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Identification and characterization of a widely expressed phosphate transporter/retrovirus receptor family

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Identification and characterization of a widely expressed phosphate transporter/retrovirus receptor family. The cell-surface receptors for gibbon ape leukemia virus (Glvr-1; [1]) and rat amphotropic virus (Ram-1; [2]) were recently demonstrated to serve normal cellular functions as sodium-dependent phosphate transporters [3, 4]. These transporters, called PiT-1 and PiT-2, respectively, are approximately 59% identical in amino acid sequence and are members of a gene family distinct from the renal type I and type II NaP_i sodium-dependent phosphate transporters. Both PiT-1 and PiT-2 are widely distributed in many tissues including kidney, brain, heart, liver, muscle, and bone marrow. Expression of both transporters is increased by phosphate deprivation. The distinct structural and functional properties of these molecules establishes them as members of a new family of phosphate transporters which may play a major role in phosphate uptake in a wide variety of cell types.

Retroviruses utilize distinct cell-surface receptors for cellular recognition and infection [reviewed in 5]. The specificity of the interaction between these cell surface receptors and the retrovirus envelope glycoproteins determines the host range of the virus. In recent years a number of receptors have been cloned based on their ability to confer susceptibility to viral infection when tranfected into normally resistant cells [1, 2, 6–10]. In some cases the normal cellular functions of the viral receptors were previously known or have been since determined. For example, CD4, the T-cell surface receptor for HIV, is an integral membrane protein involved in antigen complex recognition [6]. Elucidation of the cellular functions of several recently identified viral receptors has revealed a viral strategy which involves utilizing membrane transport proteins as surface recognition molecules. For example, the membrane receptor for ecotropic murine leukemia retrovirus was shown to be a cationic amino acid transporter [11, 12]. More recently, receptors for gibbon ape leukemia virus (Glvr-1; [1]) and amphotropic murine retrovirus (Ram-1; [2]) were demonstrated to serve normal cellular functions as sodium-dependent phosphate transporters in many tissues [3]. In this role they will be referred to as PiT-1 and PiT-2, respectively. These two molecules are approximately 60% identical in amino acid sequence, and they exhibit no significant overall sequence homology with the type I or type II renal sodium-dependent phosphate transporters [13]. PiT-1 and PiT-2 are thus the first identified members of the type III phosphate transporter gene family. Here we will briefly review the structure, distribution, electrophysiology, retroviral receptor functions, and regulation of these transporters.

Molecular structure

The Pit-1 (Glvr-1; Genbank L20859) and PiT-2 (Ram-1; Genbank L19931) sequences share weak homology (approximately 30% identity) with Pho-4^+, a gene implicated in phosphate uptake in *Neurospora crassa* [14]. PiT-1 and PiT-2 are also distantly related to partially characterized genes from *Eschericia coli*, *Streptomyces halstedii*, and *Methanobacterium thermoautotrophicum* [15]. The PiT-1 and PiT-2 sequences share less than 20% identity with the mammalian type I and type II NaPi renal transporter sequences [16, 17]. The human PiT-1 and rat PiT-2 share 59% sequence identity (Fig. 1). The human homologue of PiT-2 [10] is 61% identical with human PiT-1. Thus, PiT-1 and PiT-2 are members of an ancient phosphate transporter gene family. More careful analysis will be required to establish whether any sub-domain sequence similarities exist between the type I and type II renal transporters and the PiT family which might suggest a common evolutionary origin.

The predicted amino acid sequences encoded by the PiT cDNAs are characteristic of integral membrane proteins with multiple potential transmembrane domains. Hydropathy analysis suggests the presence of at least 10 potential transmembrane-spanning sequences, with a large hydrophilic domain near the center of each molecule (Fig. 2). This predicted topological profile differs from that of the renal transporters, which are modeled to contain between six and eight transmembrane domains [16, 17]. This central hydrophilic domain contains several consensus phosphorylation sites and may be located intracellularly (see below).

Tissue distribution and chromosome location

In contrast to type I and type II Na-dependent phosphate transporters which are expressed almost exclusively in kidney, both PiT-1 and PiT-2 are widely distributed. Using probes derived from the non-homologous central hydrophilic domains, Northern blotting detected both transcripts in many rat tissues [3]. The size of each mRNA is approximately 4 kb, and each displays a distinct but overlapping tissue distribution. Both transcripts were found in relatively high abundance in total mRNA prepared from kidney, although the regional localization in this tissue has not yet been determined. High levels of expression are also seen in tissues outside the kidney, including liver, lung, striated muscle, heart,

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MATLITSTTAATAASGPLVDYLWMLILGFIIAFVLAFSVGANDVANSFGTAVGSGVVTLKQ 61
                  MAMDGYLWMVILGFIIAFILAFSVGANDVANSFGTAVGSGVVTLRQ 46

ACILASIFETVGSVLLGAKVSETIRKGLIDVEMYNSTQGLLMAGSVSAMFGSAVWQLVASF 122
ACILASIFETTGSVLLGAKVGETIRKGIIDVNLYNETVETLMAGEVSAMVGSAVWQLIASF 107

LKLPISGTHCIVGATIGFSLVAKGQEGVKWSELIKIVMSWFVSPLLSGIMSGILFFLVRAF 183
LRLPISGTHCIVGSTIGFSLVAIGPKGVQWMELVKIVASWFISPLLSGFMSGVLFILIRMF 168

ILHKADPVPNGLRALPVFYACTVGINLFSIMYTGAPLLGFDKLPLWGTILISVGCAVFCAL 244
ILTKEDPVPNGLQALPLFYAATIAINVFSIMYTGAPVLGL-SLPIWAIALISFGVALLFAF 228

IVWFFVCPRMKRKIEREIKCSPSESPLMEKKNSLKEDHEETKLSVGDIENKHPVSEVGPAT 305
FVWLFVCPWMKRKIAGRLEKESALS--RASDESLRKVQEAESPVFKELPGAKASDD---SA 284

VPLQAVVEERTVSFKLGDLEEAPERERLPSVDLKEETSIDSTVNGAVQLPNGNLVQFSQAV 366
VPLTSLAGEAAGASEGTSAGNHPRASYGRALSMTHGSAKSPISNGTFGFEGHMRND----- 340

SNQINSSGHSQYHTVHKDSGLYKELLHKLHLAKVGDCMGDSGD--KPLRRNNSYTSYTMAI 425
-------GHV-YHTVHKDSGLYKDLLHKIHVDKGPEEKPAQENNYRLLRRNNSYTCYTAAI 393

CGMPLDS-FRAKEGEQKGEEMEKLTWPNAD-SKKRIRMDSYTSYCNAVSDLH-SASE--ID 481
CGMPVHATFRASDTSSAPEDSEKLVGDTVSYSKKRLRYDSYSSYCNAVAEAEIEAEEGGVE 454

MSVKAAMGLGDRKGSNGSLEEWYDQDKPEVSLLFQFLQILTACFGSFAHGGNDVSNAIGPL 542
MRLASELTDPDQPHEDPAEDEKEEKDSAEVHLLFHFLQVLTACFGSFAHGGNDVSNAIGPL 515

VALYLVYDTGDVSSKVATPIWLLLYGGVGICVGLWVWGRRVIQTMGKDLTPITPSSGFSIE 603
VALWLIYKQGGVTQEAATPVWLLFYGGVGICTGLWVWGRRVIQTMGKDLTPITPSSGFTIE 576

LASALTVVIASNIGLPISTTHCKVGSVVSVGWLRSKKAVDWRLFRNIFMAWFVTVPISGVI 664
LASAFTVVIASNIGLPVSTTHCKVGSVVAVGWIRSRKAVDWRLFRNIFIAWFVTVPVAGLF 637

SAAIMAIFRYVILRM                                                679
SAAIMALLMYICGFVSSSR                                            656
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Fig. 1. *Amino acid sequence alignment of the human PiT-1 transporter (Glvr-1; [1]), top, and rat PiT-2 transporter (Ram-1; [2]), bottom.* (The Genbank accesssion numbers for Glvr-1 and Ram-1 are L20859 and L19931 respectively). A minimal number of gaps were introduced to maximize sequence identity alignment (indicated by dashes).

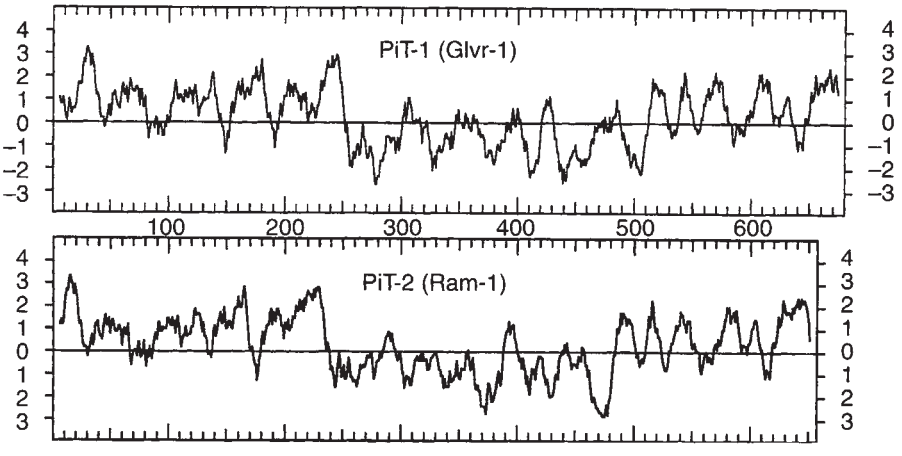

Fig. 2. *Hydropathy analysis for PiT-1 (top) and PiT-2 (bottom) using the Kyte-Doolittle algorithm.*

and brain. The greatest differences in expression levels are in heart, which expresses PiT-2 in higher relative abundance, and in bone marrow, where the converse holds true. Each transcript is also abundantly expressed in rat fibroblasts [3]. More high resolution anatomical and subcellular localization awaits the availability of specific antibodies. PiT-1 has been localized to human chromosome 2 and PiT-2 is on human chromosome 8 [10].

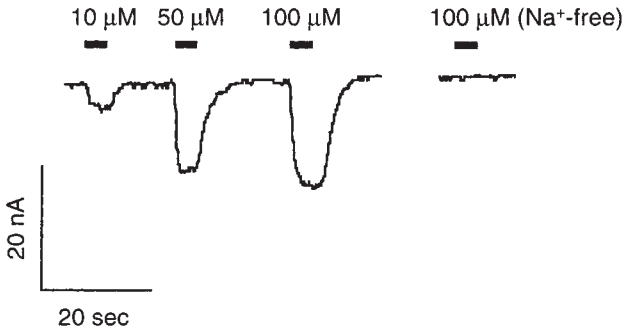

Fig. 3. *Currents induced by superfusion of varying concentrations of inorganic phosphate in an oocyte voltage-clamped at −30 mV.* P_i was superfused at the indicated concentrations for the duration indicated by bars above trace.

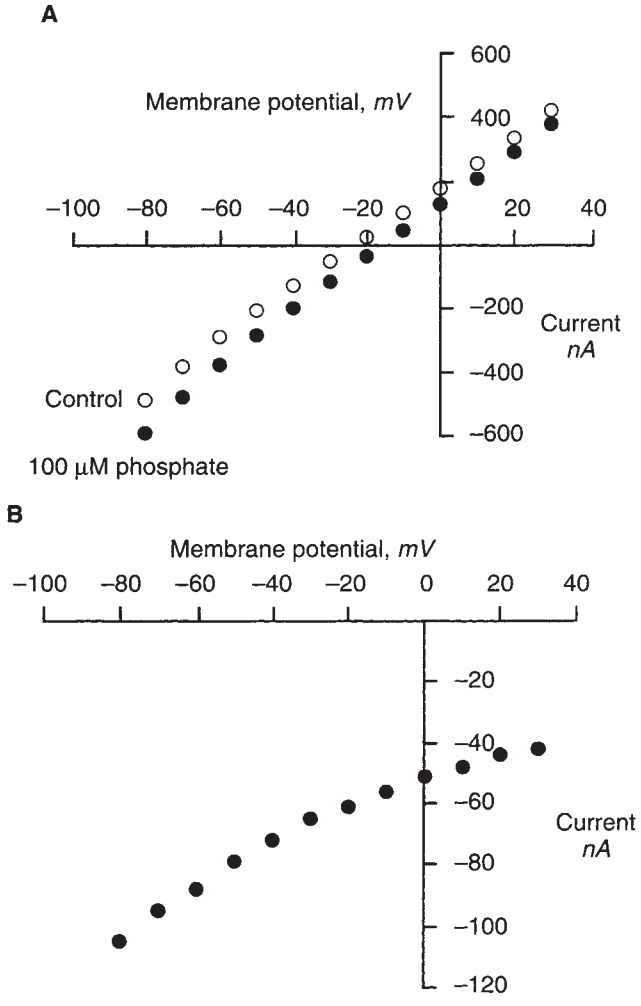

Fig. 4. *Voltage-dependence of inorganic phosphate-induced currents.* **A.** Currents were recorded during a 250 ms pulse to the indicated potential from a holding potential of −30 mV in control Ringers (○) and in the presence of 100 μM P_i (●). **B.** Voltage-dependence of transport obtained by subtraction of current recorded in absence of P_i from that recorded in the presence of P_i.

Kinetics and electrophysiology

Injection of mRNA transcribed from PiT-1 or PiT-2 results in 10- to 40-fold increase in uptake of $^{32}P_i$ relative to water-injected oocytes [3]. This uptake is nearly completely abolished by substituting Na^+ in the Ringer's solution with $Tris^+$, $choline^+$, Li^+, K^+, or NH_4^+ [3, 4]. The Na^+ concentration-dependence of PiT-1 and PiT-2 for ^{32}P uptake is sigmoidal, with an EC_{50} between 40 to 50 mM, and Hill coefficients between 1.5 and 2 (Zhang et al, manuscript in preparation). This result, together with the fact that PiT transport is rheogenic (see below), suggests that P_i is cotransported with a stoichiometric excess of Na^+ ions. The $^{32}P_i$ concentration-dependencies of PiT-1 and PiT-2 reveal that they have approximately six- to sevenfold higher apparent affinities for P_i than do the renal NaPi transporters [17]. The K_M values at pH 7.5 for uptake of $^{32}P_i$ mediated by PiT-1 and PiT-2 are 24 and 25 μM, respectively [3]. Unlike the renal transporters, which exhibit increased uptake at more alkaline pH values [17], uptake mediated by PiT-1 and PiT-2 is decreased by increasing pH [3, 4]. This effect appears to be related to the specifiy of the transporters for $H_2PO_4^-$ over HPO_4^{2-}, resulting in an increase in the apparent K_M for P_i at more alkaline pH values (Zhang et al, manuscript in preparation).

Inward currents are induced by superfusion of inorganic phosphate onto oocytes expressing PiT-1 or PiT-2, but not water-injected oocytes [3]. The sodium requirement and phosphate concentration-dependence for activation of these inward currents is the same as that for uptake of $^{32}P_i$ (Fig. 3). Thus, like the renal phosphate transporters [18], PiT-1 and PiT-2 co-transport sodium and inorganic phosphate with a stoichiometry that results in net influx of positive charge. The sigmoidal sodium concentration-dependence for inorganic phosphate uptake is consistent with cotransport of > 1 Na^+ ion. Correlation of ^{32}P uptake into *Xenopus* oocytes expressing PiT-1 with measurements of charge transfer under voltage clamp suggest that one net positive charge is translocated per atom of ^{32}P (Zhang et al, manuscript in preparation). Together with the measured values of the Hill coefficient for sodium and the charge coupling ratio, the data suggest that transport occurs with a stoichiometry of $2Na^+$:$1H_2PO_4^-$ (or $3Na^+$:$1HPO_4^{-2}$). As equilibrium thermodynamic considerations would predict, transport is increased by membrane hyperpolarization, and the inward current activated by inorganic phosphate superfusion does not reverse even at positive potentials (Fig. 4).

Viral receptor functions

The first of the two retrovirus receptors/phosphate transporters to be cloned was the human receptor for gibbon ape leukemia virus. The cDNA encoding this receptor, Glvr-1 (PiT-1), was isolated by transfecting human cDNA into mouse cells resistant to the virus and then selecting transformants which had acquired susceptibility to infection with an engineered retrovirus carrying a drug resistance gene [1]. A similar approach was utilized to isolate the rat cDNA encoding the receptor for amphotropic murine retroviruses, Ram-1 (PiT-2) [2]. The expression of each receptor/transporter is required for cellular infection by the cognate virus to occur. Relatively minor species-specific differences in protein sequence can alter the ability of a given transporter to function as a retroviral receptor even while its substrate transport properties

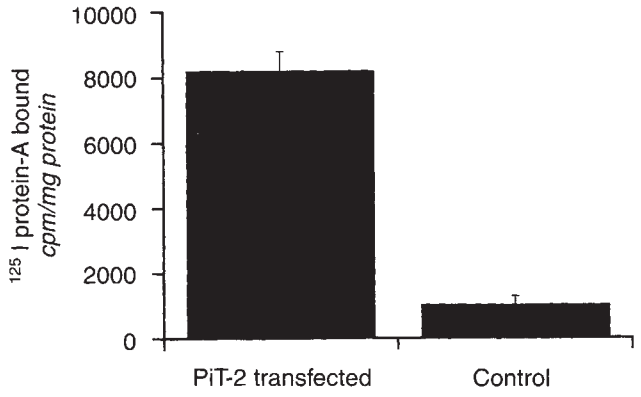

Fig. 5. *Binding of murine amphotropic retrovirus envelope protein to hamster cells expressing rat PiT-2 transporter.* CHO cells transfected with the rat PiT-2/Ram-1 cDNA exhibited increased specific binding of ^{125}I-labeled protein A relative to control (non-transfected) cells. Binding assay was performed by incubation with gp70 followed by sequential incubation with anti-gp70 and ^{125}I-labeled protein A. Data are from [3].

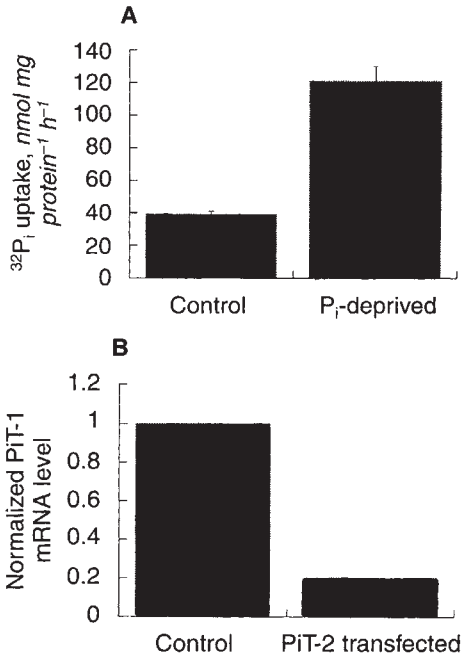

Fig. 6. *Positive and negative regulation of PiT-2 transport by phosphate levels.* **A.** Rat 208F fibroblasts grown in phosphate-free medium for 24 hours exhibited approximately threefold increase in $^{32}P_i$ uptake relative to controls. Similar increases in both PiT-1 and PiT-2 mRNA levels were seen in phosphate-deprived cells [3]. **B.** Negative regulation of PiT-1 mRNA levels in 208F cells induced to overexpress PiT-2 by transduction with PiT-2 vector. Data are from [3].

are conserved [5]. Thus, although PiT-2 is expressed in hamster cells [3], these cells are resistant to murine amphotropic virus unless transfected with the PiT-2 transporter from a susceptible species [2]. In other retrovirus receptors, site-directed mutagenesis has identified discrete amino acid sequences in putative extracellular loops that appear to interact with viral envelope proteins [5]. Transfection of CHO cells with PiT-2 confers high affinity binding of purified murine amphotropic retrovirus envelope protein to the cells (Fig. 5). Cells infected with one type of retrovirus are typically resistant to superinfection with the same virus or other viruses that utilize the same receptor [5]. This phenomenon may in part involve down-modulation of receptor. In mouse NIH-3T3 fibroblasts, which are susceptible to infection by amphotropic virus, expression of the amphotropic viral envelope glycoprotein gp70 by transfection or viral infection specifically down-modulates the component of phosphate uptake mediated by PiT-2 [3, 19]. A similar phenomenon has been observed to occur with the cationic amino acid transporter/ecotropic retrovirus receptor [20].

Regulation of transport

Phosphate deprivation results in a significant stimulation of renal reabsorption of inorganic phosphate [reviewed in 21]. This effect is correlated with an increase in type II, but not type I transporters [13]. Rat 208F fibroblasts, which contain both PiT-1 and PiT-2 mRNA, exhibit significantly increased uptake of inorganic phosphate when grown in phosphate-free medium [3]. Following a 24 hour deprivation of inorganic phosphate, uptake is increased approximately threefold (Fig. 6A). Concomitant with changes in transport, levels of both PiT-1 and PiT-2 mRNA are increased a similar amount [3]. Interestingly, PiT-1 transcript levels are significantly decreased by transfection of cells with PiT-2 (Fig. 6B), suggesting that modulation occurs via sensing of the concentration of intracellular rather than extracellular inorganic phosphate (or a metabolite thereof).

Consensus phosporylation sites for protein kinases exist within the types I and II phosphate transporters, and regulation of transporter function may occur through parathyroid hormone activation of kinase [22]. Multiple consensus phosphorylation sites are present within both PiT-1 and PiT-2 transporters, particularly in the hydrophilic internal domain (residues 250 to 450 in PiT-1). Kinase regulation of phosphate transport has been shown to occur in NIH-3T3 cells [23] which express high levels of both PiT-1 and PiT-2 [3]. Preliminary experiments suggest that PiT-2 transport is increased by activation of protein kinase C (unpublished observations), which has the opposite the effect on renal transporters [22]. Regulatory control of phosphate transport is crucial because of the diverse roles of intracellular phosphate in cell biology, and the availability of molecular clones encoding different phosphate transporter subtypes will now allow detailed molecular studies of the structural and functional bases of this regulation.

Acknowledgments

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