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Targeted Retroviral Infection of Tumor Cells by Receptor Cooperation

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Retroviruses expressing two different receptor-binding domains linked by proline-rich spacers infect only cells expressing both retroviral receptors (Valsesia-Wittman et al., EMBO J. 6:1214–1223, 1997). Here we apply this receptor cooperation strategy to target human tumor cells by linking single-chain antibodies recognizing tumor antigens via proline-rich spacers to the 4070A murine leukemia virus surface protein.

Development of targeted vectors is critical for many gene therapy applications to avoid the inappropriate modification of bystander cells. As unmodified retroviral vectors transduce a number of normal tissues in vivo (16, 21), cell surface targeting is attractive to avoid depletion of limited viral particles. To date, the only surface-targeting strategies that have allowed efficient infection by retroviral vectors in vivo are those in which the normal retroviral surface protein (SU) participates in receptor binding and fusion (10, 16, 21, 22). We described retroviruses targeted to human tumor xenografts by a chimeric SU containing a single-chain antibody (scFv) recognizing high-molecular-weight melanoma-associated antigen (HMWMAA) (18) followed by a full-length proline linker and a matrix metalloprotease (MMP) cleavage site (17). These viruses bind to HMWMAA and then are cleaved by cell surface MMPs, revealing the 4070A SU that mediates infection via its Pit-2 receptor. In the absence of MMP cleavage, the full-length proline linker prevents interaction of SU with Pit-2. However, this complex strategy is less attractive for clinical application, as human tumors will not express antigen and protease uniformly. Here we report a simpler scFv targeting approach based on receptor cooperation. This was described in studies where the 4070A Pit-2 binding domain was linked to the Moloney murine leukemia virus (MMLV) SU via proline-rich spacers (24, 25), generating envelopes that required both Pit-2 and the MMLV receptor mCAT-1 for infection.

Construction of targeted envelopes. LMH2 (14), an scFv which recognizes HMWMAA, and MFE23 (6), an scFv which recognizes carcinoembryonic antigen (CEA) (4), were fused to codon 5 of the mature 4070A SU by proline-rich linkers (Fig. 1). These linkers were derived from the proline-rich region (Pro) of 4070A SU that promotes a conformational change leading to fusion after receptor binding by the native envelope (1, 3, 15). Pro is the full proline-rich region, while Pro2 and Pro3 are truncated versions with the first 2 or 3 predicted β-turns (25). Plasmids expressing the different envelopes or a 4070A envelope expression plasmid (ALF) (8) were transfected into TELCeB6 cells which carry the MFGnlslacZ vector genome and a murine leukemia virus (MLV) Gag-Pol expression plasmid, CeB (8). Transfected cells were selected with phleomycin (50 μg/ml), and supernatant from pools of phleomycin-resistant clones was pelleted and analyzed for viral proteins by Western blotting (7) (Fig. 2). All chimeric envelopes were detected in the pellets; the MFE23 chimeras were present at a higher level, comparable to that of 4070A.

Targeted infection. Viruses were harvested from the selected producer cells in Optimen (GIBCO-BRL) at 32°C, in some cases concentrated by centrifugation at 2,500 × *g* at 4°C for 12 h, then frozen at −70°C. A375m (ATCC CRL-1619) and B-1 (20) are human melanoma cell lines, TE671 is a human rhabdomyosarcoma cell line (ATCC CRL-8805), Ecv304 is a human endothelial cell line (ATCC CRL-1998), HT1080 is a human fibrosarcoma cell line (ATCC CCL-121), and HT29 (ATCC HTB-38) and Mawi (2) are both human colonic ade-

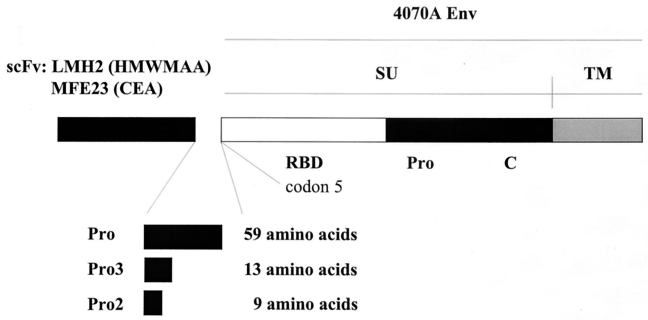

FIG. 1. Construction of targeted envelopes. LMH2 and MFE23 scFvs recognizing HMWMAA and CEA were fused to the N terminus of amphotropic 4070A MLV-SU by using three spacers derived from the Pro of 4070A SU. The Pro spacer contains all 59 amino acids of Pro, and Pro2 and Pro3 spacers are truncated versions that encode the first two or three predicted β-turns of Pro. The spacers were introduced in position +5 of the 4070A envelope. RBD, receptor binding domain; TM, transmembrane.

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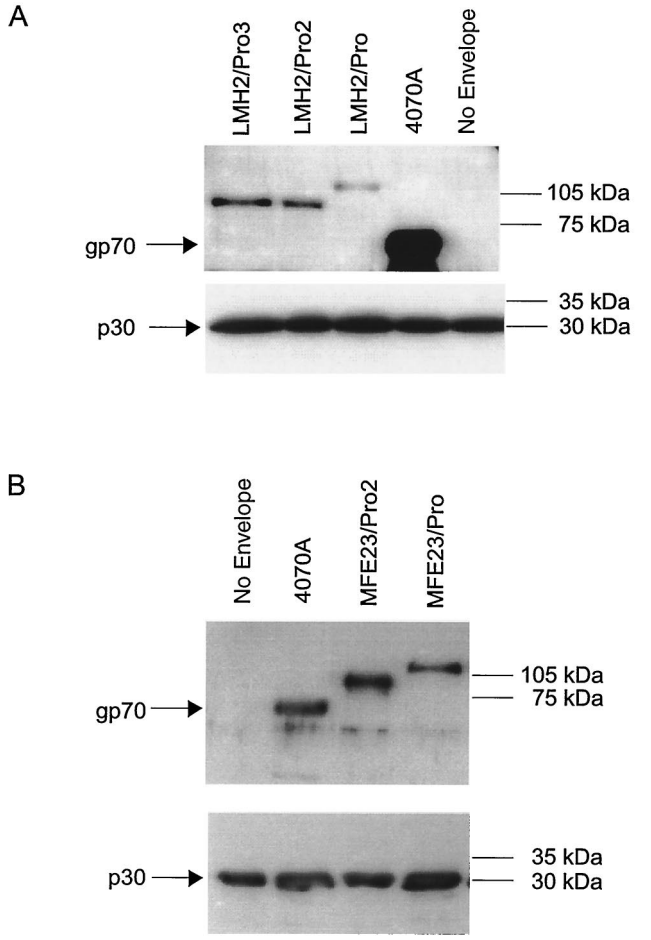

FIG. 2. Targeted envelope incorporation in retroviral particles. (A) LMH2-4070A chimeras. Concentrated supernatants from TELCeB6 cells (No Envelope) and TELCeB6 transfected with 4070A, LMH2/Pro, LMH2/Pro2, or LMH2/Pro3 envelopes were separated on a 10% sodium dodecyl sulfate–polyacrylamide gel, electroblotted, incubated with goat anti-Rauscher leukemia virus SU (gp70) and anti-Rauscher leukemia virus CA protein (p30) antisera followed by anti-goat horseradish peroxidase, and then developed with ECL (Amersham). (B) MFE23-4070A chimeras. Concentrated supernatants from TELCeB6 (No Envelope) and TELCeB6 transfected with 4070A, MFE23/Pro, and MFE23/Pro2 envelopes were analyzed as described for panel A.

nocarcinoma cell lines. Expression of HMWMAA on the target cells was determined by using LMH2 antibody (14) or CP/Mel.2 (Immune Systems Ltd.) and fluorescence-activated cell sorting. CEA expression by target cells was determined by immunoblotting with an anti-CEA antibody (Dako Ltd., Ely, Cambridgeshire, United Kingdom) or by immunostaining with anti-CEA antibody A5B7 (data not shown).

Figure 3 shows infection by viruses incubated with target cells for 4 h at 37°C in the presence of Polybrene and then washed and analyzed by X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining after 48 h (23). Figure 3A shows that viruses with LMH2 chimeric envelopes and the shorter linkers, Pro2 or Pro3, were able selectively to infect HMWMAA-positive cells. As previously reported, the LMH2 chimeric envelope with the full-length proline-rich spacer, LMH2/Pro, was not infectious (17) (note that this virus was designated scLPA in the previous paper). LMH2/Pro2 gave the best titer, up to 1,000 IU/ml unconcentrated, and was approximately 100-fold more infectious on HMWMAA-positive cells. MFE23/Pro2-enveloped vectors were also able selectively to infect CEA-expressing cells, in this case with titers over 10,000 IU/ml on CEA-positive cells and 10 IU/ml on CEA-negative cells; again, MFE23/Pro is not infectious (Fig. 3B). The higher titer with MFE23/Pro2 than with LMH2/Pro2 probably reflects the higher level of envelope incorporation (Fig. 2). We propose that the binding of the single-chain antibodies to their cell surface targets induces a conformational change within the shorter proline-rich spacers, most efficiently with the Pro2 spacer, which reveals the 4070A Pit-2 binding domain. Clearly, even the MFE/Pro2 envelope remains several logs less infectious than the wild-type 4070A envelope, which implies that

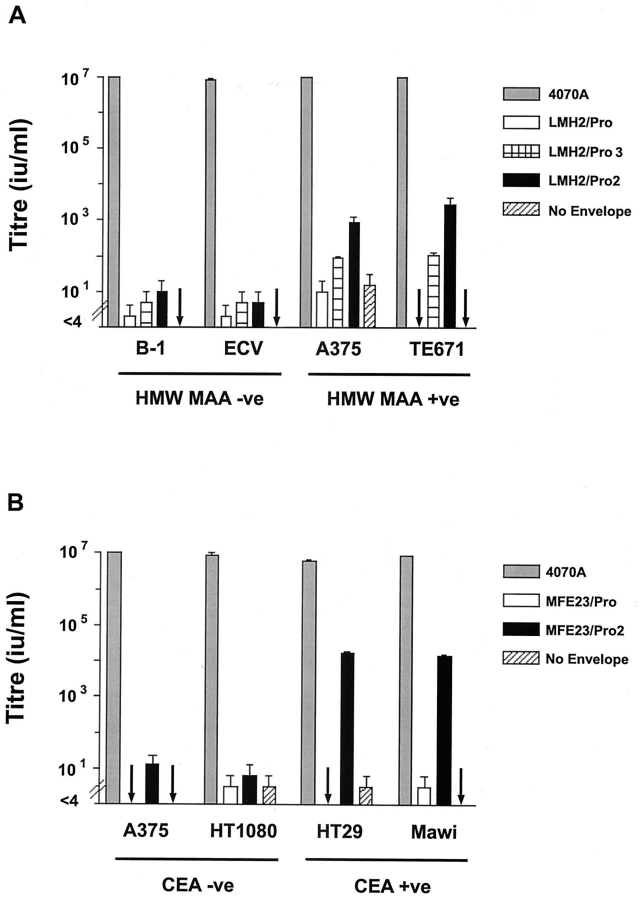

FIG. 3. Results of targeted infection are shown. (A) LMH2-4070A chimeras. Viruses with the HMWMAA-targeted chimeras LMH2/Pro, LMH2/Pro2, and LMH2/Pro3, 4070A, or no envelope were added (with 4 μg of Polybrene/ml) to HMWMAA-negative (−ve) (B-1 and Ecv304 [Ecv]) or HMWMAA-positive (+ve) (A375 and TE671) cells. (B) MFE23-4070A chimeras. Viruses with the CEA-targeted chimeras MFE23/Pro2 and MFE23/Pro, 4070A, or no envelope were added (with 4 μg of Polybrene/ml) to CEA −ve (A375 and HT1080) or CEA +ve (HT29 and Mawi) cells. Target cells were infected with serial dilutions of virus. Titer was calculated from values in the range where the number of infection events was directly proportional to the volume of virus added. Clusters of LacZ-positive cells were counted as single infection events.

TABLE 1. Enhancement of targeted infection[a]

Vector	A375m cells			HT29 cells		
	Nil	PB	LIP	Nil	PB	LIP
No Env	<4	<4	1.1×10^2	<4	5.0×10^0	1.2×10^2
MFE23/Pro	<4	<4	9.0×10^1	<4	<4	6.0×10^1
MFE23/Pro2	1.1×10^2	2.0×10^1	1.3×10^2	1.2×10^4	1.7×10^4	8.1×10^4
4070A	6.0×10^6	1.0×10^7	1.4×10^7	1.4×10^6	6.3×10^6	9.0×10^6

[a] MFE23/Pro2 vectors were incubated with 4 μg of Polybrene (PB)/ml or 10 μg of lipofectamine (LIP)/ml or with no addition (Nil) for 10 min at room temperature before addition to the target cells. Results are indicated in IU/ml.

the conformational change which follows scFv binding is not 100% efficient.

These targeted infection levels are approximately 1 log higher than those reported in a previous study with MFE23 fused to ecotropic MMLV envelope and coexpressed with wild-type ecotropic envelope (13). Khare et al. also describe a scFV which recognizes CEA fused to ecotropic MMLV envelope and coexpressed with wild-type ecotropic envelope (12). The titer that Khare et al. reported was similar to those shown here, but cocentrifugation of virus and cells was used to enhance infection (12). Table 1 shows that lipofectamine could enhance the efficiency of transduction of CEA-positive cells by MFE/Pro2-enveloped virus sevenfold without affecting its specificity, as has been previously described for other scFv-targeted viruses (17).

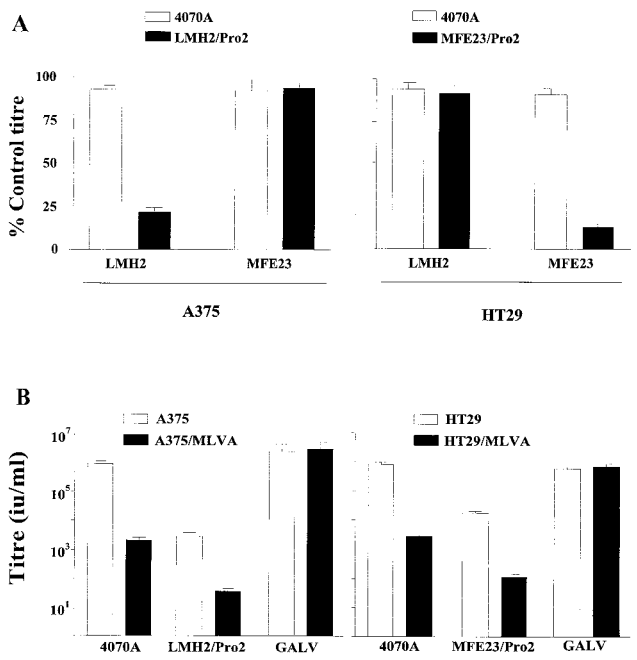

FIG. 4. Targeted infection requires antigen and Pit-2. (A) Antigen blocking. HMWMAA +ve (A375) and CEA +ve (HT29) cells were treated with 50 μg of LMH2 or MFE23 scFv per ml for 2 h. Infection by virus with 4070A envelope and LMH2/Pro2 (top panel) or MFE23/Pro2 (bottom panel) was then measured. Titer is expressed as a percentage of that of untreated cells. (B) Pit-2 blocking. HMWMAA +ve (A375) and CEA +ve (HT29) cells were infected with wild-type amphotropic MLV. After 2 weeks, A375, HT29, and infected A375/MLVA and HT29/MLVA cells were infected with viruses carrying targeted (LMH2/Pro2 or MFE23/Pro2) envelopes or 4070A or GALV envelopes, and their titers were determined.

These data differ from the receptor cooperation when two retroviral receptor binding domains were linked; in this case, the Pro linker promoted cooperation, while the Pro2 and Pro3 linkers allowed infection when only the N-terminal receptor binding domain contacted its receptor (25). To demonstrate that scFv-targeted infection required receptor cooperation, we inhibited interaction with the tumor antigen or the Pit-2 receptor. Figure 4A shows that addition of the appropriate, but not the irrelevant, scFv could inhibit targeted infection. Blocking with the monovalent scFv is incomplete, presumably because of the considerably higher avidity of viral binding. To block Pit-2, we infected target cells with replication-competent 4070A MLV, which reduces the number of Pit-2 molecules available for virus entry (11). Transduction of 4070A-infected TE671 and HT29 cells by the targeted vectors was reduced by approximately 500-fold, similar to the reduction of infection by viruses carrying the unmodified 4070A envelope (Fig. 4B).

Conclusions. Here, we have applied the receptor cooperation idea to achieve targeted retroviral infection of human tumor cells. The MFE23/Pro2 titer of 10^4 IU/ml, measured on cell lines in vitro in the presence of Polybrene, is similar to that which was previously reported for virus targeted to HMWMAA by an MMP-cleavable scFV and Pro linker (17). This virus gave reasonable infection (about 5% of cells) in HMWMAA-positive tumor xenografts (16), which suggests that MFE23/Pro2-targeted virus will be suitable for experiments in CEA-positive tumor xenografts. This level of targeted infection is the hallmark of approaches that use retroviral envelope interaction with its natural receptor to trigger efficient fusion. Previously, such approaches have included inverse targeting, where the cells that express the target molecule are not infectible (5, 7, 9), and protease targeting, where infection of the target cell requires cleavage of an incorporated domain by a protease (17, 19, 22). Both strategies are limited, either by the type of molecules that can be targeted (inverse targeting) or by the requirement for an active protease for infection (protease targeting). In the present paper, we describe a strategy where cell transduction requires only the expression of a target molecule and a retroviral receptor, which should provide a more general approach. Further modifications to improve efficiency could involve engineering scFvs to allow efficient envelope incorporation. In this study, the level of incorporation was acceptable in the case of MFE23 but suboptimal in the case of LMH2. The proline-rich linker could also be further

optimized (24), and point mutations could be introduced into the 4070A envelope to improve its stability (26).

The first two authors contributed equally to the work.

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