown). In these experiments, the level of TVA-MR1-mediated viral entry into 293T-EGFRvIII cells was 8.5%
of the level seen with 293T-TVA<sup>syn</sup> cells, and titers of approximately 10<sup>8</sup> infectious units/ml of 100-fold-concentrated virus were achieved (Table 1). In contrast, the addition of this bridge protein had no effect upon the susceptibility of the parental 293T cells to viral infection (Table 1) (Fig. 3, compare panels E and F).

To confirm that TVA-MR1 had to be bound to the EGFR-vIII protein in order to mediate enhanced levels of viral entry into 293T-EGFRvIII cells, we determined whether this effect could be blocked by the MR1 epitope-containing peptide. The MR1 epitope-containing peptide, but not the control peptide, blocked this bridge protein-dependent viral infection (Fig. 4). Therefore, TVA-MR1 is capable of facilitating targeted ALV-A entry into mammalian cells when bound to the EGFR-vIII protein.

During these experiments we found that ALV-A is capable of infecting human 293T cells and 293T-EGFRvIII cells at a low “background” level that was 1/2,000 to 1/5,000 of the level seen with 293T-TVA<sup>syn</sup> cells (Table 1 and data not shown). The addition of TVA-MR1 led to an 180-fold to 200-fold increase in the susceptibility of 293T-EGFRvIII cells to ALV-A infection but did not affect the “background” level of infection seen with 293T cells (Table 1).

**FIG. 3.** TVA-MR1 promotes ALV-A infection of 293T-EGFRvIII cells. 293T-EGFRvIII cells (A through C) and 293T cells (D through F) were incubated with extracellular supernatant that contained (+) or lacked (−) TVA-MR1 as indicated. These cells were incubated in the absence or presence of the ALV-A vector RCASBP(A)-EGFP, and then aliquots of each cell type (5 × 10<sup>4</sup> 293T-EGFRvIII cells and 10<sup>5</sup> 293T cells) were analyzed by flow cytometry.

**FIG. 4.** The TVA-MR1-EGFRvIII interaction is required for targeted viral entry. Extracellular supernatant containing TVA-MR1 was incubated with no peptide (none), the MR1 epitope-containing peptide, or the scrambled peptide before addition to 293T-EGFRvIII cells. The cells were then challenged with RCASBP(A)-EGFP and analyzed by flow cytometry 72 h later. For control purposes, viral infection of 293T-EGFRvIII cells was also assessed in the absence of any TVA-MR1.

**DISCUSSION**

In this report we have demonstrated the feasibility of using a soluble retroviral receptor–single-chain antibody bridge protein for targeting retroviral infection to a specific cell type. Cell-binding studies and peptide competition experiments confirmed that TVA-MR1 is a bifunctional reagent that can bind to a cell surface EGFRvIII protein expressed on 293T cells and to ALV-A SU. Furthermore, the binding of TVA-MR1 to the EGFRvIII protein allowed specific viral entry at a level that was approximately 7% (ranging from 1.8 to 14% in separate experiments) of that found in ALV-A infection of 293T-TVA<sup>syn</sup> cells (Table 1 and data not shown). In contrast, TVA-MR1 did not bind to or promote infection of the parental 293T cells, which are known to express cell surface EGFRs (50).

Indeed, based upon previous results that demonstrated the unique specificity of the MR1 antibody for EGFRvIII, we fully expected that TVA-MR1 would allow only ALV-A infection of cells expressing mutant, but not wild-type, EGFRs. For example, Lorimer et al. (29) have already shown that this single-chain antibody, engineered in the context of an MR1-toxin fusion protein, directs the potent killing of cells that express 400,000 EGFRvIII molecules (at a concentration of 7 to 10 ng/ml), whereas this recombinant toxin is unable to kill cells expressing 200,000 wild-type EGFR molecules even when added at concentrations as high as 1,000 ng/ml. Taken together, our studies have demonstrated that TVA-MR1 can be an efficient and specific facilitator of viral entry into cells when bound to EGFRvIII.

**TABLE 1. Infection of human 293T cell lines by the ALV-A vector RCASBP(A)-EGFP**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Viral titer&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>− TVA-MR1</td>
</tr>
<tr>
<td>293T</td>
<td>(5.96 ± 0.58) × 10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>293T-EGFRvIII</td>
<td>(5.94 ± 0.28) × 10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>293T-TVA&lt;sup&gt;syn&lt;/sup&gt;</td>
<td>(1.29 ± 0.02) × 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Titers are expressed per microliter of 100-fold-concentrated virus.
understand why certain mammalian cell types are more susceptible to ALV infection than are others, since this information may help us eliminate such infections and thus optimize the use of retroviral receptor-ligand and retroviral receptor–single-chain antibody bridge proteins for viral targeting.

The results presented in this report suggest that it might be possible both in vivo as well as in vitro, to use retroviral receptor–single-chain antibody bridge proteins as tools to deliver retroviral vectors to specific cell types that express cognate cell surface antigens. With regard to in vivo applications, it is not yet clear whether the background level of ALV-A infection that was observed in cultured human 293T cells will represent a significant hurdle for cell type-specific viral targeting: viral targeting studies, performed with transgenic lines of mice that express a transmembrane form of TVA in specific cell types, indicate that the “background” level of ALV-A infection seen with cells that lack this viral receptor is extremely low (16). Furthermore, in considering the potential utility of this system for delivering viral vectors to tumor cells, the use of ALV-based or MLV-based vectors for gene delivery affords another level of specificity since these viruses only establish proviral DNA in dividing cell types (36, 43). Indeed, several groups have already shown that it is possible to use this feature of MLV vectors to deliver genes specifically to tumor cells, even in the absence of a selective Env-targeting system (13, 23).

However, since the retroviral vectors used in these studies have a broad host range, there exists the potential for infecting other dividing cell types in addition to the target tumor cells. The use of retroviral receptor-ligand and retroviral receptor–single-chain antibody bridge proteins for viral delivery may lead to more specific viral targeting in vivo. Indeed, it will be interesting to determine the efficiency and specificity of in vivo viral targeting that can be achieved with TVA-MR1 in mouse models of human cancer that employ tumor cells expressing EGFRvIII (21). An added advantage of using retroviral receptor–single-chain antibody fusion proteins is that such bridge proteins might be useful for targeting retroviral vector infection to cells expressing a number of different cell surface factors, including those with no known ligands.

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