The Amphotropic Murine Leukemia Virus Receptor Gene Encodes a 71-Kilodalton Protein That Is Induced by Phosphate Depletion

MEI-LING CHIEN, JOHN L. FOSTER, JANET L. DOUGLAS, AND J. VICTOR GARCIA*

Department of Virology and Molecular Biology, St. Jude Children's
Research Hospital, Memphis, Tennessee 38105

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The amphotropic murine leukemia virus (MuLV) can infect cells from a number of mammals, including humans, via its specific receptor. Basic knowledge of amphotropic MuLV receptor expression is likely to be useful in the development and improvement of gene therapy protocols based on amphotropic-pseudotyped vectors. To investigate the expression of the human receptor for the amphotropic MuLV (GLVR-2, newly termed *Pit2*), we determined its mRNA levels in several cell lines and found them to vary significantly. Induction of increased levels of mRNA after removal of phosphate from the media was observed in two osteosarcoma cell lines. The increase in GLVR-2 mRNA resulted in a concomitant rise in the levels of a 71-kDa protein specifically recognized by affinity-purified antibodies against GLVR-2. Using these antibodies, we were able to confirm the intracellular topology of the large hydrophilic domain between the proposed sixth and seventh transmembrane domains of the GLVR-2 protein. This assignment is in agreement with the fourth extracellular loop being outside the cell, consistent with the proposal that the fourth extracellular loop of GLVR-2 contains the envelope binding site.

The initial step of a retroviral infection is the binding of the envelope glycoprotein to a specific cell surface receptor. The expression of this receptor on any given cell surface often confers susceptibility to viral infection. Therefore, a major determinant of the tropism of a virus is the presence of its specific receptor. The amphotropic murine leukemia virus (MuLV) has a broad tropism to infect a variety of mammalian cells (24), which has made it an important tool in gene transfer technology. In addition, amphotropic MuLV's ability to infect human cells has made it particularly useful as a vector for gene therapy. Thus, characterization of the expression of the receptor for the amphotropic MuLV has the potential to improve these applications, since modulation of receptor expression may affect transduction efficiency.

The gene encoding the receptor for the amphotropic MuLV has previously been mapped to the pericentromeric region of human chromosome 8 (6). The cDNAs for the human and rat amphotropic MuLV receptors have been cloned and designated *Glvr-2* (newly termed *Pit-2*) and *Ram-1* (newly termed *RaPit-2*), respectively (5, 17, 23). These two genes encode proteins that are 92% identical (17, 23). The receptor for the gibbon ape leukemia virus (GALV), GLVR-1 (newly termed *Pit1*), is 62% identical to GLVR-2 (23). Regardless of the homology between the two proteins, GLVR-1 does not confer infectivity by the amphotropic MuLV and GLVR-2 does not confer infectivity by GALV (5). However, the receptor for amphotropic MuLV in Chinese hamster E36 cells functions as a receptor for both amphotropic MuLV and GALV (26). Analysis of hydropathy plots suggested that GLVR-1 and GLVR-2 have 10 transmembrane domains, five extracellular loops, and a single large hydrophilic intracellular domain (8, 18, 23). *Ram-1*, GLVR-1, and GLVR-2 are homologous to the phos-

phate permease of *Neurospora crassa*, and *Ram-1* and GLVR-1 were identified as sodium-dependent phosphate symporters (8, 10, 19). Interestingly, both *Ram-1* and GLVR-1 mRNA levels appear to be negatively regulated by inorganic phosphate (P_i) in the culture medium (10).

To investigate GLVR-2 expression and its regulation, we analyzed mRNA and protein levels in several cell lines in the presence or absence of P_i . The amount of GLVR-2 mRNA was found to vary significantly in different cell lines under normal P_i conditions. However, induction of mRNA after removal of P_i from the media was readily observed in two osteosarcoma cell lines. Using antibodies generated against GLVR-2, we were able to detect the expected 71-kDa protein. In addition, we show that the increase in GLVR-2 mRNA resulted in a concomitant rise in GLVR-2 protein expression. Furthermore, studies of GLVR-2 topology by immunofluorescence, flow cytometry, and trypsin digestion of live cells indicate that the large hydrophilic domain between the sixth and seventh transmembrane domains of GLVR-2 (against which our antibodies were raised) is inside the cell. This result is in agreement with the extracellular assignment of the fourth outer domain, which has been identified as the putative envelope binding site.

MATERIALS AND METHODS

Cell lines. Human osteosarcoma cell lines 143B (thymidine kinase-negative ATCC CRL 8303), HOS (ATCC CRL 1543), and 143B/GLVR-2 (see below), the human cervical carcinoma HeLa cell line (ATCC CCL 2), 293T cells, and NIH 3T3 cells were cultured in Dulbecco's modification of Eagle's medium (DMEM; Mediatech Cellgro; Fisher, Pittsburgh, Pa.) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, Utah), 50 IU of penicillin per ml, and 50 μ g of streptomycin (Mediatech Cellgro; Fisher) per ml. Cells were maintained at 37°C in a humidified incubator with 10% CO₂. The human monocytic cell line U937 was cultured in RPMI 1640 with 25 mM HEPES (Mediatech Cellgro; Fisher) supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 IU of penicillin per ml, and 50 μ g of streptomycin per ml. For induction experiments, P_i -free DMEM (ICN, Costa Mesa, Calif.) was supplemented with dialyzed FBS (GIBCO/BRL, Grand Island, N.Y.) and MEM nonessential amino acids (GIBCO/BRL) and P_i -free RPMI 1640 (ICN) was supplemented with dialyzed FBS. These media were supplemented with 1 mM (DMEM) or 5.6 mM (RPMI 1640) P_i when necessary.

* Corresponding author. Mailing address: Department of Virology & Molecular Biology, St. Jude Children's Research Hospital, 332 N. Lauderdale, Memphis, TN 38105. Phone: (901) 495-2611. Fax: (901) 523-2622. E-mail: victor.garcia@stjude.org.

Retrovirus-mediated gene transfer. pLGLVR-2SN was constructed by inserting a *HindIII*-*AflIII* fragment (containing the entire coding sequence of GLVR-2 cDNA) from pOJ74 (23) into the *EcoRI* site of pLXSN (15) with *EcoRI* linkers. PA317 amphotropic packaging cells were established as previously described (1). Virus-containing supernatant produced by PA317/LGLVR-2SN was used to infect 143B and NIH 3T3 cells in the presence of 4 µg of Polybrene (Sigma, St. Louis, Mo.) per ml as previously described (1). Pooled colonies were analyzed for GLVR-2 expression by Western blot analysis as described previously (1). Amphotropic-pseudotyped vectors encoding GLVR-1 were produced by transiently transfecting pLGrSHD (obtained from A. D. Miller) in conjunction with an amphotropic packaging construct into 293T cells (20). The resultant virus supernatant was used to transduce NIH 3T3 cells to obtain a pool of selected NIH 3T3/GLVR-1 cells. These cells were tested for susceptibility to infection by GALV vectors produced from PG13/LTatSN cells, which were obtained as described above for PA317/LGLVR-2SN except that PG13 cells (14) were used instead of PA317. This functional assay confirms expression of GLVR-1 in these cells.

Production and affinity purification of anti-GLVR-2 antibodies. The sequence encoding the large hydrophilic domain of GLVR-2, presumed to be intracellular, was synthesized by PCR with 5'-TCCCCCGGGTGGATGCGGAGGAAATAACAGG-3' and 5'-CCCAGATTCAAACCTCGGGTGCCTCTTCTCC-3' as 5' and 3' primers, respectively. The amplified DNA was cloned into pMalF (7) to obtain a fusion protein between the maltose binding protein (MBP) and this portion of the receptor. MBP-GLVR-2 fusion protein was purified as previously described (7) and used as an antigen to immunize rabbits. The *BamHI*-*EcoRI* fragment from pMalFGLVR-2 including the GLVR-2 sequences was cloned into pET28a (Novagen, Madison, Wis.) to obtain a six-histidine-tagged recombinant protein [(His)₆GLVR-2]. The His-tagged protein was expressed in BL21(DE3) pLysS bacteria and purified on a nickel column according to the manufacturer's (Novagen) instructions. Anti-GLVR-2 antibodies were affinity purified on a column of (His)₆GLVR-2 coupled to AffiGel-15 (Bio-Rad, Hercules, Calif.) and eluted with 100 mM glycine, pH 2.5.

Northern blot analysis. Total RNA was extracted from cells by acid-phenol-guanidinium thiocyanate extraction (3) with RNA STAT-60 according to the manufacturer's (Tel-Test, Friendswood, Tex.) instructions. Fifteen micrograms of total RNA from each sample was fractionated on a 1.5% agarose-2.2 M formaldehyde gel. RNA was blotted with a TurboBlotter (Schleicher & Schuell, Keene, N.H.) onto Nytran membranes and cross-linked with a UV transilluminator for 1 min (Chromato-vue model 0-63; Ultra-Violet Products, San Gabriel, Calif.). Blots were prehybridized at 60°C for 1 h in a solution containing 6× SSPE (1× SSPE is 150 mM NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA), 5× Denhardt's solution (1× Denhardt's solution is 0.02% [each] polyvinylpyrrolidone, Ficoll [type 400; Pharmacia], and bovine serum albumin [fraction V; Sigma]), 0.5% sodium dodecyl sulfate (SDS), and 100 µg of denatured salmon sperm DNA per ml. Hybridization to ³²P-labeled DNA probe was done at 60°C overnight in the same solution. Blots were rinsed briefly at room temperature in 0.1× SSPE-0.5% SDS and washed at 65°C in the same solution in a shaking water bath for 1 to 2 h with several changes. Blots were exposed to Kodak XAR-5 film with intensifying screens at -70°C. Radioactivity associated with each band was quantitated with a PhosphorImager (model 425F; Molecular Dynamics). Probes used were a 1.3-kb *HindIII*-*BglII* fragment from the coding sequence of GLVR-2 cDNA (pOJ74) (23) and a 0.5-kb *XbaI*-*HindIII* fragment of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (pSP64-GAPDH).

Western blot analysis. Cells were harvested in Dulbecco's phosphate-buffered saline (D-PBS; Mediatech Cello; Fisher) and lysed in lysis buffer (1% Nonidet P-40, 20 mM Tris [pH 8], 0.15 M NaCl, 2 mM EDTA, 1 µg of aprotinin per ml, 1 µg of leupeptin per ml, 1 mM phenylmethylsulfonyl fluoride) for 10 min on ice. The lysates were clarified and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) as previously described except that samples were not heated to 95°C unless otherwise indicated (1). Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes (Hybond C; Amersham, Arlington Heights, Ill.) with a semidry electrotransfer apparatus (Bio-Rad). Membranes were blocked with 5% nonfat dry milk in TBST (10 mM Tris [pH 8], 0.15 M NaCl, 0.05% Tween 20) for 1 h at room temperature or overnight at 4°C. Membranes were then incubated with affinity-purified GLVR-2 antibodies (1:250 in TBST with 5% milk) for 1 h at room temperature, washed 3 times with TBST, and incubated with anti-rabbit immunoglobulin G (IgG) horseradish peroxidase conjugate (1:10,000 in TBST with 5% milk; Promega, Madison, Wis.) for 1 h at room temperature. After three washes in TBST, proteins were detected by enhanced chemiluminescence (ECL) as described previously (4), with *p*-coumaric acid (Sigma) as an enhancer.

Trypsin digestion of GLVR-2 in live cells. Cultured 143B/GLVR-2 cells (2 × 10⁶ per 6-cm-diameter plate) were removed with EDTA (GIBCO/BRL), centrifuged, and resuspended in 200 µl of PBS. Twenty units of TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone)-trypsin (Worthington, Freehold, N.J.) was added to cells. Samples were incubated at room temperature for 4, 9, and 19 min. Digestions were stopped by addition of 1 mM phenylmethylsulfonyl fluoride, and cells were washed with 1 ml of PBS. Cells were lysed, and Western blot analysis was performed as described above.

Immunofluorescence assay. Cells (2 × 10⁴ per well) were plated on 10-well (diameter, 6 mm) Teflon-coated slides (Cel-Line Associates, Newfield, N.J.) and

incubated for 16 h in a CO₂ incubator. For extracellular staining (at room temperature), cells were fixed after incubation with the primary antibodies. For intracellular staining, cells were first fixed with formaldehyde (3.7%) for 20 min at room temperature and washed with D-PBS and then permeabilized with 0.3% Triton X-100 (Fisher) in D-PBS for 20 min at room temperature. Affinity-purified GLVR-2 antibodies (1:50) or an equal amount of affinity-purified rabbit anti-rat IgG (Jackson ImmunoResearch) diluted in D-PBS containing 0.5% normal goat serum (Sigma) was added to the cells, and samples were incubated at 37°C for 1 h. After being washed three times in D-PBS, samples were incubated with fluorescein isothiocyanate-labeled goat anti-rabbit IgG (Jackson ImmunoResearch) diluted 1:100 in D-PBS containing 0.5% normal goat serum. For staining of unfixed cells, D-PBS with MgCl₂ · 6H₂O (0.5 mM) and CaCl₂ · 2H₂O (0.88 mM) was used. As a positive control for extracellular staining, cells were incubated with an anti-human CD71 (transferrin receptor) monoclonal antibody (Becton Dickinson, San Jose, Calif.) followed by rabbit anti-mouse IgG (Jackson ImmunoResearch, Inc.) and fluorescein isothiocyanate-labeled goat anti-rabbit IgG. Images were obtained with a Leica DM IRBE laser scanning confocal microscope.

In vitro transcription and translation and immunoprecipitation. Full-length GLVR-2 cDNA was transcribed in vitro from pOJ74 with the mMessage mMachine kit (Ambion, Austin, Tex.). The transcript was translated in vitro with the Flexi rabbit reticulocyte lysate system (Promega) in the presence of [³⁵S]methionine-cysteine with or without canine pancreatic microsomal membranes (Promega). Immunoprecipitation was performed as described previously (12), and samples were examined by SDS-PAGE on a 7% gel. The gel was treated with 1 M sodium salicylate (Fisher) for 30 min at room temperature, dried, and exposed to Kodak XAR-5 film.

RESULTS

GLVR-2 mRNA expression and induction vary in different cell lines. The mRNA level of the rat amphotropic MuLV receptor Ram-1, a homolog of GLVR-2, increases threefold in 208F cells when cultured in P_i-free medium (10). We examined GLVR-2 mRNA expression in human cell lines in response to P_i depletion. Three cell lines were tested: HeLa (cervical carcinoma), U937 (monocytic), and 143B (osteosarcoma). All three cell lines exhibited different levels of GLVR-2 mRNA expression (Fig. 1A). Interestingly, only 143B cells showed a significant increase in GLVR-2 mRNA levels after P_i removal from the culture medium. This increase in GLVR-2 mRNA levels (threefold) was similar to that reported for the rat 208F cells (10). Because 143B cells are derived from an osteosarcoma, GLVR-2 induction was tested in a second human osteosarcoma cell line, HOS. HOS cells also showed an increase in levels of mRNA (1.9-fold) in response to P_i removal from the culture medium (Fig. 1B). These results indicate that the levels of GLVR-2 expression vary in different cells and that the induction of GLVR-2 by P_i depletion from the medium appears to be cell line dependent.

To determine the time course of induction of GLVR-2 mRNA in response to P_i depletion, cells were cultured in medium with or without P_i for 16, 24, and 48 h. GLVR-2 mRNA levels increased in a time-dependent manner in the absence of P_i (Fig. 2). In the presence of P_i there appeared to be a slight drop in levels of GLVR-2 mRNA when cells reached confluence (48 h) in the culture plates (Fig. 2). Cells were not cultured in the absence of P_i for more than 48 h, because after this time the cells had a significantly reduced growth rate.

Characterization of the GLVR-2 protein. Computer analysis of the GLVR-2 primary structure predicts that it is an integral membrane protein with 10 transmembrane domains (8, 18, 23). Based on this model, a presumed intracellular domain which is located between the sixth and seventh hydrophobic domains was used to produce an MBP and a His₆-tagged GLVR-2 fusion protein (see Materials and Methods). The purified MBP-GLVR-2 fusion protein was injected into rabbits to generate antisera. Subsequently, the (His)₆GLVR-2 protein was used to affinity purify the GLVR-2-specific antibodies. These affinity-purified GLVR-2 antibodies but not preimmune con-

FIG. 1. Northern analysis of GLVR-2 expression. Total RNA was isolated from 143B, HeLa, U937 (A), and HOS (B) cells cultured in media with 1 mM P_i (5.6 mM for U937) or without P_i . (A) Duplicate samples (15 μ g per lane) were loaded for lanes 1 to 8. The blot was hybridized with a GLVR-2 probe, and the same blot was stripped and reprobed with a GAPDH probe. The positions of the 28S and 18S rRNAs in the gel are indicated. The gel in panel B was probed as described for panel A. Radioactivity associated with each band was quantitated with a PhosphorImager (Molecular Dynamics). GLVR-2 induction in 143B cells was 3-fold, and in HOS cells it was 1.9-fold. All GLVR-2 mRNA values were normalized by the radioactivity of the GAPDH band in each lane.

trol serum recognized both the MBP-GLVR-2 fusion protein used as the immunogen and the $(\text{His})_6$ GLVR-2 fusion protein used for their purification (Fig. 3A, lanes 2 to 5). In addition, the GLVR-2 antibodies immunoprecipitated the product obtained from an in vitro transcription and translation reaction of a functional full-length cDNA clone of GLVR-2 (Fig. 3B). Because of its highly hydrophobic nature, GLVR-2 was translated in the presence of microsomes. Interestingly, the presence or absence of microsomes in the reaction did not seem to affect the synthesis of GLVR-2 (Fig. 3B, lanes 1 and 2), suggesting that GLVR-2 can be efficiently translated in the absence of membranes.

Western blot analysis of cell extracts incubated with loading buffer containing SDS and β -mercaptoethanol followed by treatment at 95°C failed to show the presence of a protein band with the molecular mass expected for GLVR-2. However, under those conditions, a band which barely entered the resolving gel was specifically recognized by the purified antibodies (Fig. 3A, lane 6). A protein band with the molecular mass predicted for GLVR-2 of approximately 71 kDa was seen when cell extracts were incubated with the same loading buffer at 25°C (Fig. 3A, lane 7). Thus, heating GLVR-2 in the presence of SDS results in aggregates that cannot enter the resolving gel. Similar results have been obtained with the E1 glycoprotein of the coronavirus (13, 22). The reason(s) for this apparent aggregation is not clear but is likely due to the highly hydrophobic nature of this protein. Therefore, heating or boiling should be avoided in the preparation of cell extracts for analysis of GLVR-2 protein.

Because the GLVR-2 protein is 62% identical to the

GLVR-1 protein (23), we determined if our purified antibodies to GLVR-2 cross-react with GLVR-1. NIH 3T3 cells were transduced with retroviral vectors encoding either GLVR-1 or GLVR-2. Lysates from these cells were analyzed by Western blot analysis with affinity-purified GLVR-2 antibodies. As shown in Fig. 3C, the affinity-purified GLVR-2 antibodies recognized only GLVR-2. These results indicate an absence of cross-reactivity between the large intracellular hydrophilic domain of GLVR-1 and the GLVR-2 antibodies. These results also indicate that our observations apply only to GLVR-2 and should not be extended to GLVR-1 without further experimentation.

GLVR-2 protein expression is induced by P_i depletion. To determine whether the increase in expression of GLVR-2 mRNA induced by P_i depletion results in an increase in levels of protein, 143B and HOS cells were cultured for 48 h in media with or without P_i . Cell extracts were then analyzed by Western blot analysis. GLVR-2 protein levels were induced upon P_i depletion in 143B cells as well as in HOS cells (Fig. 4). These results demonstrate that GLVR-2 protein expression is suppressed in the presence of P_i and that upon P_i depletion both mRNA and protein levels increase.

FIG. 2. Time course of induction of GLVR-2 mRNA in response to P_i depletion. (A) Northern blot analysis of RNA from 143B cells grown in media with or without 1 mM P_i for 16, 24, and 48 h, respectively. The blot was hybridized with a GLVR-2 probe, and the same blot was stripped and reprobed with a GAPDH probe. (B) Quantitation of GLVR-2 mRNA levels shown in panel A. Filled bars correspond to samples with P_i , and dotted bars correspond to samples without P_i . The y axis indicates GLVR-2 mRNA (PhosphorImager arbitrary units)/GAPDH mRNA (PhosphorImager arbitrary units) (10^4).

FIG. 3. Characterization of the GLVR-2 protein. (A) Purified recombinant MBP-GLVR-2 fusion protein (3 μ g, lane 2; 5 ng, lane 4), His-tagged GLVR-2 protein (10 μ g, lane 3; 5 ng, lane 5), and 143B/GLVR-2 cell lysates (lanes 6 and 7, 500 μ g per lane) were separated by SDS-PAGE on a 10% gel. Lanes 1 to 3, Coomassie brilliant blue-stained gel; lanes 4 to 7, Western blot analysis with

The large hydrophilic domain between the sixth and seventh transmembrane domains of GLVR-2 is inside the cell. Indirect immunofluorescence was used to study the cellular distribution of GLVR-2 (Fig. 5). Our results show GLVR-2 staining both on the plasma membrane and in the perinuclear regions. This pattern of staining is consistent with a transmembrane protein which enters the endoplasmic reticulum upon synthesis and is transported through the Golgi apparatus to the cell membrane (Fig. 5C). The use of the transduced cells was necessary, because the endogenous levels of GLVR-2 protein expression were barely detectable by indirect immunofluorescence (Fig. 5E).

The region of GLVR-2 used to generate the rabbit antisera has been postulated to be inside the cell (8, 18, 23). When cells were stained with the GLVR-2-specific antibodies and analyzed by confocal microscopy, only fixed and permeabilized cells showed specific signal, which was not seen in the nonpermeabilized cells (Fig. 5A and C). As a control, the same nonpermeabilized cells were also stained with antibodies specific for the transferrin receptor (CD71). As expected, intact cells showed CD71 only on their surfaces (Fig. 5B). These results indicated that the GLVR-2 epitopes are not accessible to the antibodies unless the membranes are permeabilized. Cells were also stained with purified GLVR-2-specific antibodies and analyzed for fluorescence by flow cytometry. Whereas no cell surface fluorescence was detected in cells stained with GLVR-2-specific antibodies, cells stained with antibodies specific for the transferrin receptor (CD71) showed normal levels of surface expression (data not shown). These results are consistent with the intracellular location proposed for this hydrophilic domain of the GLVR-2 protein.

To further test the model of the proposed topology of GLVR-2, live 143B/GLVR-2 cells were treated with trypsin for 4, 9, and 19 min and Western blot analysis was performed on lysates from these cells. Based on the primary structure of GLVR-2, if the proposed hydrophilic domain is accessible to trypsin, the largest tryptic peptide would be smaller than 10 kDa. However if the proposed topology is correct, that is, if the hydrophilic domain is inside the cell, a peptide of 37.5 kDa would be protected from trypsin digestion. Trypsin treatment resulted in a diffused pattern and a distinct 37.5-kDa protected fragment was observed, suggesting inaccessibility of this domain to trypsin. However, incubation of disrupted cells with trypsin at 37°C for 30 min resulted in complete degradation of Pit2 (data not shown). These results further suggest that the large hydrophilic domain of the GLVR-2 protein against which our antibodies were raised is intracellular.

affinity-purified anti-GLVR-2 antibodies. The sample loaded onto lane 7 was not heated at 95°C. Protein molecular mass (M) standards (Bio-Rad) were loaded in lane 1, and their masses (in kilodaltons) are indicated on the left. The molecular masses for MBP-GLVR-2, (His)₆GLVR-2, and GLVR-2 were determined to be 73, 40, and 71 kDa, respectively. These molecular masses correspond to the sizes expected for these proteins. (B) Immunoprecipitation of GLVR-2 in vitro translation products (arrow) with either anti-GLVR-2 antibodies (α) or anti-human CD4 antibodies as a negative control. pOJ74, a plasmid containing the GLVR-2 cDNA (21), was subjected to in vitro transcription. The transcript was then translated in vitro in the presence or absence of canine pancreatic microsomal membranes (indicated at the top of each lane). Prestained molecular mass markers (in kilodaltons; Bio-Rad) are indicated on the right. (C) Western blot analysis of lysates from NIH 3T3 cells expressing either GLVR-1 or GLVR-2. The blot was probed with affinity-purified GLVR-2 antibodies and developed by ECL. Only GLVR-2 was recognized by the affinity-purified antibodies. Prestained molecular mass markers (in kilodaltons) are indicated on the left. Equal amounts of protein were loaded onto each lane.

FIG. 4. GLVR-2 protein expression in response to P_i . Cell lysates from 143B and HOS cells grown in media with or without 1 mM P_i for 48 h were analyzed by SDS-PAGE on a 10% gel. Equal amounts of protein were loaded onto each lane, and the blot was probed with affinity-purified anti-GLVR-2 antibodies and developed by ECL. Prestained molecular mass standards (GIBCO/BRL) are indicated on the right in kilodaltons.

DISCUSSION

Retroviruses have been engineered to function as vectors in gene-transfer systems and are currently employed in human gene therapy protocols (16). Amphotropic pseudotypes are very important to these applications in that these vectors can infect cells from many mammals, including humans (24). Therefore, knowledge concerning amphotropic MuLV recep-

tor expression not only contributes to fundamental retrovirology but is also important to gene therapy applications. In this study, we show that GLVR-2 mRNA expression levels vary in different human cell types. Our results also show that induction of GLVR-2 mRNA by P_i depletion is cell line specific and not apparent in all cells. These data highlight the importance of determining the extent and time course of induction of GLVR-2 to optimize transduction protocols in different cell types. Of note, the 1.3-kb GLVR-2 cDNA probe used for Northern blotting contained regions of the gene conserved between GLVR-1 and GLVR-2. However, high-stringency hybridization conditions were used to avoid cross-hybridization between the GLVR-2 probe and GLVR-1 mRNA. As confirmation, another probe which comprises the least-conserved region between the two genes was also used in some experiments, and similar patterns of induction of GLVR-2 by P_i depletion were observed in 143B cells (data not shown).

We have identified a protein band of approximately 71 kDa (in agreement with the molecular mass predicted for GLVR-2 protein) on Western blots developed with affinity-purified GLVR-2 antibodies. GLVR-2 protein formed aggregates which do not enter the resolving gel when heated to 95°C in the presence of SDS and β -mercaptoethanol. The aggregation is probably due to the highly hydrophobic nature of this protein, as has been observed with the E1 glycoprotein of the coronavirus (13, 22). Using these anti-GLVR-2 antibodies, we were able to demonstrate that increased levels of mRNA induced by P_i depletion resulted in a concomitant rise in GLVR-2 protein

FIG. 5. Immunofluorescence staining of GLVR-2. (A to D) 143B/GLVR-2 cells; (E) 143B cells. The cells in panels A and B were not permeabilized and were stained with GLVR-2-specific or CD71-specific antibodies, respectively (surface staining). The cells in panels C to E were fixed and permeabilized prior to staining with GLVR-2-specific antibodies (C and E) or control rabbit anti-rat IgG (D) (surface and intracellular staining).

FIG. 6. Trypsin digestion pattern of GLVR-2 in live cells. Cell lysates from 143B/GLVR-2 cells treated with TPCK-trypsin (20 U) for 0, 4, 9, and 19 min (lanes 1 to 4, respectively) were analyzed by Western blot analysis. The blot was probed with affinity-purified GLVR-2 antibodies and developed by ECL. The position of the 37.5-kDa tryptic peptide is indicated by the lower arrow. Prestained molecular mass markers (in kilodaltons) are indicated on the left. Equal amounts of protein were loaded onto each lane.

expression. These results confirm that regulation of GLVR-2 expression by P_i depletion occurs prior to translation. Whether the regulation is at the transcriptional or posttranscriptional level remains to be determined.

The topologies of GLVR-2, GLVR-1, and *N. crassa* pho-4⁺ have been proposed to be similar and to contain 10 transmembrane domains (8, 18, 23). Using the antibodies made against the hydrophilic domain between the predicted sixth and seventh transmembrane segments of GLVR-2 protein, we obtained immunofluorescence, flow cytometry, and trypsin digestion data that indicate that this portion of GLVR-2 is intracellular. Other studies indicate that the predicted fourth extracellular region of GLVR-1 is necessary for GALV infection (9, 21, 25). In addition, Eiden et al. (5) have shown that the predicted fourth extracellular domain of GLVR-2, which is separated from the domain containing the epitopes recognized by our antibodies by a single hydrophobic putative membrane-spanning domain, is likely to contain the virus binding site. Thus, our results are consistent with the proposed topology which places the large hydrophilic domain inside the cell and the potential virus binding domain outside the cell.

Detection and characterization of the expression of the receptor for the amphotropic MuLV has mainly relied on a functional assay based on the susceptibility of cells to infection by the virus (5, 17, 23). Adenovirus-mediated transfer of Ram-1 increases amphotropic retroviral transduction in cultured cells, suggesting that increased expression of Ram-1 enhances transduction efficiency (11). Bunnell et al. (2) reported that transduction efficiency of primary human peripheral blood lymphocytes by amphotropic retroviral vectors increases when P_i is depleted from the medium. These observations suggest that GLVR-2 expression is induced in human peripheral lymphocytes upon P_i depletion and that increased receptor expression is likely to have accounted for the increased transduction efficiency. However, a direct observation of increased receptor expression was not demonstrated. Here we have shown that P_i depletion results in induction of both GLVR-2 mRNA and protein. These results are consistent with a model in which increased expression of the receptor results in more efficient gene transfer.

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