The Receptor Attachment Function of Measles Virus Hemagglutinin Can Be Replaced with an Autonomous Protein That Binds Her2/neu While Maintaining Its Fusion-Helper Function

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Cell entry of enveloped viruses is initiated by attachment to the virus receptor followed by fusion between the virus and host cell membranes. Measles virus (MV) attachment to its receptor is mediated by the hemagglutinin (H), which is thought to produce conformational changes in the membrane fusion protein (F) that trigger insertion of its fusion peptide into the target cell membrane. Here, we uncoupled receptor attachment and the fusion-helper function of H by introducing Y481A, R533A, S548L, and F549S mutations into the viral attachment protein that made it blind to its normal receptors. An artificial receptor attachment protein specific for Her2/neu was incorporated into the membranes of pseudotyped lentivirus particles as a separate transmembrane protein along with the F protein. Surprisingly, these particles entered efficiently into Her2/neu-positive SK-OV-3 as well as CHO-Her2 cells. Cell entry was independent of endocytosis but strictly dependent on the presence of H. H-specific monoclonal antibodies, as well as a mutation in H interfering with H/F cooperation, blocked cell entry. The particles mediated stable and specific transfer of reporter genes into Her2/neu-positive human tumor cells also in vivo, while exhibiting improved infectivity and higher titers than Her2/neu-targeted vectors displaying the targeting domain on H. Extending the current model of MV cell entry, the data suggest that receptor binding of H is not required for its fusion-helper function but that particle–cell contact in general may be sufficient to induce the conformational changes in the H/F complex and activate membrane fusion.

Attachment to cellular receptors and entry into the host cell are the first steps in viral infection. Enveloped viruses with lipid bilayers surrounding the capsid infect host cells by fusing with cellular membranes in a pH-dependent or -independent way. This process is mediated by the viral glycoproteins and can be subdivided in two steps: (i) binding of the virus to its cellular receptor and (ii) fusion of viral and cellular membranes.

While in some virus families, such as Retroviridae or Rhabdoviridae, these functions can be fulfilled by a single virus envelope protein, Paramyxoviridae rely on the concerted action of two glycoproteins. The fusion protein (F) mediates fusion of the viral and cellular membranes, while the hemagglutinin-neuraminidase (HN) or hemagglutinin (H) (morbilliviruses) attaches particles to their cellular receptor. However, H and HN mediate cell entry not only through receptor attachment but also by exerting the so-called fusion-helper function (1). The two measles virus (MV) glycoproteins H and F are organized on the viral surface in a hetero-oligomeric complex of F trimers and H tetramers which already forms in the endoplasmic reticulum (2–4). Like most paramyxoviruses, MV enters cells in a pH-independent manner and fuses directly with the plasma membrane (5). However, in contrast to other family members, MV binds to the cell not via sialic acid residues but through direct protein–protein interaction. The wild-type MV clinical isolates enter cells via SLAM (6, 7) and nectin-4 (8, 9), whereas the vaccine strains additionally use CD46 as cellular receptor (10, 11).

The structure of an H dimer is best described as a propeller with two cuboidal heads, each composed of six β-blades. Binding sites for the natural MV receptors are well characterized and cluster at one side of each head (12). The heads are situated on a long stalk region, which interacts with the globular head of F (3). Receptor binding is believed to trigger rearrangements in the central stalk region of H which are then transferred to F to trigger conformational changes that ultimately expose its fusion peptide to become inserted into the cellular membrane (13–15). The rearrangements in H are thought to lower the prefusion F activation energy barrier and thereby initiate the fusion process (16). The interactions between H and F and how the H stalk transfers the conformational changes to F have recently been mapped in detail (17).

How receptor binding alters the conformation of H and thus initiates the cascade of conformational changes is less well understood, especially when taking the unusual flexibility in using alternative receptors for cell entry into account. Besides the three identified MV receptors, the repertoire of entry receptors has been further extended by H protein engineering. Introducing mutations Y481A, R533A, S548L, and F549S (Hmut) makes the virus deficient for cell entry via its natural receptors (18, 19). By fusing a targeting ligand with high affinity for a given cell surface molecule to the C terminus of Hmut, the virus is redirected in cell entry to a receptor of choice. In this way, a variety of cell surface-exposed tumor antigens have been shown to be functional as MV receptors (20). By truncating their cytoplasmic tails, lentiviral vectors (LVs) have been pseudotyped with the MV glycoproteins (FΔ30 and HmutΔ18), and the MV-based retargeting system has been transferred to this important vector type for gene therapy applications.
The list of cell surface receptors used by the MV glycoproteins has been further extended to also include surface marker proteins relevant in immunology, hematology, and neurobiology (21, 22, 23). Usually, single-chain variable fragments (scFvs) derived from monoclonal antibodies or selected by phage display library screening have been used as targeting ligands (25). More recently, designed ankyrin repeat proteins (DARPins) were used to retarget MV-pseudotyped lentiviral vectors (26). These high-affinity binders are derived from natural ankyrin proteins and consist of two or three ankyrin repeat modules flanked by N- and C-terminal capping repeats. Her2/neu-specific DARPins were selected for binding to the Her2/neu extracellular domain using large ribosome or phage display libraries (27, 28).

This unique flexibility in receptor usage prompted us to assess whether receptor attachment of H itself is required to exert the fusion-helper function. To address this question, we fused the Her2/neu-specific DARPin 9.29 or G3 (27, 28) to a transmembrane protein domain and incorporated the resulting transmembrane high-affinity binders are derived from natural ankyrin proteins used to retarget MV-pseudotyped lentiviral vectors (26). These data extend the current model of MV-mediated membrane fusion by suggesting that receptor attachment of H is not required to trigger the fusion function of F but that particle-cell contact may be sufficient.

**MATERIALS AND METHODS**

**Plasmids.** The plasmids pDisplay-D9.29 and pDisplay-D9.23 were introduced by the introducing the DARPin 9.29- or G3-coding region (kindly provided by Andreas Pilchut, Zurich University, Switzerland) from pCG-HmutΔ18-DARPin9.29 or pCG-HmutΔ18-DARPin-G3 (26) via SfiI/NotI into pDisplay (Invitrogen, Karlsruhe, Germany) which was deleted for the second NotI site by mutagenesis. The helical linker (HL7) with the amino acid sequence RGSGA(EAAAK)7ALGS (29) was introduced into pDisplay-D9.23 via NotI/SalI to generate pDisplay-DHL7-G3. For the plasmid pHmutΔ18, an additional glycosylation site at position F93 in the HmutΔ18 protein was inserted by changing the phenylalanine codon into an asparagine codon through site-directed mutagenesis.

**Cell culture.** HEK-293T (ATCC CRL-11268) and CHO-K1 (ATCC CCL-61) cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Lonza, Cologne, Germany) supplemented with 10% fetal bovine serum (FBS) (PAA, Pasching, Austria) and 2 mM L-glutamine (Biochrome, Berlin, Germany). CHO-Her2 cells (previously termed CHO-Her2-kd [26]) were cultivated in the same medium with addition of 1.2 mg/ml G418 (Invitrogen, Karlsruhe, Germany). SK-OV-3 (ATCC HTB-77) cells were grown in McCoy’s A5 medium (Sigma-Aldrich, Taufkirchen, Germany) supplemented with 10% FBS and 2 mM L-glutamine.

**Virus particle production.** Virus particles were generated by transient transfection of HEK-293T cells using polyethylenimine (PEI). Twenty-four hours prior to transfection, 2 × 10^7 cells were seeded into a 1 T75 flask. On the day of transfection, 3.6 μg of pDisplay-D9.29, 4 μg of the plasmid encoding FΔ30, 1.4 μg of the plasmid encoding H variants, 12.6 μg of the packaging plasmid pCMVΔR8.9 (30), and 1.34 μg of the transfer vector plasmid (pSEW or pSEW-luc2) (26, 31) were mixed together with 2.3 ml DMEM without additives. For virus particle lacking one of the proteins, the respective DNA amount was replaced by pCG-1. The transfection reagent mix was prepared by mixing 140 μl PEI with 2.2 ml DMEM without additives. This mixture was added quickly to the DNA mix, briefly vortexed, and incubated for 20 min at room temperature. The cell culture medium was replaced by 10 ml DMEM with 15% FBS and 2 mM L-glutamine, and the transfection mix was added, resulting in 10% FBS total. After 24 h, the medium was exchanged to DMEM with 10% FBS and 2 mM L-glutamine to remove remaining PEI/DNA complexes. Two days after transfection, the cell supernatant containing the pseudotyped viruses was filtered (0.45-μm filter) and purified by ultracentrifugation at 25,000 × g and 4°C over a 20% sucrose cushion for 3 h. The pellet was resuspended in phosphate-buffered saline (PBS). Viral particles pseudotyped with vesicular stomatitis virus G protein (VSVG) were produced by cotransfecting cells with 6.13 μg pMD.G2 (kindly provided by Didier Trono, Lausanne, Switzerland), 11.38 μg pCMVΔR8.9, and 17.50 μg pSEW.

**Transduction of cell lines and virus stock titration.** For transduction, 1 × 10^6 cells were seeded into a single well of a 96-well plate. The next day, virus particle dilutions were added to the cells, from which in advance medium was aspirated. For spinfection experiments, cells and virus dilutions were centrifuged at 700 × g and 4°C for 1 h. After 72 h, titers were calculated based on the percentage of green fluorescent cells determined by flow cytometry on a Becton Dickinson LSRII flow cytometry system (Becton Dickinson, Heidelberg, Germany) as described previously (22). Data were analyzed using FCS Express software (De Novo Software).

For titration, serial diluted viral particle solutions were added to SK-OV-3 cells. At 72 h after transduction, the percentage of enhanced green fluorescent protein (EGFP)-positive cells was determined by flow cytometry analysis. Dilutions with 2 to 20% of EGFP-positive cells were used to calculate transducing units/ml (number of transduced cells/volume virus in μl/0.001).

**Binding assay.** Virus particles (0.14 μg p24) were incubated with 1 μg monoclonal antibodies (MAbs) for 1 h at room temperature. Subsequently, 1.5 × 10^6 SK-OV-3 cells were added following 1 h of incubation at 4°C. Cell-particle complexes were then stained with antihemagglutinin (anti-HA) phycoerythrin (PE)-coupled antibody, fixed, and analyzed by flow cytometry.

**Quantiﬁcation of p24.** The quantity of p24 gag in purified virus particle suspensions was determined using the RETROtek HIV p24 antigen enzyme-linked immunosorbent assay (ELISA) kit (ZeptoMetrix Corporiation, Buffalo, NY) according to manufacturer’s instructions.

**Detergent-resistant membrane (DRM) ﬂotation assay.** Detergent-resistant membranes were isolated as described before (32). Briefly, virus particle-producing cells were scraped and lysed in ice-cold TNE buffer (25 mM Tris [pH 7.4], 150 mM NaCl, 5 mM EDTA) containing 0.5% Triton X-100. Lysates were homogenized five times through a 23-gauge needle and incubated with constant agitation for 30 min at 4°C. They were mixed with 1 ml OptiPrep (Sigma-Aldrich, Taufkirchen, Germany), transferred to ultracentrifuge tubes, and overlaid with an OptiPrep gradient (2 ml of 30% followed by 1 ml of 25, 20, 17.5, 15, 12.5, and 5% OptiPrep in TNE buffer). Centrifugation was performed for 16 h at 39,000 rpm in a Beckman SW41 rotor at 4°C. Gradient fractions were analyzed by SDS-PAGE and Western blotting.

**Western blot analysis.** Western blot analysis of concentrated virus particles was performed as described previously (26). Briefly, particles were denatured, separated by gel electrophoresis, and electrotransferred onto nitrocellulose membranes (Amer sham Biosciences, Freiburg, Germany). The membranes were incubated with rabbit anti-F serum (F 431, I:2,000), rabbit anti-H serum (H 606, I:1,000), mouse anti-HA monoclonal antibody (clone 12CA5, I:1,000; Roche, Mannheim, Germany), or mouse anti-p24 monoclonal antibody (clone 38B/7.47, I:1,000; Gentaur, Aachen, Germany) to detect F, H, D9.29, and p24 gag, respectively. Secondary antibodies conjugated with horseradish peroxidase (1:2,000; DakoCytomation, Hamburg, Germany) were used. Signals were detected using the ECL Plus Western blotting detection system (GE Healthcare, Munich, Germany).

**Immunofluorescence microscopy.** For immunofluorescence, 3 × 10^4 HEK-293T cells were seeded into a covered chamber of an 8-well LabTek plate (Nunc, Wiesbaden, Germany). After 24 h, they were trans-
Effects with 0.25 μg of pDisplay-D<sup>9,29</sup>, pCG-H<sub>mut</sub>Δ18, and pCG-FΔ30. On the next day, cells were washed with PBS once and fixed in PBS supplemented with 4% formaldehyde for 20 min at room temperature. Subsequently, cells were permeabilized with 0.5% Triton X-100 in PBS for 8 min at room temperature. After an additional washing step and blocking in 5% chicken serum in PBS for 45 min at room temperature, cells were incubated with anti-H (clone K83, 1:20) (33) and then anti-HA (clone 16B12; Covance, Munich, Germany) monoclonal antibody for 1 h at 37°C. Subsequently, cells were washed twice with PBS and analyzed using a laser scanning microscope (LSM510; Zeiss, Jena, Germany). Micrographs are shown as false-color images generated with LSM Image Browser (Zeiss, Jena, Germany).

**Immunoelectron microscopy.** Virus particle-producing HEK-293T cells were fixed with 2% formaldehyde in PBS, washed three times with PBS, and blocked with 5% chicken serum. Primary antibody directed against HA tag (1:800, clone 16B12; Covance, Munich, Germany) was added to the cells and left for 1 h at 37°C. After two additional washing steps, cells were incubated with secondary anti-mouse antibody conjugated with 10-nm gold particles (British BioCell International, Cardiff, United Kingdom) for 1 h at 37°C following glutaraldehyde fixation, epoxy embedding, and preparation of ultrathin sections. Briefly, cells were attached from the cell culture dish and embedded in agarose. After a secondary fixation step with 1% osmium tetroxide (Sigma-Aldrich, Taufkirchen, Germany) and contrasting with 2% uranyl acetate (Merck, Darmstadt, Germany), cells were dehydrated using stepwise 30%, 50%, 70%, 80%, 90%, 96%, and 100% ethanol. Finally, cells were embedded into epoxy, and ultrathin sections were generated. Samples were examined on Formvar-coated mesh copper grids (Athene; Agar Scientific, Essex, United Kingdom) using an EM 109 transmission electron microscope (Zeiss, Jena, Germany).

**In vivo analysis.** Experimental mouse work was carried out in compliance with the German animal protection law. CB17 SCID female mice (6 to 8 weeks old; Harlan Laboratories, Eysenbrupp, Germany) were engrafted subcutaneously with SK-OV-3 tumor cells into the right flank as described previously (26). Systemic vector application was performed once tumors had reached a volume of about 50 mm<sup>3</sup> (8). Experimental mouse work was carried out in compliance with the German animal protection law. CB17 SCID female mice (6 to 8 weeks old; Harlan Laboratories, Eysenbrupp, Germany) were engrafted subcutaneously with SK-OV-3 tumor cells into the right flank as described previously (26). Systemic vector application was performed once tumors had reached a volume of about 50 mm<sup>3</sup> (8). In vivo imaging, mice were intraperitoneally injected with 150 mg D-luciferin/kg body weight (Caliper Life Sciences, Mainz, Germany) and anesthetized. Imaging data were obtained 10 to 15 min after substrate injection using a noninvasive cooled charged-coupled device (IVIS Spectrum; Caliper Life Sciences). Data were analyzed using the software Living Image 4.2 (Caliper Life Sciences).

**RESULTS**

Separating receptor attachment from H protein. To separate the receptor attachment function from the H protein, we aimed at incorporating a third separate transmembrane protein into LV particles serving as a scaffold for the targeting ligand, the Her2/neu-specific DARPin 9.29. Based on previous work we decided to use the platelet-derived growth factor receptor (PDGFR) transmembrane domain, which becomes efficiently incorporated into retrovirus particles and can be fused to proteins or peptides of choice (34, 35). Fusion of the reading frame of the Her2/neu-specific DARPin 9.29 (27) to the PDGFR transmembrane domain resulted in the construct pDisplay-D<sup>9,29</sup> (Fig. 1a). We expected that the encoded D<sup>9,29</sup> when expressed in LV packaging cells together with the cytoplasmic tail-truncated MV FΔ30 and H<sub>mut</sub>Δ18 will give rise to LV particles displaying all three proteins on their surface (Fig. 1a). D<sup>9,29</sup> was well expressed on the cell surface,
which is critical for efficient incorporation into LVs (Fig. 1b and c). Moreover, it colocalized with HmutΔ18 (Fig. 1b). H and F are known to form a complex within the endoplasmic reticulum (4) and are described to reside in detergent-resistant membranes (DRMs) after transport to the cell surface (36, 37). We therefore evaluated whether D9.29 can also be found in these distinct membrane populations together with HmutΔ18 and FΔ30 by membrane flotation assay and subsequent Western blot analysis. The DRMs were present in fractions 8 to 10 as determined by use of the DRM marker protein flotillin 1 (38), with the soluble membranes in fractions 11 and 12 as indicated by use of the non-DRM marker protein CD46 (39) (Fig. 1d). FΔ30 and also a significant amount of HmutΔ18 were detected in the DRM fractions. Although the MV H protein alone has not been described to be sorted into DRMs, it is known that it is associated up to 30% with DRMs upon coexpression with MV F (37). The modifications in the F and H cytoplasmic tails thus did not alter their DRM association. Interestingly, the vast majority of D9.29 was also found in the DRM fractions, thus suggesting a colocalization with FΔ30/HmutΔ18 at the cell membrane.

Generation and characterization of virus particles. In order to generate D9.29-LV particles having incorporated D9.29, FΔ30, and HmutΔ18, HEK-293T cells were cotransfected with the corresponding plasmids and in addition the packaging plasmid pCMVΔΔ9.1 and the EGFP-encoding transfer vector plasmid pSEW (Fig. 2a). A series of particles were generated as a control: by omitting one of each of the three plasmids encoding HmutΔ18, FΔ30, or D9.29, particles with only two of the three transmembrane proteins were generated. Moreover, we generated a variant of HmutΔ18 (HmutΔ18*), in which F93 (equates to position 111 in nontruncated H) was replaced by asparagine to become deficient in H/F complex formation (3) and used this for particle generation instead of HmutΔ18. Finally, for conventional 9.29-LV particles, DARPin 9.29 has been fused to the C terminus of HmutΔ18 (Fig. 1a) (26), resulting in D9.29-deficient particles. All particles were harvested from the supernatant of transfected cells and were pelleted through a sucrose cushion and analyzed by Western blotting. For D9.29-LV particles, we found all three types of transmembrane proteins, i.e., D9.29, HmutΔ18, and FΔ30, to be efficiently incorporated (Fig. 2b). Notably, HmutΔ18 and FΔ30 were incorporated at levels similar to those in the conventional 9.29-LV particles (Fig. 2b, lanes 1 and 2). HmutΔ18 also was detected in particles (Fig. 2b, lane 6). The analysis of control particles revealed the expected protein composition (Fig. 2b, lanes 3 to 5). Immunoelectron microscopy further proved the presence and accessibility of D9.29 on the surface of released particles (Fig. 2c).

Next, we assessed whether D9.29-LV had the potential to transduce Her2/neu-positive cells. Surprisingly, SKOV-3 cells incubated with D9.29-LV, presenting the DARPin on the PDGFR transmembrane domain, showed strong EGFP fluorescence and more EGFP-expressing cells than cells transduced with 9.29-LV where the DARPin was fused to H (Fig. 3a). Quantification revealed an almost 10-fold-higher transduction efficiency with D9.29-LV than with 9.29-LV (Fig. 3b). None of the control particles, including particles containing HmutΔ18, were able to mediate any detectable transfer of the egfp gene (Fig. 3b). Calculation of titers and determination of particle numbers by p24 ELISA for four different virus stocks revealed a 26-fold-higher infectivity-to-particle num-

FIG 2 MV-pseudotyped virus incorporates D9.29. (a) Principle of virus particle generation by transfection of HEK-293T cells. Transfection with the plasmids shown gives rise to D9.29-LV particles pseudotyped with HmutΔ18, FΔ30, and D9.29. (b) Western blot analysis for incorporation of FΔ30, D9.29, p24, and HmutΔ18 or 9.29-HmutΔ18 in vector particles 9.29-LV, D9.29-LV, and variants of D9.29-LV in which FΔ30 (w/o FΔ30), HmutΔ18 (w/o HmutΔ18), or D9.29 (w/o D9.29) was omitted, respectively, or HmutΔ18 was replaced by HmutΔ18* (w/HmutΔ18*). (c) Immunoelectron microscopy of ultrathin sections of HEK-293T producer cells releasing D9.29-LV vector particles. D9.29 was detected with primary antibody directed against the HA tag and secondary anti-mouse antibody conjugated with 10-nm gold particles following epoxy embedding and ultrathin sectioning. Scale bar, 200 nm.
ber ratio ($n = 4, P < 0.01$) for D9.29-LV than for 9.29-LV and an infectivity-to-particle number ratio similar to that for the nontargeted MV-LV (Fig. 3c).

To confirm that binding to Her2/neu is necessary for efficient transduction, we performed a spinfection experiment. Here, D9.29-LV or the controls were centrifuged onto SK-OV-3 cells. This way, viruses and cells were artificially brought into close contact, potentially circumventing the need for high-affinity receptor binding. This protocol increased transduction by D9.29-LV and 9.29-LV, whereas no remarkable transduction was observed when F*H/18 or D9.29 was missing or the interaction between F and H (H*mut18) was prevented (Fig. 4). This clearly demonstrates that high-affinity receptor binding is needed for cell entry of D9.29-LV.

To validate that these findings were not just DARPin 9.29 specific, particles displaying the Her2/neu binding DARPin G3 on the PDGFR transmembrane domain were generated (DG3-LV). This DARPin binds the more membrane-proximal Her2/neu domain IV, whereas DARPin 9.29 is directed against the membrane-distal domains I to III (27, 28). Compared to particles presenting the DARPin G3 fused to H (G3-LV), D53-LV revealed lower titers. Remarkably, titers increased dramatically when the distance between DARPin and Her2/neu domain IV was compensated for after insertion of a helical linker between the PDGFR transmembrane domain and DARPin G3 (DHL7-G3-LV) (Fig. 5).

Characterization of D9.29-LV cell entry. To assess whether gene transfer mediated by D9.29-LV was dependent on the presence of Her2/neu, we transduced CHO cells stably expressing Her2/neu (26). While the parental CHO-K1 cells which expressed neither Her2/neu nor any of the natural MV receptors remained untransduced, CHO-Her2 cells were readily transduced by D9.29-LV (Fig. 6a). Although the percentage of EGFP-positive CHO-Her2 cells was lower than that of transduced SK-OV-3 cells, which is likely due to the lower susceptibility of rodent cells to LVs compared to that of human cells, the data demonstrate that cell entry of D9.29-LV is Her2/neu dependent. Hence, receptor binding in D9.29-LV particles is uncoupled from H while still mediating efficient cell entry.

After having demonstrated that D9.29-LV specifically enters Her2/neu-positive cells, we next assessed whether cell entry relied on the MV membrane fusion mechanism. First, we blocked acidification of endosomes with chloroquine to prove that virus particle entry was pH independent, as described for MV (15). Besides 9.29-LV, MV-LV virus particles pseudotyped with FΔ30/HΔ18 entering into cells pH independently via CD46 (22), as well as virus particles pseudotyped with the glycoprotein G of vesicular stomatitis virus (VSV), which enter cells pH dependently (40), were used as controls. As expected, chloroquine treatment of SK-OV-3 cells reduced the transduction rate mediated by VSVG-LV substantially (Fig. 6b). In contrast, the transduction...
rates mediated by D9.29-LV and MV-LV were slightly but significantly enhanced in the presence of chloroquine. A remarkably strong enhancement of transduction by chloroquine was observed for 9.29-LV (Fig. 6b). It is possible that blocking endocytosis allowed more virus particles to enter cells at the plasma membrane. These data show that D9.29-LV entered cells pH independently. In order to demonstrate that cell entry not only is pH independent but can be blocked by specific inhibition of the F protein, we applied the fusion-inhibitory peptide (FIP) (41,42). While FIP did not influence VSVG-LV-mediated gene transfer, that of all three types of MV-pseudotyped particles was almost completely inhibited (Fig. 6c).

Next, we assessed the role of H in cell entry of D9.29-LV by applying neutralizing H-specific monoclonal antibodies (MAbs). The selected MAbs recognize conformational epitopes on H which have been roughly mapped by competition assays and mutational analysis of MV escape variants (33,43). Two of these MAbs (L77 and K71) have recently been shown to neutralize MV-LV vectors (44). Putative MAb binding sites as well as the mutations introduced in Hmut/H900418 to ablate the natural receptor usage were localized on the dimeric globular head domain of the H protein. According to this model, the epitopes of K71 and K29 are in close proximity to the introduced mutations, while those of Nc32 and L77 are located more distantly (Fig. 7a). MV-LV particles, which served as a positive control for the neutralizing activities of the MAbs, were completely neutralized in the presence of 0.1 g of K71 or L77 and 1 g of Nc32 or K29 (Fig. 7b). In contrast, VSVG-LV was unaffected by these MAbs, thus excluding any interference with virus particle entry in general (Fig. 7b). The strongest effect on D9.29-LV was exerted by L77, Nc32 and K29 impaired gene delivery moderately, and K71 was ineffective on this particle type (Fig. 7b). The inability of K71 to neutralize Her2/neu-targeted viruses could be due to ablation of the epitope in Hmut/H900418. To confirm this, we stained cells expressing H/H900418, Hmut/H900418, or 9.29-Hmut/H900418 with all four MAbs and performed flow cytometry to determine potential differences in avidity. As expected, K71 bound only to H/H900418 and not to the mutated variants, whereas Nc32, L77, and K29 bound all three variant H proteins with similar efficiencies (Fig. 7c). Moreover, none of the MAbs impaired binding of D9.29-LV particles to Her2/neu-expressing cells, confirming that the observed neutralization by Nc32, L77, and K29 was not simply due to interference with receptor attachment (Fig. 7d). Thus, three of four tested MAbs neutralized D9.29-LV to at least some extent. Hmut/H900418 must therefore fulfill an essential role during cell entry of D9.29-LV particles.

D9.29-LV as a gene transfer vector. Finally, we assessed the potential of D9.29-LV as a novel type of cell entry-targeted vector for gene transfer. For that purpose, high titers and stable gene transfer are crucial. Funke et al. (22) showed that the ratio of H- and F-encoding plasmids in packaging cells can be optimized. Moreover, LV particles displaying three different transmembrane proteins have, to our knowledge, not been described before. We therefore optimized vector particle production in two consecutive

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**FIG 4** High-affinity receptor binding is needed for virus entry. SK-OV-3 cells were incubated with equal amounts of egfp gene-delivering virus particles 9.29-LV, D9.29-LV, and variants of D9.29-LV in which FΔ30 (w/o FΔ30), Hmut/H900418 (w/o Hmut/H900418), or D9.29 (w/o D9.29) was omitted, respectively, or Hmut/H900418 was replaced by Hmut/H900418 either without centrifugation (a) or with additional centrifugation at 700 × g for 1 h (b). Flow cytometry analysis was performed 72 h after transduction.

**FIG 5** DARPin G3 as an alternative attachment protein. SK-OV-3 cells were transduced with particles presenting the Her2/neu-specific DARPin G3 fused to H (G3-LV), to the PDGFR transmembrane domain (DG3-LV), or to the PDGFR transmembrane domain connected by a helical linker (DH7G3-LV). Representative pictures of EGFP-expressing cells were taken at 72 h after transduction, and the percentage of EGFP-positive cells was determined by flow cytometry. Scale bar, 100 μm.
of HEK-293T cells can transfer EGFP directly as protein produced in the packaging cells. In order to assess whether the observed titers were attributable to this so-called pseudotransduction, we determined the sensitivities of these vectors to reverse transcriptase inhibition, which would not affect protein transfer (45). In the presence of 3’-azido-3’-deoxythymidine, gene transfer rates dropped substantially to extents that were similar for both D9.29-LV and VSVG-LV (Fig. 8c). Moreover, SK-OV-3 cells transduced with D9.29-LV at a low or high multiplicity of infection (MOI) showed constant levels of EGFP-positive cells over the whole observation period of 20 days (Fig. 8d).

After having demonstrated that D9.29-LV transfers genes stably into receptor-positive cells, we assessed its in vivo targeting potential in a xenograft mouse model carrying a subcutaneously growing SK-OV-3 tumor. To follow tumor targeting by in vivo imaging, D3.29-LV particles were equipped with a luciferase reporter gene (D9.29-LVluc). As a control, we used particles pseudotyped with FA30 and HmutΔ18 but devoid of D9.29 (MVmut-LVluc). Particles were injected systemically through the tail vein once tumors had reached a volume of about 50 mm3. D9.29-LVluc resulted in luciferase activity already 3 days after vector administration, which steadily increased over time in the SK-OV-3 tumor (Fig. 8e). In contrast, even after 2 weeks no luciferase signals could be detected in the tumor tissue of MVmut-LVluc-injected mice (Fig. 8e). Thus, it is clear that D9.29-LV is directed to the tumor cells by D9.29 and that in its absence an MV-pseudotyped lentiviral vector containing a receptor-blinded H is not able to specifically target SK-OV-3 cells in vivo.

**DISCUSSION**

A common principle of cell entry by enveloped viruses is fusion of the viral envelope membrane with that of the host cell directly at the cell surface or after endocytotic uptake. Membrane fusion involving two different viral glycoproteins is especially complex and less well understood than that of viruses having receptor attachment and membrane fusion combined in a single protein. For MV it is assumed that fusion is triggered upon binding of H to one of the three MV receptors (SLAM, CD46, and nectin-4), leading to conformational changes in H that are passed on to F (fusion-helper function of H). Here we describe an engineered MV envelope protein complex in which receptor attachment and the fusion-helper function of H have been separated onto two membrane proteins. This is to our knowledge the first demonstration that in such a setting a paramyxovirus envelope protein complex mediates cell entry equally well as in the two-transmembrane-protein-based setting. Transfer of cell surface attachment to a third transmembrane protein has been described for the alphavirus Sindbis virus, where receptor attachment has been transferred to a membrane-anchored immunoglobulin molecule specific for the cell surface protein CD20 (46). Similar to our study, the transmembrane proteins were incorporated into lentivirus particles. In contrast to MV, however, Sindbis virus enters cells by endocytosis, and membrane fusion is triggered not upon receptor contact but by the pH drop in the endosomes.

To achieve separation of the attachment and fusion-helper functions, we made use of the option to pseudotype lentivirus core particles with the MV glycoproteins. Such particles exhibit a receptor usage identical to that of MV but can be more easily engineered and generated from plasmid DNA (22, 47). For pseudotyping, the cytoplasmic tails of F and H have been truncated by 30 and

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**FIG 6** Characterization of D9.29-LV cell entry. (a) Cell entry is Her2/neu dependent. Representative pictures and flow cytometry dot plots of CHO-K1, CHO-Her2, and SK-OV-3 cells at 72 h after transduction with D9.29-LV are shown. Scale bar, 100 μm. (b) SK-OV-3 cells (1 × 10⁶) were treated with 150 μM chloroquine before the indicated virus particles were added. Relative transduction rates compared to that for cells transduced in the absence of chloroquine were determined by flow cytometry analysis. (c) MV-pseudotyped LV particles (0.07 μg p24) and VSVG-LV (0.008 μg p24) were incubated with (+FIP) or without (control) 200 μM FIP for 1 h at room temperature and then added to SK-OV-3 cells at an MOI of 0.3. The percentages of EGFP-positive cells were determined by flow cytometry after 72 h, and transduction rates relative to that obtained with untreated particles were calculated (n = 3; means ± SD are shown; *, P < 0.05; **, P < 0.01; ***, P < 0.0001; n.s., not significant by unpaired t test).
18 amino acids, respectively. These truncated mutants have been successfully used for the retargeting of lentiviral vectors (22). Previous studies demonstrated that the cytoplasmic tail of F is not required for cell surface expression and membrane fusion (48). Likewise, the C-tail of H can be truncated by 20 amino acids without losing its fusion-helper function (48). A recombinant MV encoding the cytoplasmic tail-truncated F/H900430 and H/H900420 variants was fully infectious and replication competent, although F and H particle incorporation rates were reduced upon cytoplasmic tail truncation (48,49). While MV particle incorporation is not relevant for the LV particles investigated here, it has also been suggested that cytoplasmic tail truncation enhances the fusogenicity of F and H (49). It is thus well possible that the F/H complex in D9.29-LV particles is in a preactivated state. It is, however, also evident from our data that D9.29-LV particles require the fusion-helper function of H for cell entry. Moreover, D9.29-LV entry was independent of endosomal uptake, sensitive toward the F protein-specific fusion inhibitory peptide (FIP), and completely dependent on Her2/neu expression. Principally, our observations could also be explained through the existence of a so-far-unidentified MV coreceptor. As D9.29-LV entered both human and rodent Her2/neu-positive cells, such a coreceptor likewise had to be present on human and rodent cells. While we cannot formally exclude this possibility, we are not aware of any data supporting its existence except for a report in 2007 in which neurokinin 1 was proposed as putative coreceptor for MV F on neurons (50). We therefore conclude that attachment of particles to cells in general is sufficient to trigger fusion of the virus particle envelope membrane with the cell, without a need for H itself binding to an MV receptor.

Interestingly, Watanabe et al. (51) reported in 2010 that MV particles incorporate cyclophilins into their membrane. Putative antibody binding sites are indicated in orange, red, green, and blue. Residues mutated in HmutΔ18 to ablate natural receptor tropism are shown in yellow. Antigenic site vi is shown in purple. The virus membrane is illustrated as a gray box. (b) MV-pseudotyped LV (0.1 μg p24) and VSVG-LV (0.004 μg p24) particles were incubated with or without H-specific MAbs K71 (top left), L77 (top right), Nc32 (bottom left), and K29 (bottom right) for 1 h at room temperature and then added to SK-OV-3 cells at an MOI of 0.3. The percentages of EGFP-positive cells were determined by flow cytometry after 72 h, and transduction rates relative to that obtained in the absence of MAbs were calculated (n = 3; means ± SD are shown). (c) HEK-293T cells (1 × 10⁶) transiently transfected with pCG-HmutΔ18, pCG-HmutΔ18-DARPIn-9.29 were stained with K29, K71, Nc32, or L77. Secondary anti-mouse-PE was used for detection in flow cytometry. (d) Binding of virus particles to SK-OV-3 cells. D9.29-LV was incubated with 1 μg K29, K71, Nc32, or L77 for 1 h at room temperature, and then SK-OV-3 cells were added and left for another hour. Samples were stained against the HA tag of particles using an anti-HA PE-coupled antibody. Cells incubated only with virus or virus displaying an attachment domain unable to bind to Her2/neu (D9-LV) were used as positive and negative controls, respectively. Fluorescence of cell-particle complexes was analyzed using flow cytometry.

**FIG 7** Neutralization of D9.29-LV by H-specific monoclonal antibodies. (a) Sphere representation of the H dimer crystal structure (58) (Protein Data Bank [PDB] ID 2ZB5; modified with PyMOL). The stalk, transmembrane, and cytoplasmic regions are depicted as vertical lines, with two horizontal lines indicating disulfide bonds. Putative antibody binding sites are indicated in orange, red, green, and blue. Residues mutated in HmutΔ18 to ablate natural receptor tropism are shown in yellow. Antigenic site vi is shown in purple. The virus membrane is illustrated as a gray box. (b) MV-pseudotyped LV (0.1 μg p24) and VSVG-LV (0.004 μg p24) particles were incubated with or without H-specific MAbs K71 (top left), L77 (top right), Nc32 (bottom left), and K29 (bottom right) for 1 h at room temperature and then added to SK-OV-3 cells at an MOI of 0.3. The percentages of EGFP-positive cells were determined by flow cytometry after 72 h, and transduction rates relative to that obtained in the absence of MAbs were calculated (n = 3; means ± SD are shown). (c) HEK-293T cells (1 × 10⁶) transiently transfected with pCG-HmutΔ18, pCG-HmutΔ18-DARPIn-9.29 were stained with K29, K71, Nc32, or L77. Secondary anti-mouse-PE was used for detection in flow cytometry. (d) Binding of virus particles to SK-OV-3 cells. D9.29-LV was incubated with 1 μg K29, K71, Nc32, or L77 for 1 h at room temperature, and then SK-OV-3 cells were added and left for another hour. Samples were stained against the HA tag of particles using an anti-HA PE-coupled antibody. Cells incubated only with virus or virus displaying an attachment domain unable to bind to Her2/neu (D9-LV) were used as positive and negative controls, respectively. Fluorescence of cell-particle complexes was analyzed using flow cytometry.
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FIG 8 D9.29-LV as gene transfer vector. (a and b) Titers of D9.29-LV stocks were optimized by keeping the amount of pDisplay-D9.29 constant and varying that of pCG-Fα30 to pCG-Hmut18 (a) or keeping the amounts of pCG-Fα30 and pCG-Hmut18 constant and varying that of pDisplay-D9.29 (b) for transfection of HEK-293T cells along with pCMVΔ8.91 and pSEW. For each ratio, pseudotyped vectors were titrated on SK-OV-3 cells, and their relative titer normalized to that obtained after transfection of a 1:1 plasmid ratio (100%) is shown (n = 3; means ± SD are shown). (c) SK-OV-3 cells were transduced with D9.29-LV or VSVG-LV in the presence or absence of 10 μM reverse transcriptase inhibitor azidothymidine (AZT) to verify that vectors mediated transgene integration. Relative transduction efficiency is shown (n = 3; means ± SD are shown; P < 0.001 by unpaired t test). (d) In order to demonstrate stable transduction, SK-OV-3 cells were cultivated for 20 days after transduction with D9.29-LV at an MOI of 0.4 or 0.1. The percentage of EGFP-positive cells was determined at the indicated time points by flow cytometry analysis. (e) CB17 SCID mice were injected with 5 × 10⁶ SK-OV-3 cells into the right flank (arrowheads). Once tumors had reached a volume of about 50 mm³, D9.29-LVLuc (8 μg p24) or MVmut-LVLuc (15 μg p24) was injected into the tail vein, and luciferase signals were analyzed by in vivo imaging 1 week later. Luciferase signal intensity is expressed as photons/second/square centimeter/steradian.

demonstrated that the binding partner CD147 is involved in MV infection of HEK-293 cells, since CD147-specific antibodies significantly reduced MV infectivity. Furthermore, they observed a low level of increased MV infectivity on CHO cells that stably express CD147 on their surface in comparison to CD147-negative CHO cells. Although clear evidence for CD147 acting as an MV receptor on epithelial cells is lacking, this process can possibly be seen as a natural counterpart of the D9.29-Her2/neu interaction in our engineered particles. What clearly differs in both settings are the affinities of the protein-protein interactions, which are high for D9.29- and DG3-Her2/neu (low nanomolar range) (27,28) and presumably orders of magnitude lower for CD147/cyclophilin B (52). Consequently, D9.29-LV and DG3-LV particles were substantially more active in entering cells in the absence of natural receptor attachment.

Structural analysis of H head domains and SLAM cocrystals recently revealed new insights into the mechanism of fusion triggering. Two discrete spatial organizations of the tetrameric H together with SLAM were identified and were suggested to represent a prefusion form of H immediately after receptor binding and a postfusion receptor-bound form (53). The reorganization that takes place from the pre- to postfusion form is interpreted as shift of the noncovalent dimer-dimer interface (13). Another recent study by Navaratnarajah et al. (54) tested a model proposing that the monomers of the covalently linked H dimer move relative to each other and thereby transmit the signal from H to F. They could show that the introduction of disulfide bonds across the dimer interface prevented membrane fusion and that addition of reducing agents reversed this effect. Our data show that conformational and/or structural changes can be triggered even if receptor binding is carried out not by H but by an additional protein. The fact that D9.29-LV was not able to enter cells in the absence of H or when the H/F complex formation was impaired (HmutΔ18) (Fig. 3a) indicates a need for H to interact with F in order to achieve membrane fusion. This was further supported by the sensitivity of D9.29-LV toward three H-specific monoclonal antibodies that did not interfere with Her2/neu attachment (Fig. 7b). Tahara et al. identified the so-called antigenic site vi, which is located at the bottom, membrane-proximal site of the H blade (Fig. 7a) and seems to be involved in H/F interaction (55). The epitope of L77, which was most efficient in neutralizing D9.29-LV, is adjacent to the vi site, thus further supporting the active role of H in initiating the membrane fusion process of D9.29-LV particles.

These findings support the idea that it is not direct binding of an MV receptor to H that induces the conformational change but that short-range changes in the microenvironment of H and F...
(13), such as the close proximity of the target cell membrane as mediated by attachment of D9.29 to Her2/neu, drive irreversible changes and ultimately membrane fusion. Taking the colocalization of D9.29 with the MV glycoproteins into account, D9.29 possibly replaces one of the H dimers physically and functionally or closely associates with the MV complex, thereby transcompleting the receptor binding-deficient H_HΔ18. This is reminiscent of work by Brindley et al. (56) demonstrating transcomplementation of the MV fusion complex by combining an H dimer deficient for F triggering but able to bind to MV receptors with an H dimer competent for F triggering but lacking receptor binding function. The authors concluded that unilateral receptor docking to one of the dimers is sufficient for MV fusion (56).

Besides serving as a tool to investigate the mechanism of MV-mediated membrane fusion, the system described here may prove useful as a novel targeting concept for lentiviral vectors. D9.29-LVmediated membrane fusion, the system described here may prove useful for functional genomics studies in tumor biology. Although the flexibility of this approach in terms of targeting surrogate systems and oncolytic viruses are preferred, D9.29-LV may prove useful for functional genomics studies in tumor biology. Although the flexibility of this approach in terms of targeting surface receptors other than Her2/neu will have to be assessed in future studies, our data on the DARPin G3, which binds more membrane proximally to Her2/neu than 9.29, show that vectors based on this targeting concept can be optimized by altering the length of the linker between the PDGFR transmembrane domain and the displayed targeting domain.

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