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A Family of Retroviruses That Utilize Related Phosphate Transporters for Cell Entry

DANIEL G. MILLER[1,2,3] AND A. DUSTY MILLER[1,2]*

Fred Hutchinson Cancer Research Center, Seattle, Washington 98104,[1] and Department of Pathology[2] and Medical Scientist Training Program,[3] University of Washington, Seattle, Washington 98195

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The amphotropic murine retrovirus receptor Ram-1 shows significant sequence similarity to the gibbon ape leukemia virus (GALV) receptor Glvr-1, and both of these cell surface virus receptors normally function as sodium-dependent phosphate symporters. However, Ram-1 from humans or rats does not serve as a receptor for GALV, and Glvr-1 from humans does not serve as a receptor for amphotropic virus. Here we show that the murine retrovirus 10A1 can enter cells by using either Glvr-1 or Ram-1. Furthermore, we have constructed Ram-1/Glvr-1 hybrid receptors that allow entry of both GALV and amphotropic virus. While GALV and amphotropic virus are in separate interference groups when assayed on human cells, they do interfere with each other in cells expressing the hybrid receptor. These results indicate a close functional relationship between retroviruses that utilize members of this newly defined receptor family and provide a molecular explanation for nonreciprocal and cell type-specific interference observed for some retrovirus classes.

Seven retroviral receptors have been identified to date. These molecules are membrane-bound proteins which specifically associate with the retroviral envelope gene product and are required for virus entry. The receptors for human and simian immunodeficiency viruses (CD4) (8, 22, 40), avian leukosis virus type A (Tva1) (50), and bovine leukemia virus (Blvr) (2) are organized with a hydrophilic globular extracellular domain linked to an intracellular portion by a single membrane-spanning sequence. Cell surface receptors for feline immunodeficiency virus (CD9) (15, 49), ecotropic murine leukemia viruses (MCAT) (1), amphotropic murine leukemia viruses (Ram-1) (30, 45), and the common receptor (Glvr-1) for gibbon ape leukemia virus (GALV) (33), feline leukemia virus group B (44), and simian sarcoma-associated virus (41) span the cell membrane multiple times on the basis of hydropathy analysis. MCAT, Glvr-1, and Ram-1 are transport proteins, with MCAT serving as a cationic amino acid transporter (21, 46), while Ram-1 and Glvr-1 are both sodium-dependent phosphate symporters (20). Of the receptors cloned to date, only Ram-1 and Glvr-1 show significant sequence similarity.

The utilization of diverse receptors for cell entry is the primary factor that determines the distinct host ranges of the murine leukemia viruses. Ecotropic retroviruses are able to infect only rodent cells, while amphotropic retroviruses can infect cells from many species. Xenotropic retroviruses are generally unable to infect mouse cell lines, with the exception of some wild-mouse strains, but can infect cells from other species. Polytropic viruses are the product of recombination events between ecotropic viruses and endogenous mouse proviral sequences (4, 11, 14) and are able to infect nonrodent cell lines as well as most rodent cell lines. The 10A1 retrovirus class includes a single membrane that was the result of a recombination between 1504A amphotropic virus and an endogenous provirus found in Swiss mice (38). 10A1 virus exhibits the wide host range characteristic of the parent amphotropic retrovirus but also infects amphotropic virus-resistant CHO cells (34).

* Corresponding author. Mailing address: Fred Hutchinson Cancer Research Center, 1124 Columbia St., Seattle, WA 98104. Phone: (206) 667-2890. Fax: (206) 667-6523.

Virus interference analysis allows the grouping of retroviruses on the basis of cell surface receptor usage. Two types of anomalies result from using interference criteria to classify the murine retroviruses. First, virus groupings depend on the cell type used for interference analysis (6, 9, 41). For example, xenotropic retrovirus interferes with superinfection by polytropic virus in SC-1 mouse cells (6), suggesting that these viruses use the same receptor. However, xenotropic virus infection has no effect on superinfection by polytropic virus in NZB mouse cells (6, 39), suggesting that these viruses use different receptors. Nonreciprocal interference is the second type of anomaly observed in retroviral interference studies (9, 39). For example, NIH 3T3 mouse cells infected with 10A1 retrovirus are resistant to infection by amphotropic retrovirus, but the same mouse cells infected with amphotropic retrovirus are completely susceptible to infection by 10A1 virus (39). These results suggest that 10A1 virus is able to use an unidentified receptor in addition to the receptor used by amphotropic viruses.

In this study, we show that 10A1 retrovirus can use either Ram-1 or Glvr-1 as a receptor for cell entry, providing a molecular explanation for nonreciprocal interference between 10A1 and amphotropic viruses. We have also constructed hybrid molecules between Glvr-1 and Ram-1 to study determinants required for virus entry and found that some hybrids can serve as receptors for both amphotropic and GALV pseudotype retroviral vectors, supporting the hypothesis that retroviruses can use alternative receptors in different cell types. These results provide insight into the evolution of new retrovirus classes with altered host range and receptor utilization.

MATERIALS AND METHODS

Nomenclature. A small p before a virus or vector name indicates the plasmid form of the virus, while the absence of this prefix indicates the virus in either the proviral or viral form. Cells that contain a virus or vector are indicated by the cell name followed by a slash and the name of the virus or vector, e.g., NIH 3T3/10A1 or PA317/LXSN. A retroviral vector in its viral form is indicated by the vector name followed, in parentheses, by the name of the helper virus or packaging

cells used to pseudotype the vector, e.g., LAPSN(10A1) or LAPSN(PA317). Throughout this report, the pseudotype of a retroviral vector refers only to the viral envelope proteins present on the vector virions. For example, vectors produced by PG13 cells will be referred to as having a GALV pseudotype because of the presence of GALV Env proteins in the virions, even though the Gag-Pol proteins in these virions are derived from Moloney murine leukemia virus.

Cell culture. Mammalian cells were grown in α-modified minimal essential medium (CHO cells) or Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells used include CHO-K1 cells (19), NIH 3T3 (TK^-) cells (47), 208F rat embryo fibroblasts (36), PA317 amphotropic retrovirus-packaging cells (26), and PG13 GALV-based retrovirus-packaging cells (27). CHO cell-conditioned medium was prepared by incubation of the culture medium with confluent monolayers of CHO cells for 24 h. Conditioned medium was filtered (0.45-μm pore size) and frozen at −70°C.

Retroviruses. The pB6 plasmid containing the 10A1 retrovirus (34) was a gift of Alan Rein, National Cancer Institute Frederick Cancer Research and Development Center, Frederick, Md. This permuted clone was cut with *Sal*I restriction endonuclease, religated to generate intact 10A1 provirus circles, and transfected into NIH 3T3 cells expressing the LAPSN retrovirus vector (30). Cells were passaged for 2 weeks in 2 μg of Polybrene (Sigma) per ml to facilitate virus spread. Mv 1 Lu mink cells (ATCC CCL 64) producing GALV SEATO strain were a gift from Maribeth V. Eiden, National Institutes of Health, Bethesda, Md. Replication-competent amphotropic virus AM-MLV, a hybrid virus constructed by replacing the *env* gene of Moloney murine leukemia virus with that of 4070A amphotropic virus, was harvested from NIH 3T3 cells transfected with the plasmid pAM-MLV (26). Replication-competent wild-type virus stocks as well as retrovirus vector stocks were prepared, and vector titer was determined as described elsewhere (28, 29).

Virus infections. Virus infections were performed in the presence of fresh medium containing 4 μg of Polybrene (Sigma) per ml. Cells were fixed and stained for alkaline phosphatase expression encoded by the LAPSN vector as described elsewhere (10). To improve infection of CHO cells by GALV, the cells were treated with 0.2 μg of tunicamycin (Sigma) per ml for 19 h prior to infection (28, 31) and were passaged in medium containing 2 μg of Polybrene (Sigma) per ml for 3 weeks. Cells were reinfected with GALV (after tunicamycin treatment) three times over the next 2 weeks in an attempt to infect all cells in the population.

Construction of Glvr-1/Ram-1 receptor hybrids. Chimeric receptors between the human *glvr-1* and the rat *ram-1* cDNAs were constructed by first making two point mutations in the *ram-1* sequence. One mutation was at bp 600 and altered the sequence from GTACAC to GTATAC to create an *Acc*I restriction site without changing the native amino acid sequence. The second mutation was made at bp 1476 and altered the nucleic acid sequence from CTGCAA to CTGCAG to create a *Pst*I site, again without altering the amino acid sequence. These two sites, already present in the *glvr-1* sequence, were used to divide each gene into three sections and create hybrid molecules in the Bluescript plasmid backbone pBSKS II (+) (Stratagene, La Jolla, Calif.). The hybrids were then cloned into LXSN for expression analysis in mammalian cells.

The hybrid construct labeled GGrG contains the *glvr-1* sequence up to bp 1647 and then the *ram-1* sequence underlined in the oligonucleotide below, followed by the *glvr-1* sequence starting with *glvr-1* bp 1675. The sequence of the oligonucleotide used for this mutation was CTGGTTGCTTT ATATTTGGTTTATAAACAAGGCGGAGTTACACAAGA AGCTGCAACACAATATGG. Roman type corresponds to the *glvr-1* sequence, and the underlined sequence corresponds to the *ram-1* sequence (Ram-1 amino acids 523 to 530). Point mutations and the GGrG construct were made by site-directed mutagenesis (25).

Generation of cell lines expressing retrovirus receptors and receptor hybrids. Receptor and hybrid receptor constructs were cloned into the retroviral expression vector LXSN (29). NIH 3T3 cell lines that stably expressed the receptor constructs were made by transduction with vectors produced by transient transfection of PA317 cells as described elsewhere (28). CHO cell lines expressing the receptor constructs were made in a similar way, except that PG13 packaging cells expressing the GALV envelope gene were used for transient expression of receptor constructs instead of PA317 cells and CHO cells were treated with 0.2 μg of tunicamycin (Sigma) per ml for 19 h prior to transduction to facilitate vector entry (28, 31). Transduced cells were selected in 1.5 mg (NIH 3T3 cells) or 1.0 mg (CHO cells) of G418 (GIBCO) per ml (total weight, about half of which is active), and polyclonal populations were assayed for receptor construct function. For some experiments, the receptor constructs were expressed transiently following calcium phosphate-mediated transfection (5).

RESULTS

Interference analysis indicates that the 10A1 retrovirus can enter cells by using the amphotropic receptor as well as another unidentified receptor distinct from those used by other classes of murine retroviruses (39). The expanded host range of 10A1 virus can be conferred by as few as six amino acid changes in the amphotropic envelope glycoprotein (35), suggesting that the additional receptor utilized by 10A1 may be related to the amphotropic receptor Ram-1. The GALV receptor Glvr-1 exhibits about 60% amino acid identity with Ram-1, making Glvr-1 a possible candidate for the additional receptor utilized for cell entry by 10A1 virus.

The most unequivocal method to test whether Ram-1 and/or Glvr-1 can mediate entry of 10A1 virus into cells would be to introduce the receptors into cells that are normally resistant to infection by 10A1 virus and assay for the ability of the modified cells to be infected. Unfortunately, all of the cell lines that we tested were susceptible to infection by retroviral vectors pseudotyped by 10A1 virus. However, we had previously identified protein factors in CHO cell-conditioned medium that blocked CHO cell transduction by retroviral vectors having either an amphotropic or GALV pseudotype but did not block transduction of cells from species other than hamsters (31, 32). We hypothesized that the same inhibitory factors might also block CHO cell transduction by vectors pseudotyped with 10A1 retrovirus if this virus utilizes Ram-1 or Glvr-1 for entry into CHO cells. Use of conditioned medium to block endogenous hamster virus receptors might then allow testing for activity of potential receptors from other species in CHO cells, since the inhibitors present in the conditioned medium from CHO cells are apparently hamster specific.

Transduction of CHO cells by vectors pseudotyped with 10A1 retrovirus is inhibited by conditioned medium from CHO cells. We measured transduction rates in CHO cells by using a retroviral vector encoding alkaline phosphatase (LAPSN) pseudotyped by replication-competent 10A1 retrovirus [LAPSN(10A1)]. CHO cell transduction by the LAPSN(10A1) vector was reduced 36-fold by including conditioned medium from CHO cells during infection (Table 1).

TABLE 1. Inhibition of transduction by a 10A1-pseudotyped retroviral vector by medium conditioned by CHO cells[a]

Cells	Vector titer (FFU/ml)[b]		Fold decrease in titer
	Standard medium	Conditioned medium[c]	
CHO-K1 (hamster)	5.1×10^3	1.4×10^2	36
NIH 3T3 (mouse)	8.8×10^6	1.2×10^7	0.7
208F (rat)	2.3×10^6	4.7×10^6	0.5

[a] Cells were seeded at 2×10^4/well (diameter, 3.5 cm) in multiwell dishes on day 1. On day 2, the cells were transduced with the retroviral vector LAPSN(10A1), and on day 4, the cells were stained for alkaline phosphatase-positive clusters (foci) of cells. Values are averages of duplicate dishes. The experiment was repeated twice with similar results.

[b] FFU, focus-forming units.

[c] One milliliter of the 2 ml of medium present during transduction was replaced with conditioned medium from CHO cells.

TABLE 2. Utilization of Ram-1 or Glvr-1 by a 10A1-pseudotyped retroviral vector for cell entry[a]

Receptor	Species	Vector titer (FFU/ml)[b]
Ram-1	Human	1×10^6
	Rat	3×10^5
Glvr-1	Human	6×10^5
	Mouse	7×10^5
None		<500

[a] Retrovirus receptor cDNAs were expressed by using the retroviral vector LXSN. CHO cells were seeded at 2×10^4/well (diameter, 3.5 cm) in multiwell dishes on day 1. On day 2, cells were cotransfected with 2.5 μg of β-galactosidase expression plasmid and 2.5 μg of the receptor expression construct. On day 3, one set of dishes was stained for β-galactosidase to assess transfection efficiency, while the other set was infected with 2 μl of the LAPSN vector pseudotyped with the 10A1 retrovirus in the presence of 50% medium conditioned by CHO cells. On day 4, cells were stained for alkaline phosphatase, and foci of stained cells were counted. Transfection efficiencies were similar for all constructs, as measured by β-galactosidase staining. Values are averages of duplicate dishes. The experiment was repeated twice with similar results.

[b] FFU, focus-forming units.

Mouse NIH 3T3 and rat 208F cells showed no decrease in transduction by the LAPSN(10A1) vector when the conditioned medium was included during infection (Table 1). This hamster cell-specific reduction in LAPSN(10A1) vector titer is analogous to that seen for amphotropic and GALV pseudotype vectors in CHO and other hamster cell lines (31, 32). Since amphotropic and GALV pseudotype vectors, but not xenotropic or ecotropic pseudotype vectors (31), are affected by the protein factor, this result is consistent with the hypothesis that the 10A1 virus can utilize Glvr-1 to enter CHO cells.

10A1 pseudotyped retroviral vectors can utilize Ram-1 or Glvr-1 for cell entry. The observations that LAPSN(10A1) transduction of CHO cells could be blocked by the addition of CHO cell-conditioned medium and that this inhibition was specific to CHO cells provided us with a system to analyze cloned receptor genes for the ability to serve as receptors for 10A1 retrovirus. Human Ram-1, rat Ram-1, human Glvr-1, and mouse Glvr-1 cDNAs were cloned downstream of the long terminal repeat promoter in the retroviral expression vector LXSN. Each construct was mixed 1:1 with a plasmid containing a β-galactosidase gene and was transfected into CHO cells. One set of dishes was stained for β-galactosidase 2 days after transfection, while a duplicate set was infected with the LAPSN(10A1) vector in the presence of CHO cell-conditioned medium and stained for the presence of alkaline phosphatase activity. Transfection efficiencies were similar for all constructs as determined by β-galactosidase expression. In agreement with previous interference studies, either human or rat Ram-1 could serve as receptors for a 10A1 pseudotype vector in CHO cells (Table 2). In addition, the human and mouse Glvr-1 expression constructs promoted entry of LAPSN(10A1) (Table 2). These results show that 10A1 virus can use either Ram-1 or Glvr-1 for entry into cells. It is interesting that 10A1 can use the mouse homolog of Glvr-1 that does not mediate entry of GALV into mouse cells. This is the first example of the mouse Glvr-1 receptor homolog functioning as a retrovirus receptor.

These data provide a molecular explanation for the mechanism of nonreciprocal interference. Since the 10A1 virus uses the amphotropic receptor, 10A1 interferes with superinfection by amphotropic viruses. However, in a cell initially infected with an amphotropic virus, the GALV receptor would still be available and sufficient for 10A1 virus entry.

Receptor domains that determine infectibility by GALV or amphotropic virus. The similarities between Glvr-1 and Ram-1 provided an opportunity to define the molecular determinants which permit entry of amphotropic virus and GALV. Using site-directed mutagenesis, we made two point mutations in the rat Ram-1 sequence. The first was made at bp 600 and creates an *Acc*I site without altering the amino acid sequence of the protein. The second mutation was made at bp 1476 to create a *Pst*I site while again leaving the native amino acid sequence unaltered. Glvr-1 already contains both of these restriction sites, allowing us to cut both genes into three sections and reconstruct hybrid molecules by using different combinations of these three sections (Fig. 1, left). These hybrid molecules were inserted downstream of the long terminal repeat promoter in the retroviral expression vector LXSN and cotransfected with a β-galactosidase expression plasmid into mouse NIH 3T3 cells for GALV receptor analysis (NIH 3T3 cells are resistant to GALV infection) or into CHO-K1 cells for amphotropic receptor analysis (CHO-K1 cells are resistant to amphotropic vector infection). One set of dishes was stained for β-galactosidase activity, while a duplicate set of dishes was infected with the LAPSN retrovirus vector pseudotyped with GALV envelope (produced by using PG13 packaging cells) or LAPSN pseudotyped with amphotropic envelope (produced by using PA317 packaging cells). Two days after vector infection, cells were stained for alkaline phosphatase activity.

Only hybrid receptor constructs containing the 3′ portion of Glvr-1 allowed infection by the GALV pseudotype vector in mouse NIH 3T3 cells (Fig. 1, constructs GGG, RGG, RRG, and GRG). This 3′ section contains the eight amino acids shown to be important for conversion of the inactive mouse Glvr-1 homolog to a molecule which functions as a GALV receptor (17, 43). While most of the 3′ end of Glvr-1 (amino acids 520 to 679) and Ram-1 (amino acids 493 to 656) is conserved between the two proteins (79% identity), these eight residues are highly divergent and most likely important for alteration of Ram-1 to function as a GALV receptor. Indeed, replacement of an 8-amino-acid region in Glvr-1 that includes this determinant with the corresponding region of Ram-1 (Fig. 1, construct GGrG) abolished its ability to promote entry of GALV.

Glvr-1 molecules containing the 5′ portion of Ram-1 or the 3′ portion of Ram-1 allowed infection by an amphotropic vector in CHO cells (Fig. 1, constructs GRR, GGR, RGG, and RRG). Glvr-1 constructs containing only the middle portion of Ram-1 were inactive as amphotropic retrovirus receptors (Fig. 1, construct GRG). Since a Glvr-1 molecule containing the 3′ section of Ram-1 can function as an amphotropic receptor, we

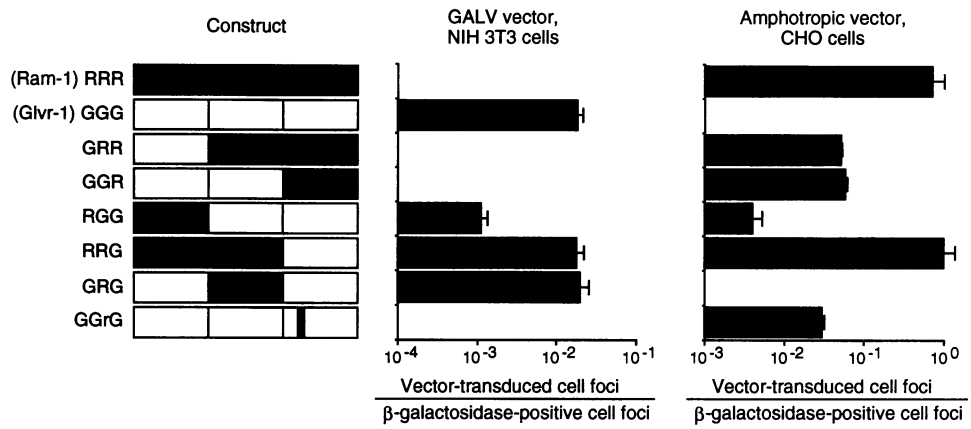

FIG. 1. GALV and amphotropic pseudotype vector transduction of cells expressing hybrid Ram-1/Glvr-1 receptors. CHO or NIH 3T3 cells were seeded in 3.5-cm-diameter dishes at 2×10^4 cells per dish. On day 2, hybrid constructs cloned in the retrovirus vector LXSN were cotransfected (1:1) with a plasmid encoding β-galactosidase (2.5 μg each). Parallel dishes were stained for β-galactosidase, while the other set was infected with the retrovirus vector LAPSN pseudotyped by an amphotropic envelope [LAPSN(PA317)] or GALV envelope [LAPSN(PG13)] at a multiplicity of infection of about 2. Cells were stained for alkaline phosphatase activity on day 5. Values are the number of vector-transduced (alkaline phosphatase-positive) foci divided by the number of β-galactosidase-positive foci. Results are the averages of duplicate dishes. The experiment was repeated three times with similar results.

tested the possibility that replacement of the nonconserved eight amino acid residues important in the GALV receptor with the corresponding region of Ram-1 would convert Glvr-1 to an amphotropic receptor. Indeed, this hybrid protein (Fig. 1, construct GGrG) allowed amphotropic virus entry but at levels 100-fold below wild-type levels. This result shows that amino acid residues 523 to 531 of Ram-1 (550 to 558 of Glvr-1) are important but not fully sufficient for conversion of a GALV receptor to an amphotropic virus receptor.

Certain Glvr-1/Ram-1 hybrid proteins function as receptors for both amphotropic and GALV pseudotype vectors. Interestingly, several hybrid constructs were able to serve as receptors for both GALV and amphotropic murine retroviruses. The best example of this is construct RRG, which functions as well as Glvr-1 does to mediate GALV pseudotype vector infection and better than Ram-1 for amphotropic vector infection. With the single exception of GRG, the common feature of such hybrids is that they contain a Glvr-1 sequence in the 3′ third of the hybrid molecules (Fig. 1, constructs RGG and RRG). This finding demonstrates that single molecules can serve as receptors for viruses that normally fall into separate interference groups.

GALV and amphotropic virus exhibit interference in cells expressing hybrid Glvr-1/Ram-1 proteins that function as receptors for both viruses. We tested whether cell lines expressing molecules which function as receptors for both GALV and amphotropic murine leukemia virus might reveal interference between GALV and amphotropic murine leukemia viruses. NIH 3T3 mouse cells are normally uninfectible by GALV, but expression of human Glvr-1 in NIH 3T3 cells makes them susceptible to transduction by GALV pseudotype vectors. We generated NIH 3T3 cells lines that expressed human Glvr-1 or the RRG hybrid (Fig. 1) by transduction of the cells with the retroviral vector LhGlvr1SN (the human Glvr-1 cDNA cloned into LXSN) or LRRGSN (RRG inserted into the LXSN vector) followed by selection of the cells in G418. Cells expressing LXSN without an insert were similarly generated. We assayed these cell lines for susceptibility to transduction by the LAPSN vector pseudotyped with either GALV envelope proteins [LAPSN(PG13)] or amphotropic virus envelope proteins [LAPSN(PA317)] (Fig. 2). Transduction events were scored by staining infected cells for alkaline phosphatase activity. NIH 3T3 cell lines expressing Glvr-1 or the hybrid receptor construct RRG were fully susceptible to transduction by the GALV pseudotype retrovirus vector (Fig. 2, bars 6 and 10), while cells expressing the empty vector were resistant (Fig. 2, bar 2). All three cell lines were fully susceptible to the LAPSN vector when packaged with an amphotropic envelope (Fig. 2, bars 1, 5, and 9). We next infected these cell lines with wild-type AM-MLV amphotropic virus

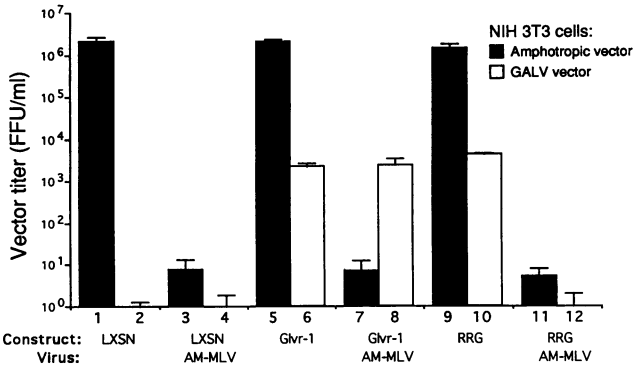

FIG. 2. Effect of AM-MLV infection on vector transduction of NIH 3T3 cells expressing the Glvr-1 or RRG retroviral receptor. NIH 3T3 cells stably expressing various receptor constructs cloned in the retrovirus vector LXSN and the same cells infected with the amphotropic murine leukemia virus (AM-MLV) were seeded in 3.5-cm-diameter dishes at 2×10^4 cells per dish. RRG is the Ram-1/Glvr-1 receptor hybrid in Fig. 1. The following day, the cells were infected with the LAPSN vector pseudotyped with amphotropic envelope [LAPSN(PA317)] or GALV envelope [LAPSN(PG13)] proteins. Medium was replaced with fresh medium the following day, and cells were stained for alkaline phosphatase 48 h after virus addition. Values are the averages of duplicate dishes. The experiment was repeated twice with similar results. FFU, focus-forming units.

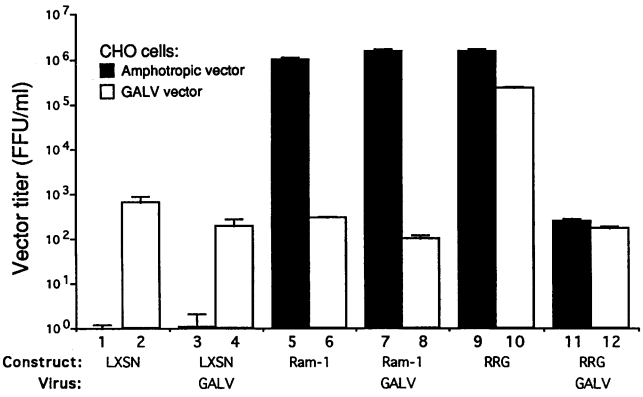

FIG. 3. Effect of GALV infection on vector transduction of CHO cells expressing the Ram-1 or RRG retroviral receptors. CHO cells stably expressing various receptor constructs cloned in the retrovirus vector LXSN and the same cells infected with GALV (SEATO strain) were seeded in 3.5-cm-diameter dishes at 2×10^4 cells per dish. RRG is the Ram-1/Glvr-1 receptor hybrid in Fig. 1. The following day, the cells were infected with the LAPSN vector pseudotyped by amphotropic envelope [LAPSN(PA317)] or GALV envelope [LAPSN(PG13)] proteins. Medium was replaced with fresh medium the following day, and cells were stained for alkaline phosphatase 48 h after virus addition. Values are the averages of duplicate dishes. The experiment was repeated twice with similar results. FFU, focus-forming units.

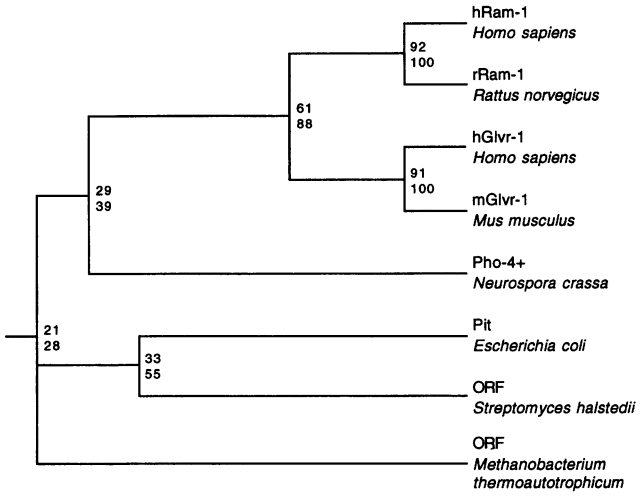

FIG. 4. Amino acid sequence similarities between phosphate transporters. The top number at each branch point gives the overall identity between sequences (averaged over all sequences following the branch point) with gaps to provide optimal alignment, while the bottom number gives the identity without gaps in a 40-amino-acid block identified by a multiple alignment program (MACAW; National Center for Biotechnology Information) as exhibiting the most significant uninterrupted similarity between the proteins. Distances in the figure are based on averages of the overall and 40-amino-acid-block identities.

and asked if this virus, which normally is classified in an interference group separate from GALV, could now interfere with GALV pseudotype vector transduction. The completeness of amphotropic retrovirus spread in the culture was demonstrated by the acquired resistance of these virus-infected cells to transduction by the amphotropic pseudotype LAPSN vector (Fig. 2, bars 3, 7, and 11). The GALV pseudotype LAPSN vector was able to efficiently transduce NIH 3T3 cells that expressed Glvr-1 and were productively infected with AM-MLV (Fig. 2, compare bars 6 and 8). However, cells expressing the RRG receptor hybrid show interference between the amphotropic murine leukemia virus and the GALV pseudotype LAPSN vector (Fig. 2, compare bars 10 and 12).

The reciprocal experiment was conducted in CHO cells that are normally resistant to amphotropic vector infection. The LXSN retrovirus vector was used to express Ram-1 (LRam1SN) or the hybrid RRG receptor (LRRGSN) in CHO cells by transduction of the cells with LRam1SN or LRRGSN followed by selection of the cells in G418. CHO cells expressing Ram-1 or the RRG construct could be transduced by the LAPSN vector having an amphotropic pseudotype (Fig. 3, bars 5 and 9), but cells expressing the empty vector could not (Fig. 3, bar 1). All three cell lines were transduced by the GALV pseudotype LAPSN vector (Fig. 3, bars 2, 6, and 10). The titer of the GALV pseudotype LAPSN vector was low in cells expressing only the endogenous hamster receptor (Fig. 3, bars 2 and 6) because of the factor secreted from CHO cells that binds the endogenous receptor and inhibits GALV pseudotype vector infection, while CHO cells expressing the RRG receptor were highly infectible, because the hamster factor does not block either human or rat Ram-1 and thus would not be expected to block this hybrid receptor. Prior infection of the three cell lines with GALV inhibited GALV pseudotype LAPSN transduction in all cases (Fig. 3, bars 4, 8, and 12), although the inhibition was small in cells expressing only the endogenous hamster GALV receptor (Fig. 3, bars 4 and 8). Reduced inhibition can be explained by the presence of the hamster factor that strongly inhibits GALV pseudotype vector infection even in cells not infected with GALV. GALV-infected CHO cells expressing Ram-1 were fully susceptible to amphotropic vector transduction, showing no interference between GALV and vectors pseudotyped with amphotropic envelope proteins (Fig. 3, compare bars 5 and 7). However, GALV-infected CHO cells expressing the hybrid receptor RRG were resistant to amphotropic vector transduction, documenting interference between GALV and the amphotropic LAPSN vector (Fig. 3, compare bars 9 and 11).

Additional related phosphate transporters. A search for proteins related to Ram-1 and Glvr-1 by using the BLAST network service at the National Center for Biotechnology Information revealed related molecules in several distant species. Similarity of Ram-1 and Glvr-1 to a putative phosphate transporter of *Neurospora crassa* (Pho-4^+ [24]) was found, as has previously been reported (16). In addition, Ram-1 and Glvr-1 show significant similarity to a recently sequenced proton-driven phosphate symporter in *Escherichia coli* (GenBank accession U00039) designated Pit (an acronym for P_i transport) (37) as well as to proteins encoded by partially sequenced open reading frames in *Streptomyces halstedii* (EMBL accession L05390) and the archaebacterium *Methanobacterium thermoautotrophicum* (EMBL accession X15364). Figure 4 shows the relationship between these proteins, for which we propose the family name Pit. Although the actual functions of the *Streptomyces* and *Methanobacterium* proteins are unknown, these results indicate that Pit family members are widespread and may perform similar functions in all organisms, including eubacteria, archaebacteria, and eukaryotes.

DISCUSSION

We have shown that nonreciprocal interference observed between amphotropic and 10A1 retroviruses is a consequence of the ability of 10A1 virus to use either the amphotropic virus receptor Ram-1 or the GALV receptor Glvr-1 for cell entry, while the amphotropic virus can use only Ram-1. No more than six amino acid changes in Env are required to expand the host range of an amphotropic virus to that of the 10A1 virus (35). That such minor changes can expand the range of receptors used by the virus to include Glvr-1 as well as Ram-1 is probably due to the similarity between these receptors. Likewise, other examples of nonreciprocal interference may reveal additional receptor similarities.

We have shown that relatively small changes in receptor sequence can generate single proteins that serve as receptors for viruses that normally belong to independent interference groups. Furthermore, expression of these modified receptors in cells results in cell lines which show interference between these viruses. These results provide an explanation for cell type-dependent interference, which can be due to receptor modifications that allow an expanded repertoire of envelope specificities. Other virus pairs that interfere as a function of cell type are likely to utilize related but distinct receptors in some cell types.

The gene products of Ram-1 and Glvr-1 are sodium-dependent phosphate symporters (20). The importance of these proteins is indicated by the presence of related proteins in organisms as distant as fungi and bacteria. Ram-1 and Glvr-1 show no significant similarity to several other sodium-dependent phosphate symporters (NaPi-1, -2, -3, and -4 and NPT1) cloned from mammalian kidney (7, 23, 42, 48). NaPi-2, -3, and -4 are homologous transporters from different species, while NaPi-1 and NPT1 are homologous transporters from rabbits and humans that are unrelated to the other group at the sequence level. Unlike the broad distribution of Ram-1 and Glvr-1 in animal tissues (20), the NaPi family of transporters are tissue specific and localize to the brush border of the renal proximal tubule (3, 23, 48).

We have defined a family of retroviruses that use Ram-1 and Glvr-1 for cell entry; this family includes amphotropic, GALV, FeLV-B, and 10A1 retroviruses. While Ram-1 and Glvr-1 have similar sequences and functions, these proteins are clearly encoded by different genes that localize to different chromosomes in mice and in humans (chromosomes 8 and 2, respectively) (12, 13, 18) and display very different patterns of tissue-specific expression (20). These differences may exert selective pressure to drive the evolution of viruses with altered receptor specificity in animals. Indeed, these pressures are likely responsible for the generation of the highly oncogenic 10A1 virus from the weakly oncogenic amphotropic virus in mice (38).

While results described here clarify the molecular bases for several anomalies associated with the interference grouping of retroviruses, these results will also complicate retrovirus classification. For example, viruses that use Ram-1 and/or Glvr-1 might all be grouped together or each could define its own group as a result of minor differences in receptor utilization. This situation could become much worse if viruses can be identified that use dissimilar receptors for cell entry, such as a virus that uses both amphotropic (Ram-1) and ecotropic (MCAT) retrovirus receptors. Future retrovirus classification schemes must account for these complexities.

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