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Fungal Phosphate Transporter Serves as a Receptor Backbone for Gibbon Ape Leukemia Virus

LENE PEDERSEN,[1]* MARJA VAN ZEIJL,[2] STEPHEN V. JOHANN,[2] AND BRYAN O'HARA[2]

Department of Molecular and Structural Biology, University of Aarhus, DK-8000 Aarhus C, Denmark,[1] and Department of Molecular Biology, Wyeth-Ayerst Research, Pearl River, New York 10965[2]

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Pit1, the receptor for gibbon ape leukemia virus (GALV), is proposed to be an integral membrane protein with five extracellular loops. Chimeras made between Pit1 homologs differing in permissivity for infection and between Pit1 and the related protein Pit2 have shown that the fourth extracellular loop plays a critical role in infection. However, further elucidation of the roles of the extracellular loops in infection is hampered by the high level of sequence similarity among these proteins. The sodium-dependent phosphate transporter, Pho-4, from the filamentous fungus *Neurospora crassa* is distantly related to Pit1 and -2, showing an amino acid identity of only 35% to Pit1 in the putative extracellular loops. We show here that Pho-4 itself does not function as a receptor for GALV. Introduction of 12 Pit1-specific amino acid residues in the putative fourth extracellular loop of Pho-4 resulted in a functional GALV receptor. Therefore, the presence of a Pit1 loop 4-specific sequence is sufficient to confer receptor function for the mammalian retrovirus GALV on the fungal phosphate transporter Pho-4.

Infection of a cell by retroviruses is dependent on the presence of a specific receptor on the cell surface. The cellular receptor for gibbon ape leukemia virus (GALV) has been cloned from humans, hamsters, mice, and rats (3, 4, 13, 22, 23). In addition, the human, hamster, and rat cellular receptors for amphotropic murine leukemia virus (A-MLV) have been cloned (12, 20, 23). These highly related proteins are sodium-dependent phosphate symporters (5, 14, 24), and the human homologs are now designated Pit1 (GALV receptor) and Pit2 (A-MLV receptor). Pit1 also functions as a receptor for simian sarcoma-associated virus and feline leukemia virus subgroup B (FeLV-B) (18). A hydropathy plot of Pit1 predicts 10 transmembrane domains and five extracellular loops (3).

Sequences in the C-terminal part of the putative fourth extracellular loop of Pit1 were shown to be critical for receptor function for GALV and FeLV-B (4, 11, 15, 17). Furthermore, a stretch of 9 amino acids, referred to as region A, in the C-terminal part of the putative fourth extracellular loop of Pit1 (positions 550 through 558) was sufficient to confer receptor specificity for GALV, but not FeLV-B, on Pit2, when substituted for the corresponding sequence in Pit2 (15). Eiden and colleagues showed that replacement of a lysine with a glutamic acid in position 522 in the fourth extracellular loop of Pit2 was sufficient to allow the chimera to support infection by GALV (1). Moreover, the same group recently showed that a Pit2 chimera encoding the entire fourth extracellular domain and flanking regions of the *Mus musculus molossinus* Pit1 homolog also supported infection by GALV (16). These results demonstrate the importance of sequences in the fourth extracellular loop for infection by GALV. However, Pit1 and -2 exhibit 79% amino acid identity in their predicted extracellular loops, and therefore the possibility that GALV, as we have shown for FeLV-B (15), is dependent for infection on sequences other than those in loop 4, sequences which are present in both Pit1 and -2, cannot be excluded. In order to obtain further information on the role of Pit1 loop-4 sequences in GALV infection and to investigate the importance of sequences other than those in loop 4 of Pit1, we constructed chimeras between Pit1 and the distantly related protein Pho-4 from the filamentous fungus *Neurospora crassa* (3, 7). Pit1 and Pho-4 are 31% identical at the N terminus (Pit1 residues 1 to 261 and Pho-4 residues 1 to 250) and 38% identical at the C terminus (Pit1 residues 512 to 679 and Pho-4 residues 418 to 590); in both proteins, these regions are highly hydrophobic and are separated by a long hydrophilic region with a much lower level of homology (Pit1 residues 262 to 511 and Pho-4 residues 251 to 417) (3). Pit1 and Pho-4 have very similar hydropathy plots and are likely to have similar topology in the membrane (3). Moreover, it was recently shown by Versaw and Metzenberg that Pho-4, like Pit1 and -2, is a sodium-dependent phosphate transporter (21). In the putative extracellular loops, Pho-4 shows only 35% amino acid identity to Pit1, and Pho-4–Pit1 hybrids therefore offer the possibility of extending our analysis of what is important for GALV receptor function.

We show here that Pho-4 does not support infection by either GALV or A-MLV. However, introduction of the C-terminal part of Pit1 loop-4 sequences into Pho-4 was sufficient to make Pho-4 a functional GALV receptor. Thus, a sequence comprising 12 amino acids of Pit1 loop 4 is sufficient to confer receptor specificity for the mammalian retrovirus GALV on the distantly related protein Pho-4 from the fungus *N. crassa.*

MATERIALS AND METHODS

Construction of expression plasmids. pBJ005 (7) contains the open reading frame of Pho-4. An *Nco*I-*Dra*III fragment containing the open reading frame of Pho-4 was cloned into the *Sph*I site of pUC118. The two introns contained in Pho-4 were removed by using in vitro site-directed mutagenesis according to the method of Kunkel et al. (6), generating pOJ60. Pho-4 was then subcloned as a *Ban*II-*Sma*I fragment into the *Eco*RV site of pcDNA1A^{R}tkpA (20), and the plasmid was designated pOJ72. Pho-4 chimeras were constructed by replacing sequences in the five extracellular loops of Pho-4 with Pit1 sequences by site-directed mutagenesis (6). First, by using pOJ60 as a template, amino acids 450 through 463 of Pho-4 were replaced with amino acids 545 through 556 in loop 4 of Pit1, resulting in the plasmid pOJ73. pOJ73 was then used as a template to mutate loop 2 (replacing amino acids 117 through 149 of Pho-4 with amino acids 132 through 158 of Pit1), loop 3 (replacing amino acids 210 through 221 of Pho-4

* Corresponding author. Mailing address: Department of Molecular and Structural Biology, University of Aarhus, C. F. Møllers Allé, Bldg. 130, DK-8000 Aarhus C, Denmark. Phone: 45 8942 2702. Fax: 45 8619 6500. E-mail: LP@mbio.aau.dk.

with amino acids 219 through 229 of Pit1), or loop 5 (replacing amino acids 531 through 557 of Pho-4 with amino acids 624 through 649 of Pit1). The expression constructs were then made by subcloning an *Msc*I-*Pst*I fragment of each mutant into the *Msc*I and *Nsi*I sites of pOJ72. Designations for plasmids and chimeras were as follows: pOJ79, Pho/P1(4) (Pho-4 containing sequences from Pit1 loop 4); pOJ87, Pho/P1 (2+4); pOJ89, Pho/P1 (3+4); and pOJ91, Pho/P1 (4+5). pOJ60 was used as a template to mutate loop 1 by replacing amino acids 33 through 46 of Pho-4 with amino acids 48 through 60 of Pit1. A *Hin*dIII-*Msc*I fragment harboring the mutated sequence was cloned into the *Hin*dIII and *Msc*I sites of pOJ79, resulting in the expression plasmid pOJ94, encoding the chimera Pho/P1 (1+4). Construction of the pcDNA1A^{R}tkpA-derived expression plasmids pOJ74 and pOJ75, encoding Pit2 and Pit1, respectively, has been described elsewhere (15).

Cell culture. NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. CHO K1 (ATCC CCL-61) and D17 (ATCC CCL-183) cells were both grown in α-modified minimal essential medium supplemented with 10% fetal bovine serum. GALV (SEATO) and A-MLV (4070A) pseudotypes of the LacZ-encoding vector G1BgSvN (8) were derived from the producer cell lines PG13GBN (ATCC CRL-10686) (8, 10) and PA317GBN (ATCC CRL-9078) (8, 9), respectively. Both producer cell lines were grown in Dulbecco's modified Eagle's medium–10% calf serum.

Transient transfection and infection assay. NIH 3T3 or CHO K1 cells were seeded at 4×10^4 cells/60-mm-diameter dish. Next day, the cells were transfected by the calcium phosphate-DNA precipitation method (2). Each precipitate contained 10 μg of a CsCl-purified expression plasmid and 5 μg of CsCl-purified pUC19 plasmid as carrier in 1 ml. From each precipitate, aliquots of 200 μl, corresponding to 2 μg of expression plasmid, were added to 60-mm-diameter dishes with CHO K1 and/or NIH 3T3 cells. Three independent precipitates were made per construct to be tested. Forty-eight hours after transfection, the cells were challenged with 1.5 ml of filtered supernatant (filter pore size, 0.45 mm) from PG13GBN (NIH 3T3) or PA317GBN (CHO K1) in the presence of Polybrene (8 μg/ml). Four hours later, fresh media were added and the cells were incubated for another 2 days, at which time they were fixed in 0.05% glutaraldehyde and assayed for β-galactosidase activity with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as a substrate (19). Plates were examined under a light microscope for positively stained (blue) cells, and the number of blue cells per plate was counted.

Virus titer. The titers of the virus pseudotypes were determined on D17 cells for PG13GBN supernatant and on NIH 3T3 cells for PA317GBN supernatant, as described previously (15). Both pseudotypes gave titers of 10^5 CFU/ml.

In vitro transcription and translation. In vitro transcription and translation on expression plasmids encoding Pho-4 (pOJ72), Pit1 (pOJ75), Pit2 (pOJ74), the Pho/P1(4) chimera (pOJ79), the Pho/P1 (1+4) chimera (pOJ94), the Pho/P1 (2+4) chimera (pOJ87), the Pho/P1 (3+4) chimera (pOJ89), and the Pho/P1 (4+5) chimera (pOJ91) were carried out in the TNT Coupled Reticulocyte Lysate Systems (Promega) as described by the manufacturer.

RESULTS

A stretch of nine amino acids, referred to as region A, in the C-terminal part of loop 4 of Pit1 (positions 550 through 558) (Fig. 1 and 2) was shown previously to be sufficient to confer receptor function for GALV on Pit2, which does not support infection by GALV (15). Pit1 and -2 are 79% identical in their putative extracellular loops; therefore, the possibility that other sequences, present in both Pit1 and -2, are necessary for GALV receptor function could not be excluded. In order to extend our analysis of what is sufficient for GALV receptor function, we tested whether Pit1 sequences could confer GALV receptor function on the more distantly related protein Pho-4, which is predicted to have a similar topology in the membrane as Pit1 (3, 7, 21); Pho-4 and Pit1 show only 35% amino acid identity between their putative extracellular loops (3, 7).

Expression plasmids of Pho-4 and Pho-4–Pit1 chimeras were constructed and compared with Pit1 for their ability to allow GALV infection. Also, Pho-4 was tested for its ability to confer permissivity for A-MLV infection. To test whether Pit1 loop-4 sequences are sufficient to support infection by GALV in a Pho-4 backbone, we constructed the chimera Pho/P1(4), in which 14 amino acids in the C-terminal part of the fourth extracellular loop of Pho-4 were replaced with 12 amino acids representing the Pit1 loop-4 sequences least conserved between Pit1 and Pho-4 (positions 545 through 556) (Fig. 1). The chimeras Pho/P1 (1+4), Pho/P1 (2+4), Pho/P1 (3+4), and

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Loop 1

Pho-4 27-46     NDVANSWATSVAARSVTYLQ
Pit1  42-61     NDVANSFGTAVGSGVVTLKQ
Pit2  27-46     NDVANSFGTAVGSGVVTLRQ

Loop 2

Pho-4 107-149   RFGLPVSTTHSIMGGVIGMGIAAVGADGVQWVGSSINDGVVSV
Pit1  122-158   FLKLPISGTHCIVGATIGFSLVAKGQEGVKW------SELIKI
Pit2  107-143   FLRLPISGTHCIVGSTIGFSLVAIGTKGVQW------MELVKI

Loop 3

Pho-4 210-222   SYKVTLTNPEIAG
Pit1  219-230   PLLGFDKLP-LWG
Pit2  204-214   PVLGLV-LP-MWA

Loop 4

Pho-4 438-465   NDIANAIGPYATVFQLWKDGALPEKGKA
Pit1  533-558   NDVSNAIGPLVALYLVYDTGDVS--SKV
Pit2  505-530   NDVSNAIGPLVALWLIYKQGGVT--QEA

Loop 5

Pho-4 524-558   KLPVSTTQCITGATVGVGLCSGTWRTINWRLVAWI
Pit1  617-650   GLPISTTHCKVGSVVSVGWLRSK-KAVDWRLFRNI
Pit2  589-622   GLPVSTTHCKVGSVVAVGWIRSR-KAVDWRLFRNI
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FIG. 1. Sequence comparison between Pho-4, Pit1, and Pit2 putative extracellular loops. Numbers at the left of the sequences correspond to the positions of the first and last amino acids shown. Sequences exchanged between Pho-4 and Pit1 in the chimeras Pho/P1(4), Pho/P1 (1+4), Pho/P1 (2+4), Pho/P1 (3+4), and Pho/P1 (4+5), shown in Fig. 2, are underlined.

Pho/P1 (4+5) would address whether the presence of Pit1 sequences from one other extracellular loop in addition to loop 4 would affect receptor function for GALV. Again, the parts exchanged were those least conserved between Pit1 and Pho-4 (Fig. 1). The amino acids exchanged in the chimeras are indicated in Fig. 1.

The ability of these chimeras to confer permissivity for infection by GALV was investigated by transfecting plasmid DNAs encoding the chimeras into nonpermissive cells and assaying the susceptibility of these cells to infection with virus pseudotypes of LacZ-encoding vectors. The results obtained are shown in Table 1 and Fig. 2.

Pho-4 cannot function as a receptor for GALV or A-MLV. NIH 3T3 cells transfected with empty vector alone or Pho-4-encoding plasmid (pOJ72) showed no infection with GALV in the experiment shown in Table 1. In the experiment shown in Fig. 2, there was a background infection with GALV at a level

TABLE 1. Permissivity for infection in cells expressing Pit1, Pit2, or Pho-4[a]

Plasmid	Protein	Permissivity (%)[b] for infection with:	
		GALV	A-MLV
pOJ75	Pit1	100 ± 28	1.5 ± 0.3
pOJ74	Pit2	<0.002[c]	100 ± 10
pOJ72	Pho-4	<0.002	<0.0008
pcDNA1A^{R}tkpA	NA[d]	<0.002	<0.0008

[a] NIH 3T3 or CHO K1 cells were transfected with DNA of the indicated constructs and were challenged, respectively, with GALV or A-MLV pseudotypes of LacZ-encoding vectors as described in Materials and Methods.

[b] Data are averages of three independent transfections; however, the different virus pseudotypes were tested on the same three precipitates of a given construct. Data shown are standardized numbers ± standard deviations of the actual means. The number of blue cells per 60-mm-diameter dish transfected with the wild-type receptor for a given virus was assigned a value of 100% (16,000 and 44,000 blue cells per dish for GALV and A-MLV, respectively).

[c] Based on one blue cell per three 60-mm-diameter dishes.

[d] NA, not applicable (empty vector).

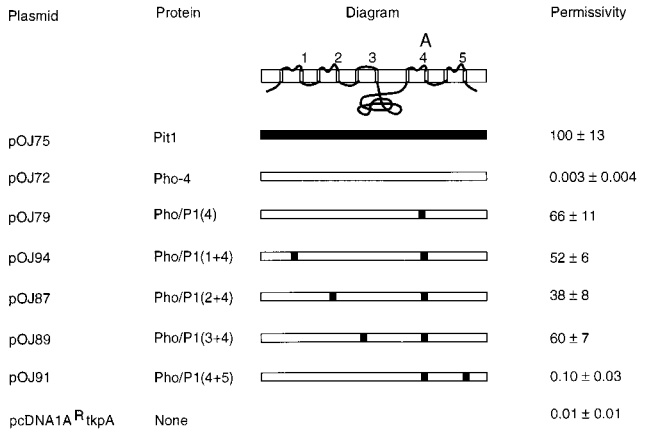

FIG. 2. Permissivity for infection by GALV in NIH 3T3 cells transiently expressing Pit1, Pho-4, or chimeras. On top is shown the predicted topology in the membrane of Pit1 and Pho-4 (3); the five putative extracellular loops are numbered 1 through 5. The position of region A (4, 15) is indicated. Pit1 is represented by a black bar, and Pho-4 is represented by a white bar. Black squares in the individual chimeras indicate the loops in which the Pho-4 sequence is replaced with the corresponding Pit1 sequence. In all chimeras, 14 amino acids in Pho-4 loop 4 are replaced with 12 Pit1-specific amino acids. In addition, in Pho/P1(1+4), 13 Pho-4 amino acids in loop 1 are replaced with 13 Pit1 amino acids; in Pho/P1 (2+4), 33 Pho-4 amino acids in loop 2 are replaced with 26 Pit1 amino acids; in Pho/P1 (3+4), 12 Pho-4 amino acids in loop 3 are replaced with 11 Pit1 amino acids; and in Pho/P1 (4+5), 27 Pho-4 amino acids in loop 5 are replaced with 26 Pit1 amino acids. pcDNA1A^{R}tkpA is the empty vector. Data are averages of three independent transfections and are shown as standardized numbers ± the standard deviations of the actual means. The number of blue cells (16,000) per 60-mm-diameter dish transfected with Pit1 was assigned a value of 100%. The experiment was repeated two times.

0.01% of that obtained with Pit1-encoding plasmid; however, the level of infection observed in cells transfected with Pho-4-encoding plasmid (pOJ72) was less than the background level. Both experiments supported the conclusion that Pho-4 did not allow infection with GALV. Moreover, Pho-4 did not support infection with A-MLV, in that no blue cells were observed in either mock- or pOJ72-transfected CHO K1 cells challenged with A-MLV pseudotype virus (Table 1). As previously observed (15), we find that Pit1 supports infection with A-MLV, albeit with low efficiency; Pit1-expressing cells challenged with A-MLV supported infection at a level 1.5% of that obtained in cells expressing Pit2 (Table 1).

Pit1 loop 4 sequences are sufficient to allow Pho-4 to function as a receptor for GALV. Replacement of 14 amino acids in the C-terminal part of the putative fourth extracellular loop of Pho-4 with 12 Pit1 loop-4-specific amino acids [chimera Pho/P1(4)] was sufficient to confer receptor specificity for GALV on Pho-4. This chimera supported infection by GALV at a level 66% of the infection level achieved with Pit1. Pho-4 chimeras harboring parts of Pit1 loop 1, 2, 3, or 5 did not support GALV infection (data not shown). Introduction of Pit1 sequences from extracellular loop 1, 2, or 3 in addition to loop 4 had no major influence on receptor function for GALV in that chimeras Pho/P1 (1+4), Pho/P1 (2+4), and Pho/P1 (3+4) gave infectibilities comparable to that of chimera Pho/P1(4). Introduction of Pit1 sequences in the putative extracellular loop 5 of Pho-4 in addition to loop 4, chimeras Pho/P1 (4+5), reduced the receptor function of the chimera in NIH 3T3 cells to only 10 times above the background level. In *Mus dunni* tail fibroblast (MDTF) cells, chimera Pho/P1 (4+5) supports infection by GALV at 0.6% of the level obtained with Pit1 (data not shown). In vitro transcription-translation products of the chimera Pho/P1 (4+5) and those of the chimeras Pho/P1(4), Pho/P1 (1+4), Pho/P1 (2+4), and Pho/P1 (3+4), proteins supporting high levels of GALV infection, comigrated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 3), indicating that Pho/P1 (4+5) does encode full-length protein.

DISCUSSION

Pit1 and Pit2 are the human receptors for GALV and A-MLV, respectively (13, 20), and show 62% amino acid identity overall (20). Pit1 is predicted to have 10 transmembrane domains and five extracellular loops (3). Introduction of 9 amino acids from the C-terminal part of Pit1 loop 4 (positions 550 through 558) into the corresponding site in Pit2 loop 4 allows the chimeric protein to support infection by GALV (15). Moreover, a single substitution of glutamic acid for lysine in position 522 in Pit2 loop 4 creates a functional GALV receptor (1). Pit1 and -2 show 79% amino acid identity in their putative extracellular loops, and therefore the possibility that sequences other than those in the C-terminal part of loop 4 are also critical for infection by GALV, but could not be identified in the above-mentioned chimeras due to their presence in both proteins, cannot be excluded. It was therefore necessary to investigate whether C-terminal sequences from Pit1 loop 4 were sufficient to confer receptor specificity for GALV on a more distantly related protein. The sodium-dependent phosphate transporter Pho-4 from the filamentous fungus *N. crassa* was predicted to have a similar topology in the membrane as Pit1 (3). Pho-4 shows about 25% overall amino acid identity with Pit1 and -2 and an amino acid identity of only 35% with Pit1 in the putative extracellular loops (3, 21). In this work, chimeras were made in which sequences in Pho-4 were replaced with Pit1 sequences and tested for their ability to support infection by GALV, in order to extend our analysis of what is important for GALV receptor function.

Pho-4 was not found to support infection with either A-MLV or GALV (Table 1; Fig. 2). However, replacement of 14 amino acids in the putative fourth extracellular loop of Pho-4 with 12 amino acids from Pit1 loop 4 (positions 545 through 556) [Pho/P1(4)] resulted in a functional GALV receptor (Fig. 2). Therefore, sequences in the putative fourth extracellular loop of Pit1 are sufficient to confer receptor specificity for GALV on the distantly related protein Pho-4. Introduction of

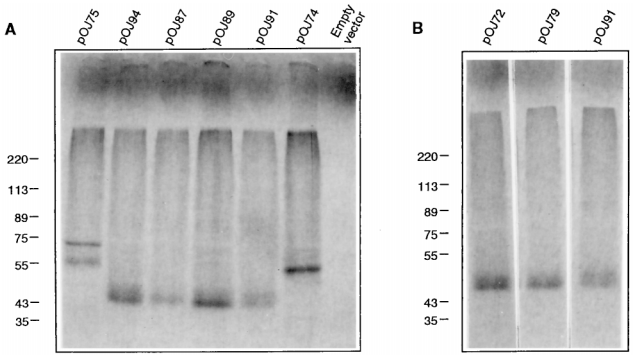

FIG. 3. Comparative analysis of in vitro-translated Pho-4–Pit1 chimeras by SDS-PAGE. The plasmid DNAs used in the individual assays are given above each lane. The numbers at the left of each panel represent the positions of molecular size standards (Rainbow marker; Amersham) in kilodaltons. (A) In vitro translation products encoded by pOJ75 (Pit1), pOJ94 [Pho/P1(1+4)], pOJ87 [Pho/P1 (2+4)], pOJ89 [Pho/P1 (3+4)], pOJ91 [Pho/P1 (4+5)], and pOJ74 (Pit2). As a control, a reaction with empty vector was performed. (B) In vitro translation products encoded by pOJ72 (Pho-4), pOJ79 [Pho/P1 (4)], and pOJ91 [Pho/P1 (4+5)].

Pit1 sequences in loop 1, 2, or 3 of Pho-4 in addition to those in loop 4 did not alter the receptor function for GALV. However, introduction of Pit1 loop-5 sequences into Pho-4 in addition to loop-4 sequences decreased the receptor function dramatically. SDS-PAGE of in vitro translation products of the chimeras showed no differences in SDS-PAGE migration between chimera Pho/P1 (4+5) and chimeras which supported a high level of infection by GALV (Fig. 3). Moreover, the low level of infection with GALV supported by chimera Pho/P1 (4+5) in NIH 3T3 (Fig. 2) and MDTF cells indicates that at least some of the chimeric proteins reach the cell surface and can function as GALV receptors. At present, we do not know whether the low infection level supported by this chimera is due to, e.g., incorrect folding in the membrane of the protein or whether the presence of Pit1 loop-5 sequences in this chimera interferes with the recognition by GALV of other sequences, necessary for its infection, in the chimeric protein. In summary, sequences in the putative fourth extracellular loop of Pit1 are sufficient to confer receptor specificity for GALV upon the distantly related protein Pho-4 from the fungus *N. crassa.*

It is possible that in addition to loop 4, other structures of the proteins, conserved between Pit1 and Pho-4, also play a role in the receptor function for GALV. These structures would also be expected to be present in the Pit2 homologs naturally supporting GALV infection, such as Pit2 from hamsters (23), and in a mutant Pit2 where a single amino acid substitution of glutamic acid for lysine at position 522 in the fourth extracellular domain is sufficient to render Pit2 functional as a GALV receptor (1). All sequenced Pit1 and -2 homologs show nearly complete amino acid conservation in the N-terminal parts of the putative first, second, fourth, and fifth extracellular loops (3, 12, 13, 20, 22, 23). It is interesting that although Pho-4 only shows an approximately 25% overall amino acid identity to Pit1, there is complete amino acid identity between Pho-4 and Pit1 in the 6 most N-terminal amino acids of loop 1 (Fig. 1). In the 10, 9, and 7 most N-terminal amino acids of loops 2, 4, and 5, Pit1 and Pho-4 show 50, 78, and 71% amino acid identity, respectively (Fig. 1). These highly conserved amino acids in the family of Pit proteins can be speculated to play a common role in the receptor functions of these proteins for the retroviruses for which they function as receptors, e.g., in the process of fusion.

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