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Cell-surface receptors for gibbon ape leukemia virus and amphotropic murine retrovirus are inducible sodium-dependent phosphate symporters

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ABSTRACT **Cell surface receptors for gibbon ape leukemia virus (Glvr-1) and murine amphotropic retrovirus (Ram-1) are distinct but related proteins having multiple membrane-spanning regions. Distant homology with a putative phosphate permease of *Neurospora crassa* suggested that these receptors might serve transport functions. By expression in *Xenopus laevis* oocytes and in mammalian cells, we have identified Glvr-1 and Ram-1 as sodium-dependent phosphate symporters. Two-electrode voltage-clamp analysis indicates net cation influx, suggesting that phosphate is transported with excess sodium ions. Phosphate uptake was reduced by >50% in mouse fibroblasts expressing amphotropic envelope glycoprotein, which binds to Ram-1, indicating that Ram-1 is a major phosphate transporter in these cells. RNA analysis shows wide but distinct tissue distributions, with Glvr-1 expression being highest in bone marrow and Ram-1 in heart. Overexpression of Ram-1 severely repressed Glvr-1 synthesis in fibroblasts, suggesting that transporter expression may be controlled by net phosphate accumulation. Accordingly, depletion of extracellular phosphate increased Ram-1 and Glvr-1 expression 3- to 5-fold. These results suggest simple methods to modulate retroviral receptor expression, with possible applications to human gene therapy.**

Key issues in virology have been identification of cell-surface virus receptors, determination of receptor expression patterns, and elucidation of the effects of infection on the normal functions of these molecules. These issues are critical in the case of retroviruses because cells can become chronically infected, leading to receptor down-modulation and subsequent derangements in cellular functions. For example, the CD4 receptor for human immunodeficiency virus (HIV) is important for helper T-cell function, and interference with this normal function is an important aspect of HIV-induced disease (1). Indeed, three genes of HIV (*env*, *nef*, and *vpu*) can each mediate CD4 down-modulation (1–4).

While several retroviral receptor cDNAs have been isolated (5–11), the only functionally characterized receptors are the above-mentioned CD4 for HIV and a sodium-independent cationic amino acid transporter for ecotropic murine leukemia viruses (MLVs) (12, 13). Among the cloned receptors with unknown functions are those that mediate infections of gibbon ape leukemia virus (GALV) and of mouse amphotropic retrovirus (these receptors are termed Glvr-1 and Ram-1, respectively) (7, 10, 11). Glvr-1 and Ram-1 are distinct proteins with multiple hydrophobic potential membrane-spanning sequences. Although they contain large unrelated central domains, they share about 60% overall sequence identity and have about 25% identity with a putative phosphate permease of *Neurospora crassa* (10, 11, 14, 15). These data suggested that Glvr-1 and Ram-1 might be transporters; however, their solute specificities could not be inferred because even closely related transport proteins often carry unrelated solutes (12, 13, 16, 17).

We identified the transporter activities of Glvr-1 and Ram-1 by a systematic approach that involved expression of these proteins in *Xenopus laevis* oocytes and measurement of transmembrane currents during exposure of the oocytes to different salts and nutrients. Our results indicate that Glvr-1 and Ram-1 are sodium/phosphate symporters and that these proteins provide a major pathway for phosphate uptake into many mammalian cells. In addition, we have shown that phosphate transport rates are affected both by retroviral infection and by changes in extracellular phosphate levels.

MATERIALS AND METHODS

Cell Lines and Retroviral Vectors. Cell lines used included 208F rat embryo fibroblasts (18), PA317 cells (19), and NIH 3T3 thymidine kinase-minus mouse embryo fibroblasts (20) and derivatives. Cells were grown in Dulbecco's minimal essential medium with 10% (vol/vol) fetal bovine serum. Retroviral vectors encoding neomycin phosphotransferase and rat Ram-1, human Glvr-1, the mouse ecotropic receptor Rec-1, or human clotting factor IX were made by cloning the respective cDNAs into the retroviral vector LXSN (21). Vectors were transfected into PA317 retrovirus packaging cells (19), and transiently produced virus was harvested after 2 days and was used to transduce 208F rat fibroblasts as described (21) except for the clotting factor IX virus, which was harvested from a previously described stable vector-producing cell line (22). Transduced cells were grown in the presence of G418 to select for cells expressing the vectors.

Ion-Transport Assays in *Xenopus* Oocytes Injected with Retrovirus Receptor RNAs. The coding regions of the rat Ram-1 (10) and human Glvr-1 (7) cDNAs were cloned into pGEM-7Z (Promega) with their 5′ ends adjacent to the SP6 promoter. For mRNA synthesis, the plasmids were linearized and transcribed with SP6 polymerase in the presence of $m^7G(5')ppp(5')G$ caps according to the manufacturer's directions (Pharmacia). *Xenopus laevis* oocytes were injected with 50 nl of mRNA (1 ng/nl) or with an equal volume of H_2O and were incubated for 4–6 days at 17°C; then two-microelectrode voltage-clamp recordings or radiolabel uptake assays were performed at room temperature as described (23).

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Abbreviations: GALV, gibbon ape leukemia virus; Glvr-1, cell surface receptor for GALV; Ram-1, cell surface receptor for amphotropic murine retrovirus; HIV, human immunodeficiency virus; MLV, murine leukemia virus; Mo-MLV, Moloney MLV.

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Voltage clamp recordings were made at −60 mV in recording solution containing 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM Hepes (pH 7.5). Sodium-free recordings and uptake assays were made by replacement of Na^+ with $Tris^+$. Uptake measurements at pH 8.5 were in solution buffered with 5 mM Hepes containing Tris. For phosphate-uptake assays, carrier-free $H_3{}^{32}PO_4$ (Amersham) was adjusted to 10 Ci (370 GBq) per ml by using unlabeled sodium phosphate (pH 7.5). Uptake of [^{32}P]phosphate was found to be linear for >2 hr. After 30–60 min of uptake, oocytes were washed three times, lysed with 1% SDS, and analyzed for radioactivity by liquid-scintillation spectroscopy. [^{32}P]Phosphate uptake rates are given in pmol/min per oocyte. The transport kinetic parameters were determined by least-squares fitting data points representing the average uptake from three or four oocytes to the Michaelis–Menten expression. K_m values expressed are the average of values determined in four separate experiments.

Phosphate Transport Assays in Mammalian Cells. Mammalian cells were seeded at 2–4 × 10^5 cells per 3.5-cm-diameter dish in medium with or without phosphate. Phosphate transport was measured 1 day later as follows. The culture medium was replaced with phosphate-free medium (Dulbecco's minimal essential medium without phosphate plus 5% dialyzed fetal bovine serum) supplemented with [^{32}P]phosphate at five concentrations from 10 μM to 1 mM. Each dish of cells was exposed to an equal concentration of [^{32}P]phosphate (9000 Ci/mmol of $H_3{}^{32}PO_4$ in water; NEN/DuPont) plus unlabeled phosphate (K_2HPO_4/KH_2PO_4, pH 7.5) to achieve the required total phosphate concentration. After 20 min of incubation at 37°C in a 5% CO_2/95% air atmosphere (medium pH = 7.5), the cells were washed twice with ice-cold phosphate-buffered saline and solubilized in 0.5% sodium dodecyl sulfate, after which portions were analyzed for radioactivity by scintillation spectroscopy and for protein by the fluoraldehyde protein assay (Pierce). Phosphate uptake was linear over 1 hr under these conditions.

RNA Preparation. Rat tissues were rapidly frozen after dissection, except for bone marrow and spleen from which single-cell suspensions were prepared. RNA was prepared from frozen tissues by the guanidine thiocyanate–phenol/chloroform extraction procedure (24) after tissue homogenization in guanidine thiocyanate by using a Polytron homogenizer (Brinkmann). RNA was prepared from spleen and bone marrow cells and from cultured cell lines by using a guanidine thiocyanate lysis/LiCl precipitation procedure (25). RNAs were stored in water at −70°C.

RESULTS

Ram-1 and Glvr-1 Transport Phosphate in Oocytes and in Mammalian Cells. To determine the normal functions of Glvr-1 and Ram-1, we prepared and injected capped synthetic mRNA into *Xenopus laevis* oocytes and screened for electrogenic transport activity by exposure of voltage-clamped oocytes to potential substrates. No specific currents were obtained with mixtures of vitamins, amino acids, or other common nutrients. However, an inward current was observed when oocytes injected with Glvr-1 mRNA were exposed to phosphate. This inward current was absent in oocytes injected with H_2O (Fig. 1*A*) or an irrelevant RNA encoding the human glutamate transporter (data not shown) and was abolished when extracellular sodium was replaced by Tris (Fig. 1*A*). Similar but smaller sodium-dependent phosphate currents were observed in oocytes injected with synthetic Ram-1 mRNA (data not shown).

Measurement of [^{32}P]phosphate uptake into oocytes expressing Ram-1 or Glvr-1 was used to examine the transporters' specificity, sodium requirements, pH dependence, and concentration dependence. Fig. 1*B* shows phosphate uptake into oocytes expressing Ram-1 or Glvr-1 in comparison with uptake into H_2O-injected oocytes. Uptake of phosphate mediated by both transporters was nearly abolished when extracellular sodium was replaced by Tris (Fig. 1*B*). The concentration dependence of phosphate uptake behaved in accord with Michaelis–Menten kinetics: for Glvr-1 the K_m was 24.1 ± 5.5 μM (n = 4), and for Ram-1 the K_m was 25.3 ± 6.0 μM (n = 4) (Fig. 1*C*). Uptake of 10 μM phosphate by

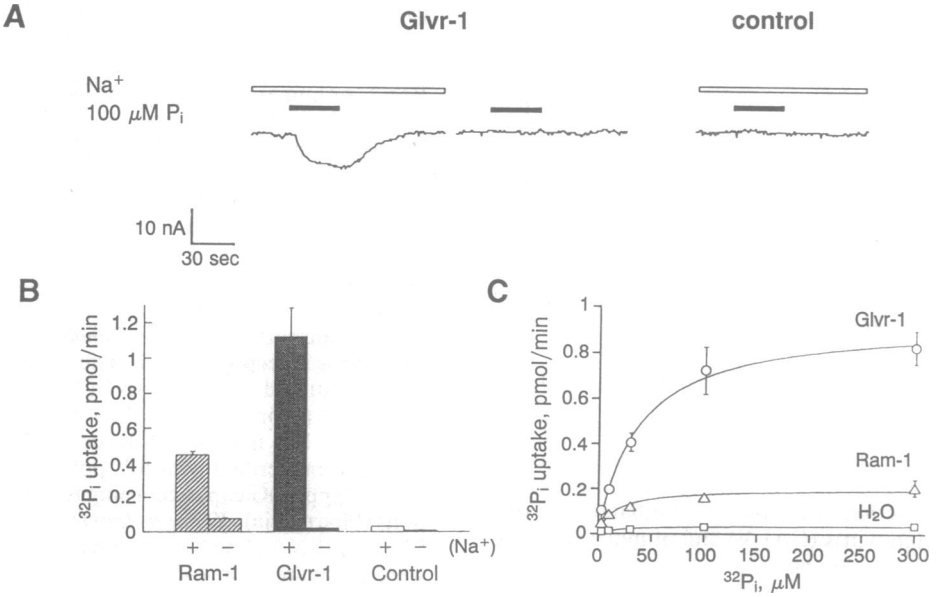

FIG. 1. Phosphate uptake induced by injection of Glvr-1 and Ram-1 mRNA into *Xenopus* oocytes. (*A*) Two-electrode voltage-clamp recording of oocytes injected with Glvr-1 mRNA (*Left*) or H_2O (*Right*). The inward current evoked in the cells expressing Glvr-1 by application of 100 μM phosphate (solid bar) is dependent on sodium (hollow bar). No phosphate-dependent currents were seen in water-injected oocytes (*Right*). Voltage-clamp recordings were performed with a holding potential of −60 mV. (*B*) Uptake of 100 μM [^{32}P]phosphate into *Xenopus* oocytes injected with Glvr-1 or Ram-1 mRNA. Oocytes were incubated in recording solution containing [^{32}P]phosphate for 60 min followed by lysis and scintillation spectroscopy. Uptake rates are given per oocyte. (*C*) Concentration-dependence of [^{32}P]phosphate uptake. Points (mean ± SEM, n = 4) were fitted by least squares to a function of the form $V = [V_{max}[P_i]/(K_m + [P_i])]$.

oocytes expressing Ram-1 or Glvr-1 was not significantly inhibited by a 10-fold excess of unlabeled sulfate [133 ± 24% and 81 ± 13% of control, respectively (n = 3)], demonstrating a specificity for phosphate over sulfate. Increasing pH inhibited uptake mediated by both Ram-1 and Glvr-1. At pH 8.5, the uptake of 10 μM phosphate was 41 ± 13% and 55 ± 4% of the respective uptake rates at pH 7.5 (n = 3).

We extended these results by using mammalian cells. Rat fibroblasts transduced with retroviral vectors encoding Ram-1 and Glvr-1 exhibited 3- to 5-fold higher rates of phosphate uptake compared with unmodified cells or cells transduced with vectors encoding other proteins (Fig. 2). K_m values for receptor-specific phosphate uptake were 200 μM (Ram-1) and 70 μM (Glvr-1) (n = 2), similar to previous values for mammalian cells (26, 27). Differences between these values and those obtained in oocytes could result from uncontrolled variables, such as membrane potential or post-translational receptor modifications.

Amphotropic Retrovirus Envelope Expression Inhibits Phosphate Transport. We examined phosphate transport in cell lines that expressed different retroviral Env proteins (Fig. 3). All cell lines were derived from the same NIH 3T3 thymidine kinase-minus cell parent, and all expressed Mo-MLV retrovirus Gag-Pol proteins. Phosphate transport was reduced by 50–60% in mouse fibroblasts that expressed amphotropic Env compared with cells that expressed no Env. Phosphate transport was unaffected in cells expressing the ecotropic Env protein, as expected because ecotropic retroviruses interact with a different cellular receptor that transports basic amino acids (12, 13). Little or no effect on phosphate transport was observed in cells expressing the GALV Env protein. The latter result was anticipated because GALV cannot infect mouse cells, presumably because of an inability to bind the murine Glvr-1 protein (30). These results suggest that Ram-1 is a major phosphate transporter in fibroblasts and that its function is specifically inhibited in infected cells by binding of the amphotropic envelope glycoprotein. This inhibition could be due to direct interference of the Env protein with phosphate transport or to effects on Ram-1 synthesis or degradation. Similar reductions in cell-surface receptor levels are caused by retroviral envelope glycoproteins in cells infected with HIV (2) or ecotropic mouse retroviruses (31).

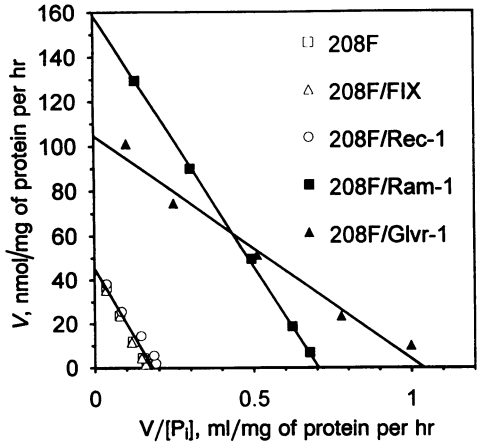

FIG. 2. Ram-1 and Glvr-1 cause increased phosphate transport in cultured 208F rat embryo fibroblasts. Retroviral vectors encoding neomycin phosphotransferase and rat Ram-1, human Glvr-1, mouse ecotropic receptor Rec-1, or human clotting factor IX (FIX) were expressed in rat 208F cells that were used for transport analysis. Eadie–Hofstee plots of phosphate uptake velocity (V) versus the velocity/phosphate concentration ratio ($V/[P_i]$) are shown. The Y axis regression line intercept gives the V_{max} for phosphate transport, and the slope is equal to $-K_m$.

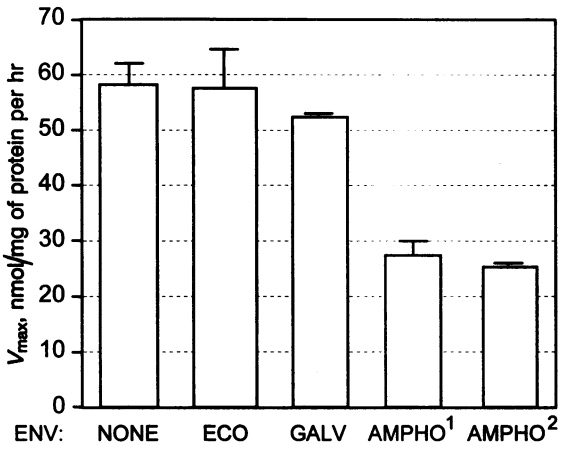

FIG. 3. Expression of amphotropic Env protein inhibits phosphate transport in NIH 3T3 mouse fibroblasts. Phosphate transport (V_{max}) was determined for NIH 3T3 cells expressing Moloney MLV (Mo-MLV) Gag-Pol proteins and the indicated Env glycoproteins. Cell lines used were GP91-22 cells expressing no Env (NIH 3T3 cells transfected with an Mo-MLV Gag-Pol expression plasmid) (28), NIH 3T3/Mo-MLV cells expressing ecotropic (ECO) env (NIH 3T3 cells transfected with the replication-competent Mo-MLV clone pMLV-K) (29), PG13 cells expressing GALV Env (GP91-22 cells transfected with a GALV Env expression plasmid) (28), NIH 3T3/AM-MLV cells expressing an amphotropic (AMPHO[1]) Env (NIH 3T3 cells transfected with hybrid Mo-MLV Gag-Pol/ampho 4070A Env replication-competent retrovirus plasmid pAM-MLV) (19), and PA317 cells expressing an amphotropic (AMPHO[2]) Env (NIH 3T3 cells transfected with a hybrid Mo-MLV Gag-Pol/ampho 4070A Env replication-incompetent expression plasmid) (19).

To provide direct evidence for binding of amphotropic Env protein to Ram-1, we measured binding of gp70 Env protein from amphotropic virus 4070A to CHO cells that express Ram-1. CHO cells are normally resistant to infection by amphotropic retroviruses or retroviral vectors but can be made susceptible by transfer of Ram-1 from a susceptible species (10, 11). Binding of gp70 Env protein was measured by incubation of cells with gp70 followed by antiserum to gp70 and by ^{125}I-labeled protein A as described (13, 32). Three independent CHO cell clones that were transduced with a retroviral vector that expresses rat Ram-1 showed binding of 8400 ± 2100, 8200 ± 600, and 6300 ± 400 cpm/mg of protein (n = 2) compared with 1030 ± 270 cpm/mg of protein (n = 4) for control CHO cells. These results indicate direct binding of amphotropic envelope protein to Ram-1.

Regulation of Phosphate Uptake in Cultured Cells. Phosphate transporter synthesis increases in response to reduced extracellular phosphate levels in bacteria (33) and fungi (14, 34), suggesting the possibility of similar regulation in mammalian cells. Indeed, exposure of 208F rat fibroblasts to phosphate-free medium for 24 hr resulted in a 3-fold increase in phosphate transport [V_{max} = 121 ± 9 without phosphate versus 39 ± 2 with phosphate (n = 2)], which was accompanied by a 3- to 5-fold increase in both Ram-1 and Glvr-1 RNA levels (Fig. 4 *Left*). Overexpression of Ram-1 in 208F cells following transduction with the LrRAMSN vector (Fig. 4 *Left Upper*) resulted in suppression of Glvr-1 RNA expression by 80% (Fig. 4 *Left Lower*). These results demonstrate both positive and negative regulation of Ram-1 and Glvr-1, presumably in response to intracellular phosphate levels.

Differential Expression of Ram-1 and Glvr-1 RNAs in Animal Tissues. Levels of virus receptor expression in different tissues influence the course of retroviral disease and are an important parameter for gene transduction studies, especially in the case of gene therapy. Ram-1 and Glvr-1 have broad but distinct tissue distributions (Fig. 4 *Right*). Expression of both Ram-1 and Glvr-1 RNAs was detected in all tissues except

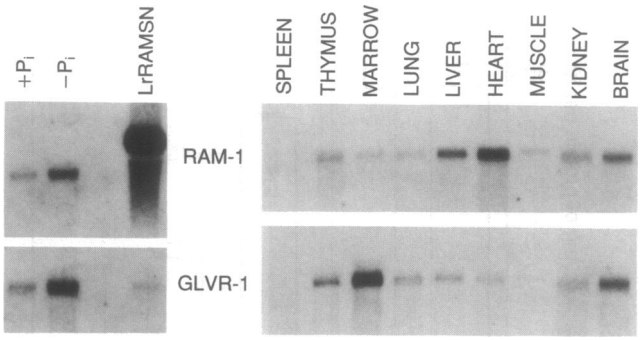

FIG. 4. Ram-1 and Glvr-1 RNA expression analysis. Probes for Ram-1 (*Hae* II to *Bam*HI, 560 bp) and Glvr-1 (*Nco* I to *Nco* I, 583 bp) were obtained from the nonhomologous central regions of the rat Ram-1 (10) and mouse Glvr-1 (15) cDNA clones. The observed sizes of the Ram-1 and Glvr-1 mRNAs are both about 4 kb by comparison to ethidium bromide-stained ribosomal RNAs (not shown), while the predicted and observed size of the LrRAMSN (retroviral vector encoding rat Ram-1) RNA is about 5.3 kb. (*Left*) RNA was prepared from 208F rat fibroblasts grown for 24 hr in medium with or without 1 mM phosphate or from 208F cells transduced with LrRAMSN and grown in the presence of 1 mM phosphate. Samples (10 μg) were analyzed by using probes for Ram-1 (*Upper*) and Glvr-1 (*Lower*). Ethidium bromide staining of rRNA confirmed equal sample loading. Radioactivity associated with each band was quantitated by phosphor imaging. (*Right*) Expression of Ram-1 and Glvr-1 RNA in rat tissues. RNA was isolated from the indicated rat tissues, and 20 μg samples were subjected to Northern analysis. The same blot was probed with Ram-1 (*Upper*), then stripped, and reprobed with Glvr-1 (*Lower*). Ethidium bromide staining of ribosomal RNAs revealed uniform RNA loading except for the muscle and spleen samples where the total rRNA levels were about one-third of those for the other lanes.

spleen. Differential expression was most evident in bone marrow, which contains little Ram-1 but abundant Glvr-1, and in heart, where the opposite was true.

DISCUSSION

We conclude that two retroviral receptors, Glvr-1 and Ram-1, are sodium-dependent phosphate transporters in a wide range of mammalian cells. Their roles in phosphate transport are suggested by expression studies in *Xenopus oocytes* and mammalian cells, by the specific inhibition of phosphate uptake that occurs in mouse fibroblasts after expression of amphotropic envelope glycoproteins, and by increases in Glvr-1 and Ram-1 expression that accompany removal of extracellular phosphate from cultured fibroblasts. Phosphate is a critical nutrient for cells and is required for nucleic acid and lipid synthesis, energy metabolism, signal transduction, and regulation of protein enzymatic activities.

An important feature of this study is the use of *Xenopus* oocytes in voltage-clamp conditions and the screening of potential transport solutes by measurements of currents. Under voltage-clamp conditions, exposure of oocytes expressing Glvr-1 or Ram-1 to phosphate resulted in inward currents, demonstrating that these transporters are electrogenic and mediate net cation influx. Removal of external sodium abolished the phosphate-dependent inward current, in accord with the observed sodium dependence for uptake of [^{32}P]phosphate (Fig. 1). These properties of Glvr-1 and Ram-1 indicate that they mediate the coupled influx of a stoichiometric excess of sodium relative to phosphate. This result is in agreement with the general finding that the electrochemical gradient of sodium is the major driving force for phosphate uptake across the plasma membrane of animal cells, while phosphate uptake in prokaryotes, plant cells, and mitochondria is driven by a proton gradient (26). Mammalian cell internal phosphate levels (75 milliequivalents per liter) are maintained at about 10-fold higher levels than external levels (4 milliequivalents per liter) in opposition to an electrochemical gradient that favors anion movement out of cells. Further studies will be required to determine whether the increased phosphate uptake at pH 7.5 relative to 8.5 is due to preferential uptake of a protonated form of phosphate or to direct effects on the transporter.

Sodium-dependent phosphate transporters (NaPi-1, -2, -3, and -4) have been cloned from rabbit, rat, human, and opposum kidney (35–37). NaPi-2, -3, and -4 are homologous transporters from different species, while NaPi-1 is unrelated to the others at the sequence level. These transporters are localized to proximal tubules of kidney cortex and are believed to mediate phosphate reuptake from glomerular filtrates at the polarized epithelial brush border membranes. Although these transporters display a substrate specificity and phosphate uptake affinity similar to Glvr-1 and Ram-1, they exhibit no sequence similarity. In addition, phosphate uptake mediated by the kidney transporters is increased at elevated pH (36), while uptake mediated by Glvr-1 or Ram-1 is decreased. Based on the relatively broad tissue distributions of Glvr-1 and Ram-1, we propose that these retroviral receptors are likely to be the major carriers of phosphate from interstitial fluids into most cell types, in contrast with the renal transporters that exhibit a discrete localization at the apical membranes of epithelial cells. The hypothesis that Glvr-1 and Ram-1 are major phosphate transporters is supported by the substantial down-modulation of phosphate uptake that occurs in mouse fibroblasts expressing amphotropic envelope glycoproteins and by the large coinductions of phosphate transport and of Glvr-1 and Ram-1 mRNA levels in cultured fibroblasts deprived of extracellular phosphate. Phosphate transport in perfused rat heart (38) is dependent on extracellular sodium and exhibits a K_m for phosphate of 240 μM, similar to the sodium requirement, and 200 μM K_m for phosphate uptake found here for Ram-1, supporting the hypothesis that Ram-1 is a major phosphate transporter in the heart.

The broad tissue distributions of Glvr-1 and Ram-1 are also consistent with their roles in mediating blood-borne infections of multiple tissues. For example, GALV has been isolated from brains and hematopoietic tissues of infected animals and has been associated with induction of leukemias in gibbon apes (39, 40). Feline leukemia virus subgroup B, which also depends on the Glvr-1 receptor for binding and entry into cells (41), is cytopathic for feline bone marrow cells *in vitro* and has been associated with lymphoma, sarcoma, and myeloproliferative diseases (42–44). It is interesting to speculate that infections might cause severe phosphate deprivations with pathological consequences, especially in cells that rely heavily on the transporter that is used by the specific virus. The pattern of Glvr-1 RNA expression that we find is consistent with a previous study that found high levels of Glvr-1 expression in brain and thymus (15), but in that study bone marrow was not examined.

These results also have important implications for gene therapy. While mouse hematopoietic stem cells can be efficiently transduced by using ecotropic retroviral vectors, these vectors do not infect human cells, and commonly used amphotropic vectors infect primate hematopoietic stem cells far less efficiently (45). Our findings that Ram-1 is poorly expressed in bone marrow while Glvr-1 expression is highest in this tissue suggest that GALV-based vectors may allow more efficient gene transfer into bone marrow hematopoietic cells, and preliminary data showing a 2- to 3-fold improvement in the transduction rate of human granulocyte/macrophage colony-forming cells support this conclusion (46).

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