Type III sodium-dependent phosphate (Na\textsubscript{P}) cotransporters, Pit1 and Pit2, have been assigned housekeeping P\textsubscript{i} transport functions and suggested involved in chondroblastic and osteoblastic mineralization and ectopic calcification. Both proteins exhibit dual function, thus, besides being transporters, they also serve as receptors for several gammaretroviruses. We here show that it is possible to uncouple transport and receptor functions of a type III Na\textsubscript{P} cotransporter and thus exploit the retroviral receptor function as a control for proper processing and folding of mutant proteins. Thus exchanging two putative transmembranic glutamate residues in human Pit2, Glu\textsubscript{55} and Glu\textsubscript{575}, with glutamine or with lysine severely impaired or knocked out, respectively, P\textsubscript{i} transport function, but left viral receptor function undisturbed. Both glutamates are conserved in type III Na\textsubscript{P} cotransporters, in fungal Na\textsubscript{P} cotransporters PHO-4 and Pho89, and in other known or putative phosphate permeases from a number of species and are the first residues shown to be critical for type III Na\textsubscript{P} cotransport. Their putative transmembranic positions together with the presented data are consistent with Glu\textsubscript{55} and Glu\textsubscript{575} being parts of a cation liganding site or playing roles in conformational changes associated with substrate transport. Finally, the results also show that Pit2 retroviral receptor function per se is not dependent on Pit2 P\textsubscript{i} transport function.

Inorganic phosphate (P\textsubscript{i})\textsuperscript{1} is essential for cellular metabolism and skeletal mineralization. Moreover, it serves as the source of phosphate for organic cell constituents, e.g., nucleotides and a variety of phosphorylated metabolic intermediates. Two proteins that show the same transport characteristics as P\textsubscript{i} uptake across the plasma membrane in animal cells have been identified (1–3), namely the sodium-dependent phosphate (Na\textsubscript{P}) cotransporters, Pit1 (human Pit1 formerly GLVR1 (4)) and Pit2 (human Pit2 formerly GLVR2 (5)). Both proteins are characterized as type III Na\textsubscript{P} cotransporters (6) and show a broad tissue distribution being expressed in all investigated human tissues albeit at different levels (7). Furthermore, low extracellular P\textsubscript{i} levels result in up-regulated Pit1 and Pit2 expression in mammalian cells (1, 8). These observations strongly suggest that the major cellular P\textsubscript{i} demand in mammalian cells is handled by type III Na\textsubscript{P} cotransporters (1). However, recent results also point at type III transporters as playing specific roles in chondroblastic and osteoblastic mineralization (9, 10) as well as being critically involved in vascular calcification under hyperphosphatemic conditions, which are often present in diabetic patients and individuals with renal failure (11). The mechanisms underlying the bone-forming roles of type III Na\textsubscript{P} cotransporters are presently not known. Recent results, however, showed that high extracellular P\textsubscript{i} levels can induce expression of the gene for osteopontin and that the induction is dependent on Na\textsuperscript{+}-dependent P\textsubscript{i} uptake across the plasma membrane (12). Interestingly, osteopontin is involved in normal bone development and present in calcified arterial plaques (13).

Despite the important roles of type III Na\textsubscript{P} cotransporters in cellular P\textsubscript{i} uptake and increasing evidence for their critical roles in normal and pathologic calcification, nothing is known about what determines their transport function. The two known type III Na\textsubscript{P} cotransporters, Pit1 and Pit2, show about 60% amino acid identity (5), whereas orthologs of Pit1 and Pit2 exhibit more than 90% amino acid identity (5, 14, 15). A putative topological model for both proteins based on hydropathy plots predicts 10 transmembrane (TM) regions and 5 extracellular loops (5, 14, 16). No significant overall sequence similarity exists between type III cotransporters and members of the two other Na\textsubscript{P} cotransport systems referred to as types I and II (1).

Both Pit1 and Pit2 were originally identified as receptors for retroviruses belonging to the gammaretrovirus genus, and they are currently the two cellular receptors targeted in human gene therapy trials employing retroviral vectors. The human gene SLC20A1 encoding Pit1 was cloned as a receptor for gibbon ape leukemia virus (4) and spleen necrosis virus subgroup B (17), and the Pit2-encoding gene SLC20A2 from human and rats was cloned as receptor for amphotropic murine leukemia virus (AM-MLV) (5, 18); an AM-MLV-related isolate, 10A1 MLV, utilizes both transporters as receptor (19). In recent years, substantial insight into the receptor functions of Pit1 and Pit2 for their cognate viruses has been obtained (reviewed in Refs. 20 and 21).
We speculated that the retroviral receptor function might provide a functional assay for proper processing and folding of mutant type III NaPi transporters with knocked out NaPi transport function thus allowing for identification of amino acids critical for transport function. We here show that it is possible to uncouple human Pit2 retroviral receptor function from NaPi transport function and to exploit the dual function of Pit2 as NaPi transporter and retroviral receptor for identification of amino acids critical for NaPi transport. Doing this, we identified two Pit2 glutamate residues Glu55 (E55) and Glu575 (E575) as critical for Na\textsuperscript{-}-dependent P\textsubscript{T} transport function. The glutamates are positioned in putative transmembrane domains and are conserved in type III NaPi transporters, in NaP\textsubscript{i} cotransporters, and in NaPi transporters from other species (see on-line supplemental material) strongly suggesting that they also are critical for the physiological function of these transporters.

**EXPERIMENTAL PROCEDURES**

**Construction of Expression Plasmids and Mutagenesis**—The pcDNA1 \*tkpA-derived expression plasmid pOJ74 (Wyeth-Ayerst Research, Pearl River, NY) encoding human Pit2 has been described previously (24). Mutations were introduced in human Pit2 using the Altered sites II kit (Promega) and p-ALTER-1-derived vectors harboring the 5′- and 3′-ends of the SCL20A2 gene. Mutated fragments were moved from pALTER to pOJ74 with appropriate restriction enzymes, and the final plasmids were cultivated as PA317GBN cells. Filtered (0.45-micron) supernatants were stored at −80 °C, corresponding to 2 × 10\textsuperscript{4} cells/60-mm-diameter dish and 2 × 10\textsuperscript{5} cells/100-mm-diameter dish.

**Phosphate Uptake Assay**—The uptake assay was performed using a protocol modified from Tatsumi et al. (28). For NaPi, phosphate uptake measurements, oocytes were incubated briefly in phosphate-free uptake solution (100 mM NaCl, 2 mM KCl, 1 mM CaCl\textsubscript{2}, 10 mM MgCl\textsubscript{2}, 10 mM HEPES-Tris, pH 7.5) followed by two 15 min incubation in uptake solution containing 0.1 mM KH\textsubscript{2}PO\textsubscript{4} (1 Ci/nmol; American Radiolabeled Chemicals) at 18 °C. After uptake, oocytes were washed in ice-cold uptake solution containing 5 mM KH\textsubscript{2}PO\textsubscript{4} and each was transferred to a scintillation vial containing 2 ml of 10% (w/v) SDS. The radioactive decay was determined after addition of 5 ml of Optiphase Polysafe scintillation liquid (Wallac, Finland). For cation-dependent \textsuperscript{32}P\textsuperscript{32}P transport (see Table I), NaCl was substituted for 100 mM LiCl in the uptake solution.

**Surface Biotinylation of Xenopus laevis Oocytes**—For investigation of the presence of wild-type and mutant proteins in the outer membrane of oocytes, five cRNA-injected oocytes were washed three times in ice-cold PBS buffer at pH 8.0. The oocytes were then incubated 30 min at room temperature in 0.5 ml of PBS containing 0.5 mg/ml sulfo-NHS-S\textsubscript{3}biotin (Pierce) and excess biotinylation reagent was removed by four washes with ice-cold PBS. The oocytes were subsequently dissolved in lysis buffer (150 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1% Triton X-100) for 30 min on ice. Solubilized oocytes were centrifuged for 15 min at 13,000 rpm, and the supernatants containing the biotinylated proteins were incubated with 50 \mu l of streptavidin-coupled Dynabeads (Dynal) for 1 h at 4 °C. The bead complexes were washed four times by suspension in a polyvinylidene difluoride membrane (Immobilon-P, Millipore). The blot was probed with an antibody raised against the large intracellular domain of the retroviral receptor function leaving retroviral receptor function intact. The band was developed using swine anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Dako) and chemiluminescence according to the manufacturer’s protocol (ECL Western blot kit, Amersham Biosciences).

**Statistical Analysis**—\textsuperscript{32}P uptake values and receptor functions were expressed as means ± S.E. The hypothesis that two mean values were identical was tested by a two-tailed Student’s t test; values were considered different at a 95% confidence level.

**On-line Supplemental Material**—The alignments are published on-line (see supplemental material) for 2.7M, 3.7M, and 9.7M sequences from Pit1, Pit2, and related proteins from the indicated species (identified by NCBI data base accession numbers): Pit1 from Homo sapiens I52822, Cricetulus griseus I48084 and AAD28693, Rattus norvegicus BAB07789, Mus musculus AAA74887, and Felis catus AAD08495; Pit2 from H. sapiens AAA18018, C. griseus I48083, AAD28693, and AAG21845, and R. norvegicus NP_058919; sodium-dependent phosphate permeases from N. crassa P15710 and S. cerevisiae NP_009855; known and putative phosphate permeases from Drosophila melanogaster AAF90089, Caenorhabditis elegans AAA92320, Leishmania major AAA09562, and C. jejuni AAA62569; C. griseus CAG50502, C. jejuni CAG50502; and Acc. no. CAB73448, Mycobacterium tuberculosis E70731, Neisseria meningitidis CAB85332, Mesembranthemum crystallinum T12576, Arabidopsis thaliana CA66826, and Archaeoglobus fulgidus AAB89449. The glutamates in Pit2 positions 55 and 75 are conserved in all shown proteins. The glutamate in Pit2 position 91 is only present in Pit2 orthologs.

**RESULTS**

**Candidate Residues for Mutagenesis**—The objective of the present study was to identify amino acids critical for human Pit2 NaPi transport function and investigate whether it was possible to knock out NaPi transport function leaving retroviral receptor function undisturbed. Conserved amino acids in TM regions are prominent candidates for amino acids liganding monovalent cations (29–33) and, as such, are potentially critical for NaPi transport function. Moreover, transmembrane amino acids might be expected next to critical transmembrane proteins involved in entry compared with amino acids positions in extracellular loops. According to the prevailing topological model of Pit1 and Pit2 (5, 14, 16), two transmembrane glutamate residues are widely conserved in type III transporters and in related proteins from a number of species (see on-line supplemental material). They are positioned in the
putative second (E55, human Pit2 numbering) and ninth (E575, human Pit2 numbering) TM regions of Pit1 and Pit2 proteins. We analyzed the possible roles of E55 and E575 in human Pit2 transport function by initially replacing them with glutamine. Moreover, in these studies we also included a non-conserved glutamate residue only present in Pit2 orthologs (Glu91 (E91) in the putative third TM region of human Pit2) (see supplemental material). Mutant Pit2 proteins are designated with a three-letter code, where the first, second, and third letters show the identity of the amino acids in positions 55, 91, and 575, respectively; thus, wild-type Pit2 would be designated EEE.

**Exchange of Glutamate Residues E55 and E575 with Glutamine Severely Impaired NaP\textsubscript{i} Transport of Human Pit2—**The Na\textsuperscript{+}-dependent \( ^{32}\text{P} \) transport function of wild-type Pit2 and the seven Pit2 mutants QEE, EEQ, EQQ, QEQ, EQQ, EQQ, and QQQ were analyzed in *Xenopus* oocytes (Fig. 1). In the experiment shown in Fig. 1A, oocytes injected with cRNA encoding human Pit2 supported a \( ^{32}\text{P} \) uptake of 521 \( \pm \) 36 pmol/h per oocyte at pH 7.5 or about 25-fold above the \( ^{32}\text{P} \) uptake in \( H_2O \)-injected oocytes (\( p < 0.001 \)). Depending on the oocyte preparation, human Pit2 supported \( ^{32}\text{P} \) uptake varied from about 250 to 500 pmol/h per oocyte (Figs. 1A and 2A and Table I) (unpublished data).

The \( ^{32}\text{P} \) transport activity of the EQE mutant was indistinguishable from that of Pit2 (\( p > 0.8 \)) (Fig. 1A). However, exchanging the highly conserved 2.TM or 9.TM glutamate residues with glutamine (QEE and EEQ) resulted in 4- and 7-fold decreases in \( ^{32}\text{P} \) uptake, respectively, compared with Pit2 (\( p < 0.001 \)). Introducing the E91Q mutation together with the E55Q mutation (QQE) further reduced the \( ^{32}\text{P} \) uptake (compared with QEE \( p < 0.001 \)), whereas introducing the E91Q mutation together with the E575Q mutation (EQQ) led to an increased \( ^{32}\text{P} \) uptake (compared with EEQ \( p < 0.001 \)) (Fig. 1A). Noticeably, exchanging both the highly conserved E55 and E575 residues with glutamine reduced the \( ^{32}\text{P} \) uptake level —10-fold (QQQ) and 8-fold (QQQ) compared with Pit2 (\( p < 0.001 \)) (Fig. 1A). In the experiment shown in Fig. 1A, all mutants did support \( ^{32}\text{P} \) uptake levels significantly different from that of \( H_2O \)-injected oocytes (\( p < 0.002 \)). The same relative Na\textsuperscript{+}-dependent \( ^{32}\text{P} \) uptake pattern was found in an independent experiment (unpublished data); however, in this experiment as well as in a separate experiment (Table I) the \( ^{32}\text{P} \) uptake levels supported by QEQ or QQQ did not differ significantly from that of \( H_2O \)-injected oocytes. Thus, the QEQ and QQQ mutants do indeed have very low \( P \) transport activities.

The cell surface expression of the QEQ and QQQ mutants was assessed by cell surface biotinylation using the membrane-impermeant reagent, sulfo-NHS-SS-biotin, followed by SDS-PAGE and Western blotting (Fig. 3). The predominant form of the membrane-associated Pit2 and mutant proteins was dimeric (marked with arrowhead in Fig. 3) in agreement with recent results from Salamün and coworkers (36), which suggest that the active transporting form of human Pit2 is a dimer. The

![Fig. 1](image-url)  
**Fig. 1.** Na\textsuperscript{+}-dependent \( ^{32}\text{P} \) transport in *X. laevis* oocytes and retroviral receptor functions in CHO cells of Pit2 and mutant Pit2 proteins. The three-letter code represents the amino acids in human Pit2 positions 55, 91, and 575; wild-type human Pit2 would be designated EEE. A, oocytes were injected with \( H_2O \) or cRNA of the indicated constructs. Three days later, \( ^{32}\text{P} \) uptake was measured at pH 7.5 in the presence of 100 mM Na\textsuperscript{+} using 0.1 mM KH\textsubscript{2}PO\textsubscript{4} as \( ^{32}\text{P} \) source. Uptake was allowed for 60 min at 18 °C, after which the \( ^{32}\text{P} \) uptake in individual oocytes was measured. Data are mean values of the indicated numbers of oocytes (\( n \) \( \pm \) S.E. For mutant EQQ, \( p > 0.8 \) relative to Pit2; for the remaining mutants, \( p < 0.001 \) relative to Pit2. For Pit2 and all mutants, \( p < 0.002 \) relative to \( H_2O \)-injected oocytes. The \( ^{32}\text{P} \) uptake values in pmol/oocyte/h were as follows, Pit2 (EEE), 521.6 \( \pm \) 36.0; QEE, 120.6 \( \pm \) 6.7; EEQ, 530.5 \( \pm \) 38.7; EQQ, 73.6 \( \pm \) 12.0; QEQ, 52.2 \( \pm \) 7.4; EQQ, 51.7 \( \pm \) 6.6; EEQ, 172.2 \( \pm \) 16.0; QQQ, 62.6 \( \pm \) 5.4; \( H_2O \), 21.1 \( \pm \) 2.7. B-D, \( 8 \times 10^{10} \) CHO cells in 60-mm-diameter dishes were transfected with 2 \( \mu \)g of plasmid DNA encoding human Pit2 or the indicated mutant Pit2 proteins. Mock, cells transfected with empty vector DNA. Three independent precipitates were made for each construct. Forty-eight hours after transfection, \( \sim \) 20,000 AM-MLV vector pseudotypes (B), 85,000 10A1 vector pseudotypes (C), or 50,000 10A1 vector pseudotypes (D) were added per dish to three randomly picked dishes transfected with the indicated constructs. The average number (\( \pm \) S.E.) of blue (infected) cells per dish from three dishes receiving each independent precipitate is shown. The number of blue cells from dishes transfected with Pit2-expressing plasmid was assigned the value 100, which corresponded to \( \sim \) 1600 (B), 1200 (C), and 4500 (D) blue cells per dish. For all mutants relative to Pit2, \( p > 0.06 \), except in D where \( p = 0.05 \) for EEQ and QQQ, respectively. A-D: the entire experiment was repeated with independent DNA preparations, and similar results were obtained, except that the \( ^{32}\text{P} \) uptake in oocytes injected with QEQ and QQQ encoding cRNAs did not differ significantly from that of \( H_2O \)-injected oocytes.
Determinants of Type III NaP₁ Cotransport

FIG. 2. Na⁺-dependent ³²Pᵢ transport in X. laevis oocytes and retroviral receptor functions in CHO cells of Pit2 and mutant Pit2 proteins. For nomenclature see the legend for Fig. 1. A, oocytes were injected with H₂O or cRNA of the indicated constructs. Uptake of ³²Pᵢ was measured as described in the legend to Fig. 1A. Data are mean values of the indicated numbers of oocytes (n) ± S.E. *p < 0.001 relative to all mutants and H₂O injected oocytes; **p < 0.05 relative to H₂O-injected oocytes. The ³²Pᵢ uptake values in pmol/oocyte/h were as follows: Pit2 (EEE), 264.1 ± 19.2; KEE, 5.4 ± 0.4; EEE, 4.7 ± 0.7; KEK, 5.5 ± 0.7; H₂O, 8.9 ± 1.6. B and C: 8 × 10⁴ CHO cells in 60-mm-diameter dishes were transfected with 2 μg of plasmid DNA encoding human Pit2 or indicated mutant Pit2 proteins. Mock, cells transfected with empty vector DNA. Three independent precipitates were made for each construct. Forty-eight hours after transfection, ~25,000 AM-MLV vector pseudotypes (B) or 100,000 10A1 vector pseudotypes (C) were added per dish. The average number (±S.E.) of blue (infected) cells per dish from three dishes receiving independent precipitates is shown. The number of blue cells from dishes transfected with Pit2-expressing plasmid was assigned the value 100, which corresponded to ~16,000 blue cells per dish for AM-MLV vector pseudotypes (B) and 15,000 blue cells per dish for 10A1 vector pseudotypes (C). For both vector pseudotypes for all mutants relative to Pit2 p > 0.1.

QEQ and QQQ mutants were present at the oocyte cell surface in approximately half the amounts of Pit2 and in amounts comparable to that of the mutant EQE, which shows wild-type transport function (Fig. 3). Thus the severely impaired transport function of the QEQ and QQQ mutants cannot solely be explained by impaired processing of the mutant proteins to the oocyte cell surface. The presence of the mutant proteins in the outer membrane of the oocyte does, however, not exclude that the impaired transport functions are due to improper folding of the mutant proteins. We therefore investigated whether we could exploit the additional function of Pit2 as retroviral receptor to assess proper processing and folding of the mutant proteins in mammalian cells.

Viral Receptor Function of Mutant Pit2 Proteins with Gln for Glu Substitutions—Using a transient transfection-infection assay, we analyzed whether the neutral conserved Gln for Glu substitutions, which in some mutants severely impaired transport activity, affected viral receptor functions of the Pit2 mutants. Expression plasmids encoding Pit2 and mutant proteins were transfected into CHO cells, which are non-permissive and largely non-permissive for infection by AM-MLV and 10A1, respectively (Fig. 1, B–D) (19, 37, 38). The ability of these proteins to support infection by AM-MLV and 10A1 was analyzed by exposing the transfected cells to retroviral vectors encoding β-galactosidase and carrying viral surface proteins responsible for receptor recognition; vectors carrying AM-MLV and 10A1 surface proteins are referred to as AM-MLV and 10A1 vector pseudotypes, respectively. The infection levels were evaluated as the number of β-galactosidase-positive (blue) cells per 60-mm-diameter dish. Dishes with CHO cells transfected with the same precipitates were chosen at random and exposed to either 20,000 AM-MLV vector pseudotypes (Fig. 1B), 85,000 10A1 vector pseudotypes (Fig. 1C), or 850,000 10A1 vector pseudotypes (Fig. 1D). CHO cells expressing human Pit2 were permissive for infection by both vector pseudotypes.
Determinants of Type III NaPi Cotransport

Type III NaPi cotransporters, Pit1 and Pit2, exhibit dual function. Here we have shown that it is possible to knock out Pit2 transport function of human Pit2, leaving its retroviral receptor function undisturbed. Thus, it is possible to exploit the retroviral receptor function of Pit2, and possibly of Pit1, as a control for processing and folding of mutant proteins with knocked out transport function. Doing this, we identified two

(1) Thus, because QEQ showed wild-type receptor function, these results suggest that E55 and E575 in general are important for cation-dependent P1 transport of Pit2.

Exchange of Either of the Glutamate Residues in Positions 55 and 575 of Pit2 for Lysine Knocked Out NaPi Transport of Human Pit2—The observation that the QEQ mutant harboring E55Q and E575Q substitutions had retained wild-type viral receptor functions encouraged us to replace these amino acids with lysine, in an attempt to completely knock out Na+-dependent P1 transport function of Pit2. Thus glutamate was replaced with lysine in positions 55 and/or 575 in wild-type Pit2 resulting in the mutants KEE, EKK, and KEK (Fig. 2). No increase in Na+-dependent 32P uptake could be detected in oocytes injected with cRNAs encoding Pit2 with E55 and/or E575 exchanged for lysine compared with H2O-injected oocytes (Fig. 2A). All three mutant proteins were present at the oocyte cell surface in amounts comparable to wild-type Pit2 (Fig. 3), thus their lack of transport activity is not due to impaired processing to the oocyte cell surface. However, we introduced rather dramatic changes in these proteins, and, as mentioned above, the presence of the mutant proteins in the outer membrane of the oocyte does not exclude that their impaired transport functions are due to improper folding. We therefore investigated whether the additional function of Pit2 as retroviral receptor could be employed to assess proper processing and folding of these mutant proteins in mammalian cells.

Pit2 Proteins Carrying E55K and/or E575K Substitutions Retained Viral Receptor Function—The effect of E55K and E575K substitutions on Pit2 AM-MLV and 10A1 receptor function is shown in Fig. 2 (B and C), respectively; notice that the infection levels in this experiment were in general higher than those shown in Fig. 1 (B–D) due to a more efficient batch of transfection buffer. All mutants exhibited AM-MLV receptor function at wild-type Pit2 levels (for all mutants p > 0.1 compared with Pit2) (Fig. 2B). Similar results were obtained for the 10A1 receptor functions of the mutants (for all mutants p > 0.1 compared with Pit2) (Fig. 2C). However, although not significantly different from the receptor function of Pit2, the mutant KEE supported infection levels of about 60% of that obtained with Pit2 (Fig. 2C). We therefore chose to subject the 10A1 receptor functions of KEE to a more detailed analysis with independent DNA preparations, using high and low receptor amounts and high and low 10A1 vector loads. In this experiment, the Pit2 infection levels ranged from 300 to 14,000 blue cells per 60-mm-diameter dish, and no impairment in receptor function of the mutant protein could be observed (unpublished data), in agreement with the data in Fig. 2C. We conclude that the KEE, EKK, and KEK mutants have knocked out Pit2 transport functions, while exhibiting wild-type receptor functions; thus, in these mutants, the receptor function is uncoupled from the transport function. Moreover, the observation that introduction of lysine instead of either E55 or E575 knocked out human Pit2 Na+-dependent P1 transport function is in agreement with the results obtained on Pit2 mutant proteins harboring Glu for Glu substitutions, and we conclude that the two highly conserved Pit2 glutamate residues E55 and E575 are crucial determinants of human Pit2 P1 transport function.

Cation-dependent P1 Uptake of Pit2 Mutant Proteins Carrying Glutamine Instead of the Highly Conserved E55 and E575—The observation that the QEQ and QQE mutants showed severely impaired NaPi transport function, but retained viral receptor function, although that of QQE was impaired, led us to investigate whether QEQ and QQE exhibited a different cation-dependent P1 transport activity than Pit2 (Table 1). Oocytes injected with human Pit2 encoding cRNA were found to support Li+-dependent 32P uptake (compared with H2O-injected oocytes p < 0.05) (Table 1), in agreement with the fact that hamster Pit2 has been shown to sustain Li+-dependent 32P uptake when overexpressed in hamster cells (3). The QQE mutant exhibited severely impaired Li+-dependent 32P transport, whereas no Li+-dependent 32P uptake above background could be detected in oocytes expressing the QEQ mutant (Table 1). Thus, because QEQ showed wild-type receptor function, these results suggest that E55 and E575 in general are important for cation-dependent P1 transport of Pit2.
glutamate residues, E55 and E575 in human Pit2, as critical for Pit2 P$_i$ transport function. This is the first time amino acids critical for P$_i$ transport of a type III NaP$_i$ cotransporter have been identified. The two glutamates are both highly conserved in type III NaP$_i$ cotransporters and in a number of known and putative phosphate permeases from other species (see supplemental material) strongly suggesting that they also are critical for the physiological function of these transporters.

The strength of employing retroviral receptor function as a control for correct processing and folding of mutant Pit2 proteins rather than relying on protein expression in outer membranes is illustrated with the mutant QQQ. It exhibited severely impaired transport function, which could not be explained by the protein not being present in the oocyte outer membrane, but it did, however, also show significantly impaired receptor function. It is possible that impaired receptor function can be due to involvement in retroviral entry of the amino acids analyzed; however, in this case, it is not likely, because mutant proteins like QEE, EQE, QEQ, KEK, EEE, and KEK were fully functional receptors, rather we suggest that the QQQ mutant proteins in the plasma membrane exhibit disturbed processing and/or folding.

The question arises whether viral receptor function will be applicable as control when studying involvement of extracellular loop positioned amino acids in transport function of type III transporters. It is in this connection noteworthy that the amino acids so far identified as critical for retroviral receptor function of Pit1 and Pit2 orthologs are those highly variable between Pit1 and Pit2 and the NaP$_i$ transporter PHO-4 from N. crassa (19, 24, 37, 40–43), whereas the amino acids critical for NaP$_i$ uptake are expected to be found among the residues conserved in Pit1, Pit2, and related proteins from other species as shown in the present work. In line with this, we have recently shown that a Pit1 mutant with abolished receptor functions showed no impairment in P$_i$ transport function as analyzed in Xenopus oocytes. Thus, it is possible that there in general is no overlap between Pit sequences critical for Pit$_i$ transport function and Pit sequences critical for receptor function.

We do not know the exact role of Pit2 residues E55 and E575 in P$_i$ transport. However, the observation that changing either E55 or E575 for lysine was sufficient to knock out P$_i$ transport function leaving receptor function undisturbed is indeed in agreement with these residues being involved in NaP$_i$ transport function, rather than fulfilling structural roles. It is possible that E55 and E575 are involved in function-dependent conformational changes of the transporter. Our data are, however, also consistent with these glutamates being parts of a cation liganding site created by residues from different transmembrane regions as has been implicated for negatively charged residues of the melibiose permease of Escherichia coli, the rabbit Na$^+$/dicarboxylate cotransporter NaDC-1, Na$^+$/H$^+$ exchangers, the e subunit of the Propionigenium modestum F$_o$/F$_{o'}$-ATP synthase and Na$^+$,K$^+$-ATPhases (29–33, 44). However, a Na$^+$-liganding site may contain only one acidic residue, the other residues being, e.g. glutamine and serine (44). In this context it is worthy to note that at least some of the mutant Pit2 proteins with Gln for Glu substitutions in only one of the positions E55 or E575 (mutants QEE, EQE, and EQQ) still supported low levels of NaP$_i$ uptake (Fig. 1A).

In recent years, a number of transport proteins have been identified that have dual function as transporters and retroviral receptors (4, 5, 18, 45–48). For murine CAT1, which is a Na$^+$-dependent cationic amino acid transporter, it has also been shown that a glutamate residue in a putative transmembrane region was critical for the transport function but not for retroviral receptor function of the protein (49). Based on the data presented here and that of Wang et al. (49), we suggest that for proteins exhibiting dual function as transporters and retroviral receptors, their viral receptor function might in general be exploited for identification of amino acid residues critical for transport function. Using a transient transfection-infection assay, which is a fast and reliable method, for analyzing the viral receptor function as described here, indeed makes it a highly feasible approach to analyze processing/overall topology of mutant proteins by investigating their receptor function.

The presented data also have implications for understanding the role of Pit2 in retroviral entry. Addition of sulphhydryl reagents to cells was previously shown to impair Pit2 P$_i$ transport and AM-MLV infection but not virus binding (50). According to the authors, these data suggest that P$_i$-induced conformational changes in Pit2 are involved in AM-MLV entry via Pit2 (50). Our data are not in direct conflict with this interpretation, but they do show that viral receptor function is not dependent on P$_i$ transport function per se. Indeed, comparison of the data in Figs. 1 and 2 reveals that there is no correlation between Na$^+$-dependent P$_i$ transport function and retroviral receptor function of Pit2. We suggest that further important insight into both functions of the type III NaP$_i$ transporters can be achieved by combining studies on transport and receptor functions as presented here.

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