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Dissection of Gammaretroviral Receptor Function by Using Type III Phosphate Transporters as Models

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Gammaretroviruses that enter cells via binding to a surface receptor use one of two fundamental mechanisms. In the first, binding of the virus particle to its cognate receptor is followed by fusion and internalization. The second, less common mechanism requires the addition of an accessory protein in order to achieve fusion and entry into the target cells; this protein is usually the soluble form of the envelope protein containing the receptor-binding domain (RBD). For some viruses, such as amphotropic murine leukemia virus (A-MLV), particles with fusion-defective envelope proteins can enter cells in the presence of their own RBD or that of another viral envelope, regardless of its cognate receptor, suggesting that these viruses share a common entry mechanism. A notable exception is gibbon ape leukemia virus (GALV). Fusion-impaired GALV envelope mutants can be *trans*-activated for infectivity only by GALV RBDs. Using dually functional GALV/A-MLV receptors, we examined the role of receptor with respect to which RBD could overcome fusion impaired virus entry.

The human receptors for gibbon ape leukemia virus (GALV) and amphotropic murine leukemia virus (A-MLV), PiT1 and PiT2, respectively, have been identified and determined to function as two distinct multiple-membrane-spanning proteins that facilitate phosphate uptake by cells (reviewed in reference 19). PiT1 also functions as a receptor for feline leukemia virus B (FeLV-B) and FeLV-T but not as an A-MLV receptor, whereas some PiT2 orthologs have been determined to function as GALV and FeLV-B receptors (7, 8, 21). Characterization of these naturally occurring dually functional PiT2 receptor orthologs from hamster E36 (8) and *Mus molossinus* (21) cells, in concert with studies involving PiT1-PiT2 chimeric receptors, have resulted in a better understanding of the contributions of specific regions within the PiT proteins for GALV (8, 12, 20), FeLV-B (20, 22), FeLV-T (13), or A-MLV entry (11, 17, 20). For example, the region within PiT2 that allows A-MLV binding has been localized to residues 62 to 91, comprising the first extracellular loop of this multi-membrane-spanning protein (11). The region(s) required for GALV entry appears to be largely conserved between the PiT1 and PiT2 proteins as a single residue change can render PiT2 functional as a GALV receptor (8).

Studies have also been undertaken to assess the regions of the A-MLV, FeLV-B, and GALV envelope proteins that are required for receptor binding and fusion. Chimeric MLV envelope proteins containing regions of A-MLV and other MLVs that use receptors other than PiT2 have been constructed and used to assess the region within the A-MLV envelope protein that is critical for PiT2-mediated entry (reviewed in reference 19). It was established that a region at the N terminus of A-MLV, designated the receptor binding domain (RBD), is sufficient to confer PiT2 binding properties to viral vectors that incorporated chimeric MLV envelope proteins (4, 5, 18). Similarly, chimeric FeLV envelope proteins have been used to identify the SU receptor domain critical for PiT1-mediated entry by FeLV-B (6). Such an approach was not available to establish a GALV

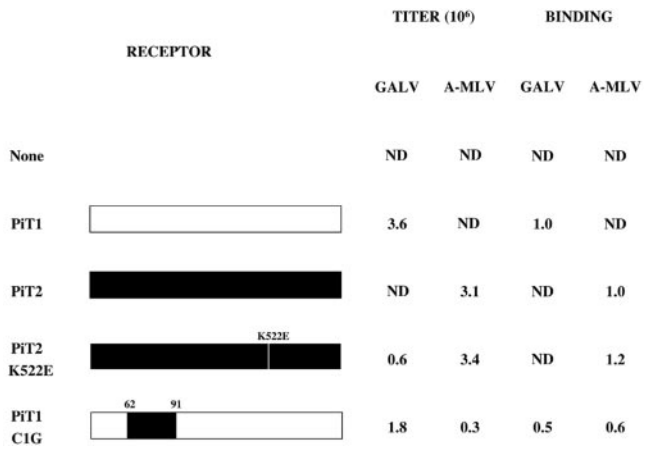

FIG. 1. Infectivity and binding properties of chimeric PiT1/PiT2 receptors. Chimeric receptors are susceptible to infection by both GALV and A-MLV. Titers are results of experiments obtained using stably transfected MDTF (for GALV) or BHK (for A-MLV) cell lines expressing PiT1, PiT2, PiT2K522E, or PiT1C1G. Titers were calculated as the number of β-galactosidase focus-forming units (BFU) per milliliter, and values are the means from at least three independent experiments. Binding results were obtained from fluorescence-activated cell sorting-based binding assays as previously described (10); GALV binding on MDTF cells expressing receptors is normalized to PiT1, and A-MLV binding on BHK cells expressing receptors is normalized to PiT2. Schematic representations of receptors are shown (white bar, PiT1; black bar, PiT2). Residue positions of substitutions are indicated. ND, not detected.

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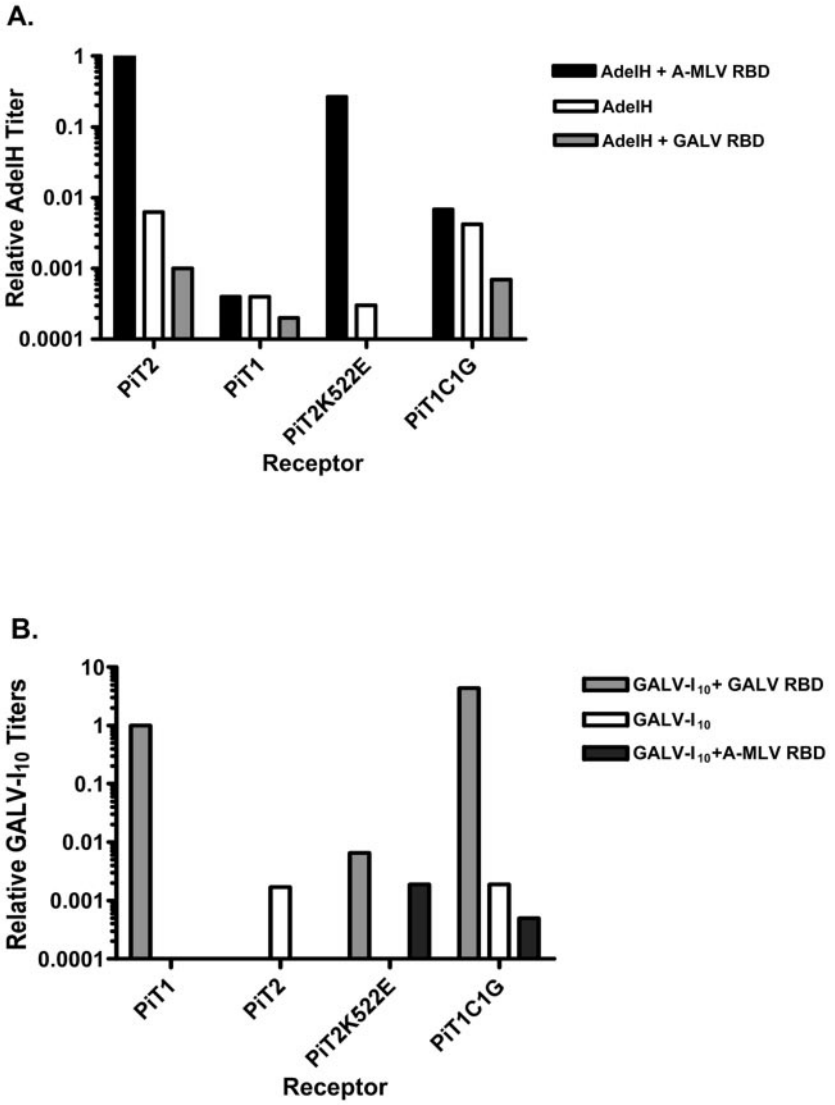

FIG. 2. (A) AdelH infection of MDTF cells expressing PiT2, PiT1, or the dually functional receptors PiT2K522E and PiT1C1G (*x* axis). AdelH-enveloped particles packaging the gene for β-galactosidase were used to infect the cells in the presence of 10 μg Polybrene per ml. Conditioned 2× medium containing A-MLV RBD, GALV RBD, or no RBD was added in an equal volume at the time of infection. Titers were calculated as the number of β-galactosidase focus-forming units (BFU) per milliliter. Titers were normalized for each receptor and RBD pair to those obtained with the same RBD on cells expressing PiT2 (*y* axis, log scale). Data are the mean values of at least three independent experiments. (B) GALV-I_{10} infection of MDTF cells expressing PiT2, PiT1, or dually functional receptors PiT2K522E and PiT1C1G (*x* axis). GALV-I_{10}-enveloped particles packaging the gene for β-galactosidase were used to infect the cells in the presence of 10 μg Polybrene per ml. Conditioned 2× medium containing A-MLV RBD, GALV RBD, or no RBD was added in an equal volume at the time of infection. Titers were calculated as the number of β-galactosidase focus-forming units (BFU) per milliliter. Titers were normalized for each receptor and RBD pair to those obtained with the same RBD on cells expressing PiT1 (*y* axis, log scale). Data are the mean values of at least three independent experiments.

envelope receptor-binding site because chimeric GALV-SU envelope proteins are not incorporated into viral vector particles (10). To date, the envelope residues required for PiT1-mediated GALV entry remain undefined.

In studies designed to further functionally dissect regions within the MLV SU region required for viral binding, fusion, and postfusion entry events, Bae and coworkers discovered that the deletion of a histidine residue that is part of a conserved PHQ motif near the N terminus of MLV SU envelope proteins had no effect on receptor binding but prevented the subsequent stages of viral entry (2). It has also been determined that the infectivity of fusion-defective mutant A-MLV lacking an intact PHQ motif can be rescued by supplying intact soluble homologous or heterologous MLV RBD in *trans* at the time of infection (3, 14–16). In contrast to fusion-defective A-MLV vectors, similarly impaired GALV vectors are not rendered infectious in the presence of soluble MLV RBDs; only soluble GALV RBD can rescue entry-impaired GALV vectors via its receptor, PiT1 (10). To gain insight into which properties of PiT1 result in its re-

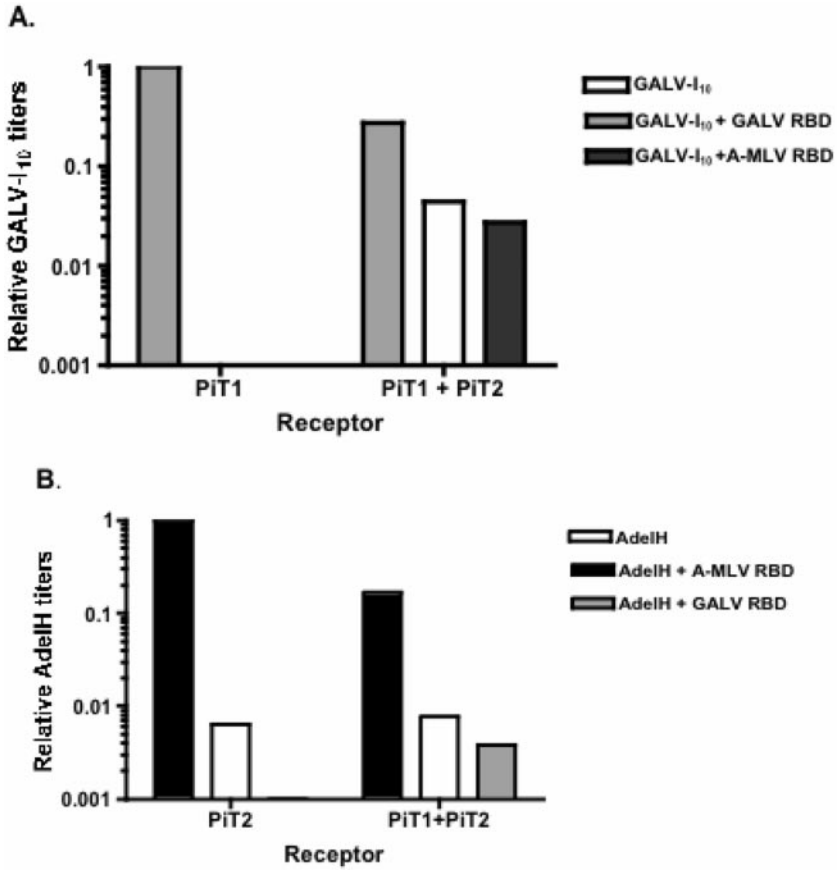

FIG. 3. Infection of MDTF cells expressing both PiT1 and PiT2. (A) GALV-I_{10}-enveloped particles packaging the gene for β-galactosidase were used to infect the cells in the presence of 10 μg Polybrene per ml. Conditioned 2× medium containing A-MLV RBD, GALV RBD, or no RBD was added in an equal volume at the time of infection. Titers were calculated as the number of β-galactosidase focus-forming units (BFU) per milliliter. Titers were normalized for each receptor and RBD pair to those obtained with the same RBD on cells expressing PiT1 (*y* axis, log scale). Data are the mean values of at least three independent experiments. (B) AdelH-enveloped particles packaging the gene for β-galactosidase were used to infect the cells in the presence of 10 μg Polybrene per ml. Conditioned 2× medium containing A-MLV RBD, GALV RBD, or no RBD was added in an equal volume at the time of infection. Titers were calculated as the number of β-galactosidase focus-forming units (BFU) per milliliter. Titers were normalized for each receptor and RBD pair to those obtained with the same RBD on cells expressing PiT2 (*y* axis, log scale). Data are the mean values of at least three independent experiments.

stricted fusion rescue properties, we compared the binding and rescue properties of PiT1 to those of dually functional receptors that mediate entry of both GALV and A-MLV.

Infectivity and binding characteristics of three PiT receptors. We compared GALV vector titers on GALV-resistant murine MDTF cells expressing PiT1, the cognate receptor for GALV, and two other GALV receptors, namely, PiT2K522E (8) and PiT1C1G (11), that have also been shown to function as receptors for A-MLV. Similar GALV titers were obtained with all three receptors when the receptors were expressed on MDTF cells (Fig. 1). In order to determine A-MLV titers for these dually functional receptors, they were expressed in A-MLV-resistant hamster BHK cells (Fig. 1). BHK and BHK-PiT1 cells are resistant to A-MLV, whereas BHK-PiT2, BHK-PiT2K522E, and BHK-PiT1C1G are susceptible to A-MLV (Fig. 1).

We next compared the abilities of these various receptors to bind soluble GALV RBD or A-MLV RBD. MDTF cells expressing PiT1 or PiT1C1G bind GALV RBD; MDTF cells and MDTF cells expressing PiT2 fail to bind GALV RBD (9) (Fig. 1). Surprisingly, MDTF cells expressing PiT2K522E, even though efficiently infected by GALV, do not bind GALV RBD. BHK cells are resistant to infection by A-MLV and are unable to bind A-MLV RBD (Fig. 1), but when expressing PiT2, PiT1C1G or PiT2K522E, these cells demonstrate A-MLV RBD binding as well as susceptibility to A-MLV infection (Fig. 1). In summary, of the dually functional receptors PiT1C1G and PiT2K522E, only PiT1C1G binds GALV, while both bind A-MLV.

Rescue of fusion-defective GALV vectors on cells expressing dually functional GALV/A-MLV receptors. Fusion-defective GALV vectors, GALV-I_{10}, lacking an intact PHQ motif at the N terminus of the envelope SU, bind PiT1 but are blocked at subsequent steps of viral entry. GALV-I_{10} can be rendered infectious in the presence of soluble wild-type GALV RBD protein, but not by soluble A-MLV RBD (10) (Fig. 2B). Conversely, fusion-defective A-MLV vectors, AdelH (16), while able to bind, can infect murine MDTF cells expressing PiT2 only in the presence of soluble A-MLV RBD, not GALV RBD (10) (Fig. 2A).

To determine which soluble RBD (GALV or A-MLV) can restore infectivity to AdelH or GALV-I_{10} (or both) in the presence of dually functional GALV/A-MLV receptors, we exposed MDTF-PiT2K522E and MDTF-PiT1C1G cells to each defective vector in the presence of either soluble GALV or A-MLV RBD using methods previously described (10). Soluble A-MLV RBD restored AdelH infectivity (3×10^3 blue focus-forming units [BFU]/ml) to MDTF-PiT2K522E cells and, to a lesser extent, to MDTF-PiT1C1G cells (5.5×10^2 BFU/ml) (Fig. 2A); however, it did not rescue GALV-I_{10} infectivity (<1 BFU/ml) on either cell line (Fig. 2B). Infectivity of GALV-I_{10} on MDTF-PiT1C1G cells, in the presence of GALV RBD, was restored to a level similar to that on MDTF-PiT1 cells (Fig. 2B). However, MDTF-PiT2K522E cells did not support GALV-I_{10} infectivity in the presence of GALV RBD (<1 BFU/ml) (Fig. 2B); this was not unexpected, given that GALV RBD binding to PiT2K522E is undetectable. Soluble GALV RBD did not rescue infectivity of AdelH on MDTF-PiT2K522E (<1 BFU/ml) and gave very low levels of rescue on MDTF-PiT1C1G cells (3×10^2) (Fig. 2A). Thus, even though PiT2K522E functions as a receptor for GALV, GALV-I_{10} fusion-defective vectors failed to infect cells expressing PiT2K522E in the presence of either A-MLV or GALV RBD (Fig. 2B).

MDTF cells expressing both PiT1 and PiT2 were used as target cells in GALV and A-MLV-RBD rescue assays to determine whether nonreciprocal *trans*-activation of GALV-I_{10} and AdelH can be achieved when these receptors are coexpressed. Nonreciprocal transactivation was not observed using MDTF-PiT1/PiT2 cells (Fig. 3). However, MDTF PiT1/PiT2 cells, unlike MDTFPiT1 cells, are susceptible to low-level infection by GALV-I_{10} (Fig. 3B).

Conclusions. PiT2K522E is identical to PiT2 except for a single residue substitution at position 522. This subtle change in the composition of PiT2 is sufficient to render PiT2K522E functional as a GALV receptor while retaining A-MLV receptor features. PiT2K522E binds A-MLV RBD (Fig. 1) and supports rescue of AdelH infectivity in the presence of soluble A-MLV RBD (Fig. 2). All of these assessed PiT2K522E features are consistent with those observed with PiT2 (Fig. 1 and 2). PiT2K522E does not function in a manner similar to PiT1 in either a GALV binding assay (Fig. 1) or a GALV-I_{10} rescue assay in the presence of GALV RBD (Fig. 2). The absence of detectable binding observed with PiT2K522E is not due to reduced levels of receptor on the cell surface, as epitope (hemagglutinin)-tagged PiT2K552E and PiT1 are detected at similar levels on the surface of MDTF cells, as demonstrated in fluorescence-activated cell sorting-based hemagglutinin binding assays (9).

The second A-MLV/GALV dually functional receptor we assessed in this study, designated PiT1C1G, consists primarily of PiT1 residues, with only 30 residues of PiT2 replacing the corresponding residues of PiT1 in the putative first extracellular domain. These substituted residues are sufficient to render PiT1 functional as an A-MLV receptor while retaining its GALV receptor function. Expression of PiT1C1G renders A-MLV-resistant hamster BHK cells susceptible to A-MLV, with titers similar to those observed on BHK-PiT2 cells (Fig. 1); these cells also bind A-MLV RBD and support A-MLV RBD rescue of AdelH (Fig. 2).

The results of these studies show that infectivity, binding, and rescue features associated with wild-type GALV and A-MLV receptors, PiT1 and PiT2, respectively, are not all required for efficient virus receptor function. For example, PIT1C1G retains all three of these PiT1-associated receptor functions while also exhibiting the PiT2 properties of infection, binding, and rescue. In contrast, PiT2K522E, while retaining all of the PiT2 receptor properties, also functions as a GALV receptor yet does not exhibit PiT1 properties for GALV RBD binding or rescue. Therefore, the strength of GALV RBD-receptor binding is not an indicator of receptor internalization properties; furthermore, all receptors that facilitate virus entry are not capable of supporting rescue of analogous fusion-defective vectors, even though the unimpaired vector utilizes wild-type PiT receptors for entry. This is the case for FeLV-T. FeLV-T uses PiT1 as a receptor and is the only known example of a naturally arising gammaretrovirus that requires an RBD (FeLiX) as a cofactor for entry (1). FeLiX, like the GALV RBD used in these rescue experiments, is a truncated form of a gammaretroviral SU lacking the C terminus. FeLiX comprises the first 273 residues of an endogenous FeLV-B envelope derivative (1) whereas the GALV RBD used in these rescue assays contains only the N-terminal 264 residues of the GALV SU. This is in marked contrast to the RBDs used in MLV rescue experiments that correspond to the entire SU region of the MLV envelope protein.

In conclusion, the ability of the PiT proteins to facilitate A-MLV and/or GALV entry is independent of the binding and rescue characteristics associated with wild-type PiT1 and PiT2, thereby suggesting that the receptor regions associated with RBD binding and rescue are not all required for efficient non-cofactor-mediated viral entry properties.

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