Functional Expression and Characterization of the Wild-Type Mammalian Renal Cortex Sodium/Phosphate Cotransporter and an ^{215}R Mutant in *Saccharomyces cerevisiae*†

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ABSTRACT: The wild-type and an R215E mutant of the rat renal cortex sodium/phosphate cotransporter type 2 (NaPi-2) were functionally expressed in the yeast *Saccharomyces cerevisiae* strain MB192, a cell line lacking the high-affinity endogenous H^+/P_i cotransporter. The expression of the mRNA molecules and corresponding proteins was confirmed by Northern and Western blot analysis, respectively. As detected by indirect immunofluorescence and antibody capture assay, both wild-type and mutant NaPi-2 proteins are expressed in the yeast plasma membrane in comparable amounts. In the presence of 5 μM phosphate, Na^+ promotes phosphate uptake into yeast cells expressing the wild-type NaPi-2 with a $K_{0.5}$ of 5.6 ± 1.1 mM. The maximum uptake of phosphate (649 ± 30 pmol/10 min) is approximately 8-fold higher than the uptake obtained with nontransformed cells (76.8 ± 8 pmol/10 min). Yeast cells expressing the R215E mutant of NaPi-2 accumulate 213 ± 9 pmol of phosphate/10 min under the same conditions. The $K_{0.5}$ for the stimulation of phosphate uptake by Na^+ is 4.2 ± 0.8 mM for the R215E mutant and thus not significantly different from the value obtained with cells expressing the wild-type cotransporter. The reduced level of accumulation of phosphate in yeast cells expressing the R215E mutant is probably due to a reduction of the first-order rate constant k for phosphate uptake: while cells expressing wild-type NaPi-2 accumulate phosphate with a k of 0.06 min^{-1}, the rate for phosphate uptake into cells expressing the R215E mutant (k) is 0.016 min^{-1} and therefore about 4-fold lower. In comparison, the rate for phosphate uptake into nontransformed cells (k) is 0.0075 min^{-1}. Phosphate uptake into yeast cells that express the wild-type NaPi-2 in the presence of 150 mM NaCl is promoted by extracellular phosphate with a $K_{0.5}$ of 45 ± 4 μM. A phosphate-dependent phosphate accumulation is also observed with cells expressing the R215E mutant, but the $K_{0.5}$ is twice as high (86 ± 5 μM) as that obtained with the wild-type cotransporter. We conclude that the yeast expression system is a useful tool for the investigation of structure−function relationships of the renal sodium/phosphate cotransporter and that ^{215}R, although not involved in Na^+ recognition, is a part of the structure involved in phosphate recognition and considerably influences the rate of phosphate uptake by the NaPi-2 cotransporter.

Phosphate metabolism and the conversion from inorganic to organic compounds comprise a key function of cell metabolism (*1*), and phosphate uptake into various tissues, cells, or cell organelles appears to be mediated by different types of phosphate transporters. Thus, the identification of phosphate transporters has been the subject of thorough investigation in recent years. Several of these phosphate transporting systems have been identified and characterized to some extent by physiological methods, and seem to operate in cotransport with either H^+ or Na^+ (*2*−*5*). Expression cloning of cRNA from rabbit kidney cortex (*6*) as well as from rat and human kidney cortex in *Xenopus laevis* oocytes (*7*) resulted in the identification of three phosphate transporting proteins (NaPi-2 type), two of them being highly homologous to each other (*7*). All three are driven by the sodium gradient, and phosphate is cotransported with the cation. Besides this fact, little is known about the mechanism of phosphate uptake or about amino acids involved in the phosphate and Na^+ translocation process.

Questions of this kind can be addressed by site-directed mutagenesis and subsequent analysis of the properties of the mutant protein, but they require the availability of suitable cell expression systems. In the case of the Na^+/P_i cotransporter, the cell system would be required to have a phosphate uptake mechanism different from the NaPi-2 type transporter protein and should allow the expression of sufficient amounts of wild-type or mutant transporters. These two requirements would ensure that the properties of low-activity mutants could still be analyzed despite the presence of endogenous phosphate uptake systems. The only expression system used thus

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far, *Xenopus laevis* oocytes, does not easily fulfill all the requirements for the investigation of mutants of the Na^+/P_i cotransporter. Not only do these cells contain an endogenous Na^+-driven phosphate uptake system, but their transfection is laborious and the time required to obtain the expressed protein rather long.

Thus, the aim of the work presented here was to develop an expression system for the Na^+/P_i cotransporter in the *Saccharomyces cerevisiae* strain MB192 (*8*). This strain of yeast lacks the high-affinity phosphate transport system usually found in yeast cells and can only grow when phosphate is present in the growth medium at relatively high concentrations (*8*, *9*). As will be shown, this yeast strain constitutes a suitable cellular system for the heterologous expression and analysis of the properties of wild-type and mutated Na^+/P_i cotransporters. The establishment of the yeast as an expression system has enabled us to obtain the first reported results concerning the significance of particular amino acid residues for the Na^+-dependent uptake of phosphate and opens up the possibility of further evaluating the structure−function relationships of the NaPi-2 type cotransporters in future investigations.

EXPERIMENTAL PROCEDURES

Materials. Oligonucleotides, linkers, and solutions for SDS−PAGE[1] were from Roth (Karlsruhe, Germany). [α-^{32}P]-dCTP (3000 Ci/mmol) for labeling of cDNA and [^{32}P]-orthophosphate (9130 Ci/mmol) were obtained from Hartmann Analytic (Braunschweig, Germany). Enzymes for molecular biology were from United States Biochemicals (Cleveland, OH) or MBI Fermentas (Vilnius, Lithuania). Growth media were from Difco (Detroit, MI). Nucleic acid purification was performed with the Jet-sorb gel extraction kit of Genomed (Bad Oeyenhausen, Germany). Immunodetection of expressed NaPi-2 was performed with the ECL kit (Amersham, Buckinghamshire, England). Nitrocellulose membranes for blotting were from Schleicher and Schuell (Dassel, Germany). Lab-Trol for protein determination is a product of Merz and Dade (Düdingen, Germany). Zymolyase-20T was purchased from Seikagaku Co. (Tokyo, Japan), and β-glucoronidase, poly-D-lysine, chemicals for the *p*-nitrophenyl phosphate assay, biotinylated sheep anti-rabbit IgG (whole molecule), F(ab′)$_2$ fragment, and alkaline phosphatase-conjugated goat anti-rabbit IgG (whole molecule) were from Sigma (St. Louis, MO). Indirect immunofluorescence detection was carried out using the ELFJ kit of Molecular Probes, Inc. (Eugene, OR). This kit also contains the CPCQ substrate. All other chemicals and biochemicals of the highest purity available were from Merck (Darmstadt, Germany) or Boehringer (Mannheim, Germany).

Strains and Media. *Escherichia coli* strain DH5αF′ (Gibco/BRL, Paisley, U.K.) and *E. coli* strain SURE (Stratagene, La Jolla, CA) were used for transformations with the plasmids pSPORT, LPSK, YEP1pT, and YEpNP2. Transformants were grown in LB medium supplemented with the appropriate antibiotics. Cells transformed with the LPSK plasmid were selected for their white color on LB-agar plates supplemented with 40 mg/L X-gal and with 9.5 mg/L IPTG. The *S. cerevisiae* strain MB192 (*8*) (haploid segregant from the diploid strain MB183 with an integrated 5.4 kb *Bam*HI−*Eco*RI fragment of pMB123; *MAT***a** *pho3-1* Δ*pho84*::*HIS3 ade2 leu 2-3,112 his3-532 trp1-289 ura3-1,2 can1*) was used for transformations with vectors YEpNP2 and YEpNP2-R215E by the lithium acetate method (*10*) and for the expression of wild-type or mutant NaPi-2. This yeast strain lacks L-tryptophan auxotrophy and grows only when yeast nitrogen base medium (YNB; 1.7 g of yeast nitrogen base without amino acids, 3.3 g/L $(NH_4)_2SO_4$, and 20 g of glucose) is supplemented with this amino acid. Tryptophan auxotrophy is provided to transformed cells by either vector YEpNP2 or YEpNP2-R215E, and transformants are therefore selected in YNB media without L-tryptophan.

Vectors. Experiments involving handling of DNA were performed according to standard published protocols (*11*). The pSPORT-1 vector that contained the cDNA encoding NaPi-2 was digested with *Not*I−*Sal*I to release a 2.5 kb fragment of cDNA that contained the entire coding region for NaPi-2 (*7*). For the insertion of the NaPi-2 cDNA into the yeast expression vector YEp1PT (*12*, *13*), the following strategy was employed. The bacterial vector pBluescript II SK (+) (Stratagene) was modified to contain a multiple cloning site in place of the multiple cloning site that is located between the *Sac*I (759) and *Kpn*I (657) restriction sites of the original plasmid. This plasmid was designated LPSK. An additional DNA fragment was inserted into the *Eco*72I site of the new multiple cloning site of LPSK by blunt end ligation. This fragment provided a *Not*I and a *Sal*I restriction site between two *Eco*RI sites. After digestion of LPSK with *Not*I and *Sal*I, the *Not*I−*Sal*I fragment containing the cDNA encoding NaPi-2 was ligated into the corresponding restriction sites of LPSK. The new plasmid was designated LPSK-NP2. After digestion of LPSK-NP2 with *Eco*RI, the cDNA fragment encoding NaPi-2 was ligated into the *Eco*RI sites of the yeast expression vector YEp1PT (*12*, *13*). The new vector, designated YEpNP2, contains a PGK promoter prior to the first *Eco*RI site, a PGK terminator after the second *Eco*RI site, and the 2 μm yeast origin of replication, and additionally provides tryptophan auxotrophy for yeast cells. It also contains a fragment of the pBR 322 plasmid from *E. coli* with its native origin of replication and provides resistance to ampicillin and tetracycline.

Mutation by Inverse PCR. The cDNA encoding the NaPi-2 cotransporter was inserted into the modified pBluescript II SK (+) plasmid LPSK mentioned in the previous paragraph, and mutations were introduced by inverse PCR (*14*, *15*) using oligonucleotides 5′-TTCAGGGAGGCTTTTGCAGGGGC-3′ and 5′-GTCAGTCCTGTC**GCCGGC**CTG-3′ as primers. The underlined sequence shows the area corresponding to the mutation R215E. The bold sequence indicates the area for insertion of a diagnostic *Nae*I restriction site. The protein sequence is not affected by this mutation. The amplified fragment was recircularized and was used for *E. coli* transformation. Plasmids from transformed cells carrying the *Nae*I restriction site were used for sequencing. Plasmids with the correct mutation were then digested with *Hin*dIII. The released *Hin*dIII−*Hin*dIII fragment of the mutated plasmid

[1] Abbreviations: CPCQ, 2-(5′-chloro-2′-phosphoryloxyphenyl)-6-chloro-4(3*H*)quinazolinone; ECL, enhanced chemiluminescence; EDTA, ethylenediaminotetraacetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IPTG, isopropyl β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; P_i, inorganic phosphate; PGK, phosphoglycerate kinase; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

that contains the R215E mutation was isolated from a 1% agarose gel and was used to replace the corresponding, nonmutant *Hin*dIII−*Hin*dIII fragment of the LPSK-NP2 plasmid. The correct orientation of the *Hin*dIII−*Hin*dIII cassette was verified by digestion with *Nae*I. After digestion with *Eco*RI, the mutated cDNA encoding NaPi-2 was purified and ligated with the YEp1PT plasmid (*12*, *13*) that had previously also been digested with *Eco*RI. The correct orientation of the insert was verified by digestion with *Eco*52I. YEp1PT plasmids harboring the mutated cDNA encoding NaPi-2 were designated YEpNP2-R215E and were used to transform *S. cerevisiae* strain MB192 (*10*).

Northern Analysis. A single colony of yeast cells transformed with YEpNP2 was grown overnight at 30 °C with vigorous shaking (200 rpm) in 5 mL of YNB medium. Thereafter, the culture was transferred to 100 mL of YNB and was allowed to grow for an additional 12 h. Nontransformed cells were grown in parallel in YNB medium supplemented with L-tryptophan. Isolation of total RNA was carried out according to the protocol of Rose et al. (*16*) with the following modifications. Lyophilized total RNA was dissolved and denatured in a buffer containing 48% (w/v) formamide. A total of 5 μg of RNA per lane was separated by electrophoresis on a 0.8% (w/v) agarose gel containing 2% (v/v) formaldehyde. Northern blot analysis was carried out according to standard protocols (*17*) on nitrocellulose membranes. Specific cDNA probes for the entire NaPi-2 coding region were labeled using the labeling kit of Pharmacia (Uppsala, Sweden) and [α-^{32}P]dCTP to yield a specific activity of 10^9 dpm/μg of DNA. After being blotted for 16 h, membranes were prehybridized for 4 h at 42 °C in a buffer consisting of 5× SSPE [0.75 M NaCl, 50 mM NaH_2PO_4, and 5 mM EDTA (pH 7.4) with NaOH], 5× Denhardt's solution (*11*), 20% (w/v) formamide, 0.1% (w/v) SDS, and 200 μg/mL herring sperm DNA. This was followed by hybridization with 50 nmol of ^{32}P-labeled NaPi-2 cDNA overnight at 42 °C. After being washed twice at room temperature and twice at 42 °C with 0.1× SSC/0.1% SDS [15 mM NaCl, 0.75 mM sodium citrate, and 0.1% (w/v) SDS (pH 7.0)], filters were exposed with intensifying screens for 2 days at −80 °C on Kodak X-Omat X-ray film.

Western Analysis of NaPi-2 Expressed in Yeast. Transformed or nontransformed yeast cells of the *S. cerevisiae* strain MB192 (*8*) were grown overnight in 100 mL of YNB medium alone or YNB medium supplemented with 0.1% (w/v) L-tryptophan. Preparation of yeast cell membrane microsomes was carried out as described previously (*12*, *18*). Microsomes were suspended in 25 mM imidazole/1 mM EDTA (pH 7.5) and homogenized on ice. The total amount of protein was determined by the Lowry method using Lab-Trol as a standard (*19*). Protein samples were dissolved in a buffer consisting of 125 mM Tris, 20% (v/v) glycerol, 2% (w/v) SDS, and 0.1% (w/v) bromophenol blue adjusted to pH 6.8, and SDS−PAGE was performed with 20 μg of protein per lane on a 7.5% (w/v) polyacrylamide gel (*20*). After separation, proteins were stained with Coomassie Brilliant Blue (Sigma). Proteins were immobilized on nitrocellulose membranes by semidry blotting at 0.8 mA/cm^2 according to the protocol of Kyhse-Andersen (*21*). Immunodetection of the expressed NaPi-2 protein was accomplished by incubation of membranes with a rabbit polyclonal anti-NaPi-2 antibody followed by a donkey anti-rabbit horseradish peroxidase-linked IgG in phosphate-buffered saline Tween [8% (w/v) NaCl, 0.2% (w/v) KCl, 4.0 mM Na_2HPO_4, 1.8 mM KH_2PO_4, and 0.1% (w/v) Tween 20, adjusted to pH 7.4], and bands were visualized by using the ECL Western blotting protocol.

Detection of NaPi-2 Expressed on the Yeast Cell Surface by Indirect Immunofluorescence. To ensure that wild-type or mutant NaPi-2 cotransporters are expressed and integrated in the plasma membrane, yeast cells of the *S. cerevisiae* strain MB192 containing the YEpNP2 or the YEpNP2-R215E plasmid were assayed for indirect immunofluorescence according to the protocols of Kilmartin and Adams (*22*) and Larison et al. (*23*), with the modifications described in detail previously (*24*). Nontransformed cells served as controls. Briefly, spheroplasts were prepared by incubating yeast cells in media containing β-glucoronidase and zymolyase-20T. Cells were then affixed in a saturating monolayer to microscope slides that had been coated previously with poly-D-lysine, and were incubated with a rabbit polyclonal anti-NaPi-2 antibody. After being washed, slides were incubated with biotinylated sheep anti-rabbit IgG. Finally, cells were treated with streptavidin linked to alkaline phosphatase and assayed for fluorescence by adding CPCQ (*23*), a substrate of alkaline phosphatase. Fluorescent cells were visualized under a fluorescence microscope using an excitation filter in the blue range of 330−450 nm and an emission filter open above 520 nm. For controls, either the primary antibody was omitted or spheroplasts from nontransformed yeast were used in the experiment.

Evaluation of the Expression Levels of Wild-Type or Mutant NaPi-2 on the Surface of Yeast Cells. To determine whether wild-type NaPi-2 and R215E NaPi-2 mutants are present in comparable numbers in the plasma membrane of transformed yeast cells, the following variation of an antibody capture assay (*25*) was employed: Spheroplasts of transformed yeast cells of the *S. cerevisiae* strain MB192 containing either the YEpNP2 or the YEpNP2-R215E plasmid were prepared as described above. Spheroplasts at saturating numbers were attached to the wells of poly-D-lysine-coated microtiter plates and incubated with an anti-NaPi-2 polyclonal antibody. For the control, the anti-NaPi-2 polyclonal antibody was omitted or spheroblasts from nontransformed yeast were used in the assay. After being washed, cells were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG and *p*-nitrophenyl phosphate following the protocol of the supplier. The amount of the product of the incubation of the alkaline phosphatase reaction, the *p*-nitrophenolate anion, can be determined by measuring the absorbance at 405 nm and employing the Lambert−Beer law $c = (E/d)\epsilon$, where E is the absorbance, c the concentration of *p*-nitrophenolate in moles per liter, and d the path length of the light beam in centimeters and the molar absorbance coefficient $\epsilon = 18\,500$ L mol^{-1} cm^{-1}.

Phosphate Uptake into Transformed Yeast Cells Expressing the NaPi-2 Cotransporter. Phosphate uptake by yeast cells was assessed by incubating transformed or nontransformed cells at room temperature in 400 mM glucose/10 mM HEPES (pH 7.5) supplemented with 5 μM [^{32}P]orthophosphate in the presence of various concentrations of NaCl. The volume of each reaction mixture was 1 mL, and osmolality was kept constant by supplementing with choline chloride. Cells were suspended at a density of 2.8×10^7/mL. This

cell density was used in all phosphate uptake experiments. After incubation for 10 min, the cell suspension was centrifuged at 12000*g* for 1 min in a benchtop centrifuge. The sedimented cells were washed twice with 1 mL of water at 4 °C and were assessed for incorporated radioactivity. The uptake of [^{32}P]orthophosphate in the absence of Na^+ was negligible after 10 min.

The phosphate concentration dependence of the phosphate uptake was measured similarly in the presence of 150 mM NaCl by incubating transformed or nontransformed cells for 10 min in glucose/HEPES buffer (pH 7.5) supplemented with various amounts of [^{32}P]orthophosphate.

To determine the reaction order, cells were incubated in glucose/HEPES buffer (pH 7.5) with 5, 10, or 20 μM [^{32}P]-orthophosphate for up to 1 h in the presence of 150 mM NaCl. At various times, 1 mL of the cell mixture was removed and the radioactivity of the cells was determined as described above. The level of uptake of P_i in the absence of NaCl was in the range of 5−10 pmol after 1 h, when 5 μM [^{32}P]orthophosphate were used extracellularly. With an extracellular [^{32}P]orthophosphate concentration of 20 μM, this value increased to 20−25 pmol. Thus, all values were corrected for these numbers. To estimate the concentration of radioactive orthophosphate in the cytosol, various aliquots of cells were spun for 10 min at 7000*g* in a hematocrit tube to determine their volume. From this volume, 26% was subtracted for the intercellular space and 12% for the space between the cell wall and plasma membrane (*26*).

Results from four determinations were averaged for each of the experiments described above. The specific activity of the probes and incorporated molar amounts were determined by internal standards and calibration curves.

RESULTS

Expression of the Na^+/P_i Cotransporter in S. cerevisiae. The YEpNP2 plasmid of 11 089 bp carrying the full-length cDNA encoding wild-type NaPi-2 (2440 bp) and the YEpNP2-R215E plasmid carrying the cDNA encoding the R215E mutant of NaPi-2 were used to transform *S. cerevisiae* strain MB192 (*8*, *10*). Cultures of these cells, and a control of nontransformed cells, were used subsequently to isolate either total RNA (*16*) or microsomal protein (*12*, *18*, *24*).

Northern analysis (*17*) of total RNA from transformed yeast cells using rat kidney cortex NaPi-2 cDNA as a hybridization probe revealed a band at 2.7 kb that was also detected in RNA isolated from kidney cortex (Figure 1A). Since an equivalent hybridization signal was not detected in the Northern hybridization with RNA from nontransformed yeast cells, one can conclude that full-length, polyadenylated mRNA is synthesized in yeast cells transformed with YEpNP2. The fact that the synthesized mRNA for NaPi-2 is translated into protein in yeast cells was verified by Western analysis with a polyclonal antibody (Figure 1B). Immunodetection of the immobilized protein by anti-NaPi-2 IgG and subsequent incubation with HRP-linked anti-rabbit IgG revealed a protein with an apparent molecular mass of approximately 75 kDa that is not present in membrane preparations from nontransformed yeast cells. This molecular mass is consistent with the NaPi-2 expressed in yeast being core-glycosylated, as is the case in kidney cortex tissue (*7*).

Insertion of Wild-Type and Mutant NaPi-2 into the Plasma Membrane of Yeast Cells. The localization of wild-type or

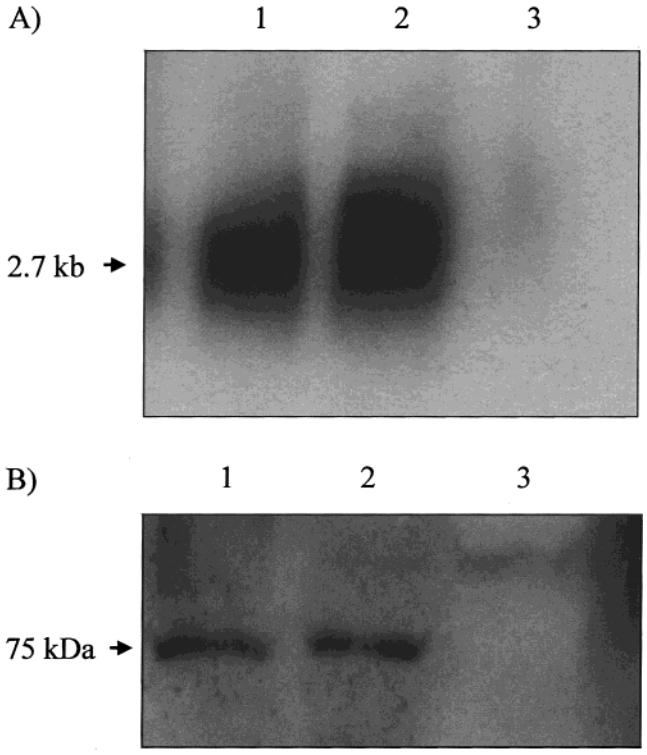

FIGURE 1: Expression of NaPi-2 in yeast. (A) Detection of NaPi-2-specific mRNA by Northern blot analysis. Total RNA was isolated from nontransformed yeast cells or cells transformed with either YEpNP2 or YEpNP2-R215E, and 5 μg per lane was separated by gel electrophoresis and transferred to nitrocellulose membranes (see Experimental Procedures). Detection of wild-type (lane 1) or mutant (lane 2) NaPi-2 mRNA was accomplished with a specific radioactive probe of full-length NaPi-2 cDNA. RNA isolated from nontransformed cells served as a control (lane 3). (B) Detection of NaPi-2 in yeast plasma membranes by Western blot analysis. Following separation on a SDS−polyacrylamide gel (20 μg of protein/lane), proteins were transferred to a nitrocellulose membrane (see Experimental Procedures). After incubation with anti-NaPi-2 antibody from rabbit, immunodetection was performed with the ECLJ system: lane 1, wild-type NaPi-2; lane 2, R215E mutant; and lane 3, nontransformed yeast.

R215E mutant NaPi-2 on the cell surface of transformed yeast was carried out by indirect immunofluorescence using the ELF kit of Molecular Probes, Inc. The protocol has been previously employed for the detection of β-galactosidase−sodium pump hybrid proteins on the surface of yeast (*23*). The principle of this detection method is based on the production of a fluorescent precipitate on the extracellular surface of the yeast cells. Yeast cells expressing either wild-type or mutant NaPi-2 were incubated first with a polyclonal antibody against the transporter and subsequently with a biotinylated secondary antibody. The successive addition of streptavidin linked to alkaline phosphatase, and the alkaline phosphatase substrate CPCQ led to the formation of a fluorescent precipitate on the surface of yeast cells expressing either the wild type or the R215E mutant of NaPi-2 (Figure 2). A similar result was not obtained with nontransformed yeast cells or when the primary antibody was omitted.

Evaluation of the Abundance of Wild-Type and Mutant NaPi-2 on the Surface of the Yeast Plasma Membrane. To ensure that possible differences in the properties of wild-type and mutant NaPi-2 were not due to differences in the number of transporters expressed and inserted in the plasma

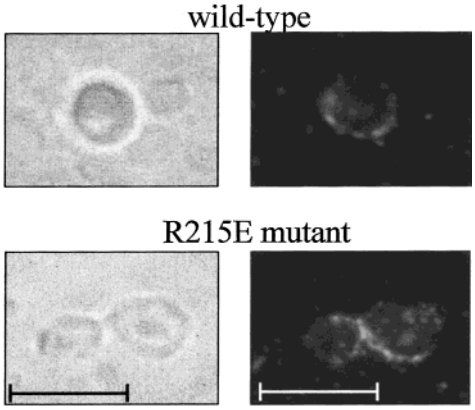

FIGURE 2: Detection of wild-type and R215E mutant NaPi-2 on the surface of yeast cells by indirect immunofluorescence. The pictures on the left show yeast cells attached to poly-D-lysine-coated slides in visible light. Wild-type NaPi-2 and the R215E mutant of the cotransporter can be detected on the surface of these cells (pictures on the right) by a primary polyclonal antibody against rat kidney cortex NaPi-2 and the ELF system that includes the fluorogenic substrate CPCQ (see Experimental Procedures). The fluorescent precipitate on the surface of the cells is not obtained when the primary antibody is omitted or when nontransformed yeast cells are used in the reaction. The bar represents 5 μm.

membrane but rather due to alterations occurring upon mutation, it was important to obtain information about the abundance of the two proteins on the yeast surface. Since radioligands that would allow the estimation of the precise number of NaPi-2 molecules are not available, we employed a modification of the ELF protocol to determine the relative abundance of the wild-type or mutant transporter on the cell surface. A saturating number of yeast cells expressing either wild-type or R215E mutants of NaPi-2 were first incubated with a polyclonal antibody against the transporter and subsequently with alkaline phosphatase-conjugated secondary antibody. As shown in Figure 3, the amount of *p*-nitrophenolate produced from *p*-nitrophenyl phosphate on the surface of yeast cells expressing either wild-type or R215E mutant NaPi-2 was the same in both cases. Hydrolysis of *p*-nitrophenyl phosphate was not achieved under these conditions with nontransformed cells.

Uptake of Phosphate by Yeast Cells Expressing Wild-Type or Mutant NaPi-2. In the presence of 5 μM phosphate (Tris form), yeast cells expressing the wild-type NaPi-2 cotransporter exhibited a Na^+-stimulated phosphate uptake (Figure 4). The $K_{0.5}$ obtained for the promotion of phosphate uptake was 5.6 ± 1.1 mM NaCl (Table 1). The maximum uptake of phosphate (649 ± 30 pmol/10 min) was approximately 8-fold higher than that obtained with nontransformed yeast cells (76.8 ± 8 pmol/10 min) (Figure 4). Yeast cells expressing the R215E mutant of NaPi-2 accumulated about 3 times more phosphate (213 ± 9 pmol/10 min) than the nontransformed cells and approximately 3 times less than the cells expressing the wild-type transporter under the same conditions. The $K_{0.5}$ obtained for Na^+ stimulation of phosphate uptake by the mutant was 4.2 ± 0.8 mM NaCl (Table 1), which was comparable to that obtained for the wild-type transporter.

Phosphate uptake in yeast cells that express the wild-type NaPi-2 in the presence of 150 mM NaCl was promoted by extracellular P_i with a $K_{0.5}$ of 45 ± 4 μM (Figure 5 and Table 1). A phosphate-dependent phosphate accumulation was also

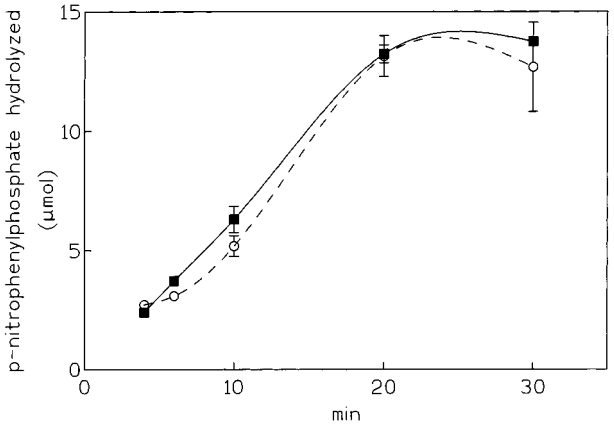

FIGURE 3: Evaluation of the abundance of wild-type and mutant NaPi-2 on the yeast cell surface. A similar, saturating number of spheroplasts expressing either wild-type (■) or the R215E mutant of the NaPi-2 cotransporter (○) were attached to the wells of poly-D-lysine-coated microtiter plates and incubated with rabbit IgG against the NaPi-2 protein (see Experimental Procedures). Afterward, cells were incubated with an alkaline phosphatase-conjugated antibody against rabbit IgG, and at the indicated times, the extent of formation of *p*-nitrophenolate from *p*-nitrophenyl phosphate was determined at 405 nm. When the primary antibody is omitted, or when nontransformed yeast cells are used in the reaction, the formation of *p*-nitrophenolate is negligible. Data points represent means ± the standard error of the mean of results from four independent experiments.

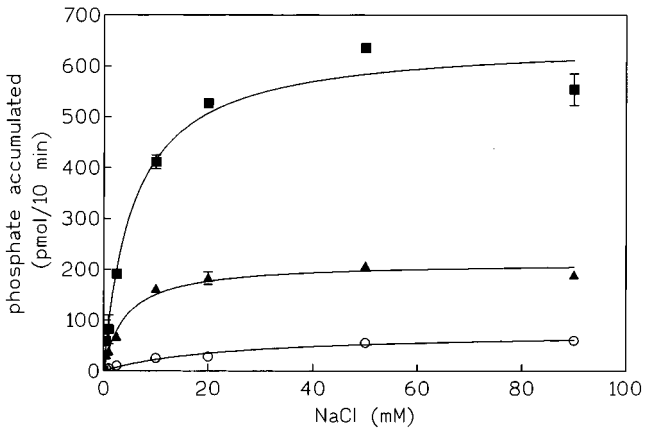

FIGURE 4: Uptake of P_i as a function of extracellular Na^+ concentration. Yeast cells expressing the wild-type NaPi-2 cotransporter show a Na^+-stimulated phosphate uptake with a $K_{0.5}$ value of 5.6 ± 1.1 mM (■). The maximum uptake of phosphate is approximately 8 times greater than that obtained with nontransformed yeast cells (○). Yeast cells expressing the R215E mutant of NaPi-2 (▲) accumulate about 3 times more phosphate than the nontransformed cells and approximately 3 times less than the cells expressing the wild-type transporter under the same conditions. The $K_{0.5}$ for the stimulation of phosphate uptake by the mutant is 4.2 ± 0.8 mM NaCl. Each value is the mean ± the standard error of the mean of results from four independent experiments and reflects the accumulation of P_i into 2.8×10^7 cells suspended in 1 mL of the reaction mixture.

observed with cells expressing the R215E mutant, but the $K_{0.5}$ for P_i (86 ± 5 μM) was considerably higher. The uptake of P_i by the nontransformed cells was also promoted by P_i, but the maximum level of incorporation of P_i (1.13 ± 0.1 nmol/10 min) was almost 6-fold lower than that obtained with cells expressing the wild-type NaPi-2 (6.35 ± 0.2 nmol/10 min) and approximately 3-fold lower than the level of uptake obtained with the cells expressing the R215E NaPi-2 mutant (2.8 ± 0.14 nmol/10 min) (Figure 5).

Table 1: Synopsis of the Kinetic Data Obtained with Nontransformed Yeast and with Cells Expressing either the Wild-Type or the R215E Mutant of the Renal NaPi-2 Sodium/Phosphate Cotransporter[a]

experiment	experimental conditions	parameter	nontransformed yeast	wild-type NaPi-2	R215E mutant of NaPi-2
Na^+-stimulated P_i uptake	5 μM P_i, 0−150 mM NaCl, 2.8×10^7 cells, incubation for 10 min	$K_{0.5}$ for Na^+ (mM)	nd[b]	5.6 ± 1.1	4.2 ± 0.8
		maximum P_i uptake (μmol)	76.8 ± 8	649 ± 30	213 ± 9
P_i-stimulated P_i uptake	0−400 μM P_i, 150 mM NaCl, 2.8×10^7 cells, incubation for 10 min	$K_{0.5}$ for P_i (μM)	nd	45 ± 4	86 ± 5
		maximum P_i uptake (nmol)	1.13 ± 0.1	6.35 ± 0.2	2.8 ± 0.14
time course of P_i-stimulated P_i uptake	5, 10, or 20 μM P_i, 150 mM NaCl, 2.8×10^7 cells, incubation for 1 h	rate constant k for P_i uptake (min^{-1})	0.0075	0.06	0.016

[a] Values are means ± the standard error of the mean of results from four independent experiments. [b] Not determined.

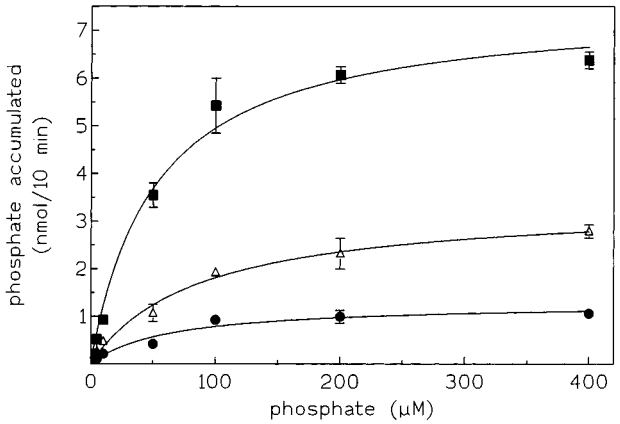

FIGURE 5: Uptake of phosphate as a function of extracellular P_i concentration. Phosphate uptake in yeast cells that express the wild-type NaPi-2 (■) is promoted by extracellular P_i with a $K_{0.5}$ of 45 ± 4 μM. With cells expressing the R215E mutant (△), the equivalent value is 86 ± 5 μM. The uptake of P_i by the nontransformed cells (●) is also promoted by P_i, but the maximum level of incorporation of P_i is 1.13 ± 0.1 nmol/10 min and therefore almost 6-fold lower than that with cells expressing the wild-type NaPi-2 (6.35 ± 0.2 nmol/10 min) and approximately 3-fold lower than that obtained with the cells expressing the R215E NaPi-2 mutant (2.8 ± 0.14 nmol/10 min). Each value is the mean ± the standard error of the mean of results from four independent experiments and reflects the accumulation of P_i into 2.8×10^7 cells suspended in 1 mL of the reaction mixture.

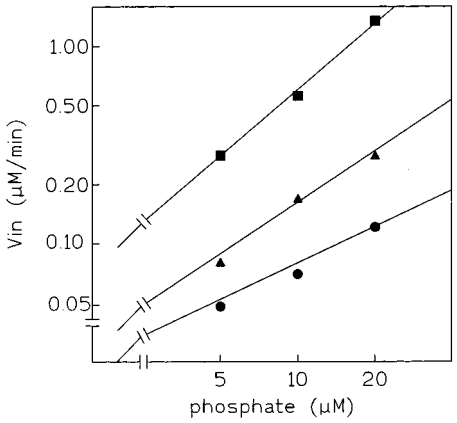

FIGURE 6: Determination of the reaction order n and the reaction rate constant k. To determine the order of the reaction for phosphate uptake, yeast cells were incubated with 5, 10, or 20 μM [^{32}P]-orthophosphate (Tris form) in the presence of 150 mM NaCl and the phosphate uptake was measured for 1 h. Since the phosphate uptake is linear (not shown), the initial velocity (V_{in}) is described by the formula $V_{in} = kA^n$, whereas k is the rate constant, A the concentration of phosphate, and n the order of the reaction. A plot of log V_{in} against log A gives a straight line with the slope n. In all three cases, n is approximately 1 and therefore phosphate uptake follows first-order reaction kinetics. Thus, k can be calculated from the formula $k = V_{in}A^{-1}$. The rate constant for phosphate uptake into cells expressing the wild-type NaPi-2 cotransporter is 0.06 min^{-1} (■). The rate for phosphate uptake into cells expressing the R215E mutant is 0.016 min^{-1} (▲). Finally, the rate for phosphate uptake into nontransformed cells is 0.0075 min^{-1} (●).

To determine the order of the reaction for phosphate uptake, yeast cells were incubated with 5, 10, or 20 μM [^{32}P]-orthophosphate (Tris form) in the presence of 150 mM NaCl, and phosphate uptake was measured for 1 h. Over this entire period of time, the initial velocity (V_{in}) for phosphate uptake was linear (not shown) and could be described by the formula $V_{in} = kA^n$, where k is the rate constant, A the concentration of phosphate, and n the order of the reaction. A plot of log V_{in} against log A gives a straight line with the slope n. With cells expressing the wild-type NaPi-2 cotransporter, the slope of the straight line was 1.12 (Figure 6). With nontransformed cells or with cells expressing the R215E mutant of the cotransporter, the values were 0.8 and 0.9, respectively (Figure 6). Thus, in all three cases, the uptake of phosphate followed first-order reaction kinetics, and therefore, $k = V_{in}A^{-1}$. Calculated from this formula, the rate constant for phosphate uptake into cells expressing the wild-type NaPi-2 cotransporter is 0.06 min^{-1} (Table 1). The rate constant for phosphate uptake into cells expressing the R215E mutant is 0.016 min^{-1}, and for phosphate uptake into nontransformed cells 0.0075 min^{-1}.

DISCUSSION

The aim of the study presented here was to develop a cellular expression system for the investigation of structure−function relationships of the Na^+/P_i cotransporter that might lead to an understanding of the mechanisms involved in the simultaneous translocation of Na^+ and phosphate. Preference for an expression system was given to the yeast *S. cerevisiae* for several reasons. First, yeast genetics are well-known, and growth conditions or transfection methods with plasmids are well-established. Second, yeast has been used on many occasions as a heterologous expression system for the successful expression of various mammalian plasma membrane proteins. Third, and perhaps most importantly, yeast strains are available that lack the high-affinity plasma membrane H^+/P_i cotransporter, one of the yeast's endogenous phosphate transporters.

The decision regarding which amino acids should be selected for mutation to obtain information about their role

in Na^+ and phosphate recognition was more difficult, since no information about areas of the protein involved in the translocation process is yet available. Thus, a selection was made by taking into consideration the predicted two-dimensional insertion of the NaPi-2 protein within the plasma membrane (*7*). According to this prediction, which is based on hydropathy plots (*27*), a large hydrophilic loop between M3 and M4 membrane spans is localized on the extracellular side of the plasma membrane. Within this stretch of the protein (^{186}P−^{346}D) are seven arginine and five lysine residues, some of which might participate in P_i recognition (*1*). Although this high number of positively charged amino acids does not make the decision much easier, preference was given to ^{215}R, which is localized in close proximity to two other arginines within the peptide ^{210}**R**TDF**RR**AF^{217}, assuming that this cluster of positive charges might be involved in interactions of the NaPi-2 cotransporter with phosphate.

Full-length cDNA molecules (2440 bp) encoding either the wild type of rat renal cortex NaPi-2 cotransporter or the R215E mutant were inserted into the yeast expression plasmid YEp1PT, and each of the resulting YEpNP2 or YEpNP2-R215E plasmids of 11 089 bp was then used for transformation of yeast cells. Northern analysis with total RNA from yeast cells transformed with either YEpNP2 or YEpNP2-R215E using rat kidney cortex NaPi-2 cDNA as a hybridization probe revealed a band at 2.7 kb (Figure 1A). Since an equivalent hybridization signal cannot be detected in the Northern hybridization with RNA from nontransformed yeast cells (Figure 1A) but is detected in RNA isolated from kidney cortex (not shown), one has to conclude that full-length, polyadenylated mRNA is synthesized in yeast cells transformed with YEpNP2 or YEpNP2-R215E. Furthermore, the synthesized mRNA for wild-type or mutant NaPi-2 is translated into protein. This was demonstrated by probing microsomal proteins from transformed yeast cells in a Western blot with a polyclonal anti-NaPi-2 IgG (Figure 1B). The proteins that interact with the antibodies each have a relative molecular mass of approximately 75 kDa. Since the predicted molecular mass of NaPi-2 is approximately 69 700 g/mol, the slightly higher molecular mass detected in the Western blot is consistent with the expressed wild-type or mutant NaPi-2 in yeast being core-glycosylated, as is the native protein from kidney cortex. A protein with a similar molecular mass is not detected in microsomal preparations from nontransformed yeast cells (Figure 1B).

Detection of the expressed cotransporters in microsomal preparations, however, does not guarantee that the proteins are indeed inserted in the plasma membrane of the yeast cells. Using a polyclonal antibody against rat kidney cortex NaPi-2 in an indirect immunofluorescence detection method, it was possible to detect wild-type and mutant cotransporters on the surface of transformed yeast cells (Figure 2). This method, which also involves the use of a secondary antibody coupled to alkaline phosphatase by biotin−streptavidin, allows the indirect visualization of plasma membrane-embedded NaPi-2 cotransporters by using the fluorogenic substrate of alkaline phosphatase, CPCQ. The precipitation of fluorescent product is only observed with yeast cells that express either the wild-type or the R215E mutant of NaPi-2. Since the fluorescent precipitate is not obtained for nontransformed yeast cells or for transformed cells when the primary antibody is omitted, one has to conclude that a fluorescent signal indicates that the cotransporters are inserted into the yeast plasma membranes.

The relative abundance of the wild-type or mutant NaPi-2 cotransporters was estimated by applying an antigen capture assay (*25*). The method is similar to the one used for the detection of the cotransporters on the surface of the yeast plasma membrane. Here, however, the substrate of the alkaline phosphatase is *p*-nitrophenyl phosphate, which is hydrolyzed by the enzyme to *p*-nitrophenolate and P_i. The amount of *p*-nitrophenolate that is produced is directly dependent on the amount of alkaline phosphatase present on the surface of the yeast plasma membrane and therefore also dependent on the amount of NaPi-2 cotransporters. Thus, by assessing the time course of *p*-nitrophenolate production, one can attain information about the relative abundance of wild-type and mutant NaPi-2 on the surface of the yeast plasma membrane. The results depicted in Figure 3 show that wild-type and mutant NaPi-2 are present on the surface of the plasma membrane of yeast cells in very comparable numbers, since no differences in *p*-nitrophenolate production were observed between the two cell lines. When the primary antibody was omitted, or nontransformed yeast cells were used in the same experiment (not shown), *p*-nitrophenyl phosphate hydrolysis did not occur, demonstrating the specificity of the reaction.

To determine whether the wild-type NaPi-2 is functional in the yeast and to investigate a possible effect of the R215E mutation on the activity of the cotransporter, [^{32}P]orthophosphate uptake into yeast cells was investigated under various conditions. With nontransformed yeast cells, phosphate uptake is only slightly stimulated by Na^+ (Figure 4). Although phosphate transport across yeast plasma membranes has not yet been fully elucidated, it appears that P_i uptake by yeast cells is driven by a H^+ rather than by a Na^+ gradient (*28*−*30*). Two different transporting systems have been identified as being involved in supplying the cell with P_i; one of them is a high-affinity and the other a low-affinity H^+/P_i cotransporter (*30*). The high-affinity cotransporter, however, is not present in the yeast strain MB192 used here (*8*, *9*). Whether the Na^+ stimulation of phosphate uptake observed here for nontransformed cells reflects the existence of an additional endogenous Na^+/P_i cotransporter in the yeast plasma membrane, or whether the endogenous low-affinity H^+/P_i cotransporter can also be activated by Na^+, or whether the observed Na^+ activation is due to other, indirect mechanisms cannot be determined at this stage.

With yeast cells expressing the wild-type NaPi-2, Na^+ promotes phosphate uptake with a $K_{0.5}$ of 5.6 ± 1.1, and the level of phosphate accumulation in the cells reaches a maximum that is approximately 8 times higher than that observed with nontransformed cells (Figure 4 and Table 1). This very significant increase in the level of phosphate accumulation suggests that the NaPi-2 cotransporter is expressed in a functional form. In contrast, however, to the results obtained with renal brush border membrane vesicles from rats (*31*) and other species (*32*), the Na^+-stimulated phosphate uptake by the yeast does not follow sigmoidal but rather simple saturation kinetics. From experiments performed in *Xenopus* oocytes, the sigmoidal kinetics were interpreted to indicate that two sodium ions are required in a stoichiometric relation to one phosphate ion to promote

the transport process (*7*). Although plausible, the sigmoidal curves might also be due to the interaction of the NaPi-2 cotransporter with other cellular proteins involved in the regulation of P_i uptake. These proteins might be present in *Xenopus* oocytes but are more likely to be absent in the yeast. This interpretation is supported by the recent identification of such NaPi-2-stimulating proteins in *Xenopus* (*32*). Alternatively, since uptake was determined after 10 min in our experiments in contrast to the 1 min with *Xenopous* oocytes, one could suggest that the longer incubation time might mask sigmoidicity. Nevertheless, the $K_{0.5}$ value of 5.6 ± 1.1 mM determined for the sodium stimulation of phosphate uptake into transformed yeast cells clearly differs from the K_d of 42 ± 7 mM determined for Na^+ stimulation of phosphate uptake into *Xenopus* oocytes (*7*). The $K_{0.5}$ in yeast, however, is similar to the $K_{0.5}$ value of 5.9 ± 0.7 mM determined for Na^+ stimulation of phosphate uptake into goat brush border membrane vesicles (*3*).

Cells expressing the R215E mutant of NaPi-2 also exhibit Na^+-stimulated P_i uptake (Figure 4 and Table 1). The $K_{0.5}$ for Na^+ is 4.2 ± 0.8 mM and therefore within the same range as that obtained with cells expressing the wild-type cotransporter (Table 1). Thus, the mutation does not seem to influence the relative affinity of the transporter for Na^+. The maximal amount of accumulated P_i, however, is about 3-fold lower than that obtained with cells expressing the wild-type NaPi-2 cotransporter. From the experiments whose results are depicted in Figures 2 and 3, it appears to be unlikely that the difference is due to the abundance of the mutant cotransporter on the surface of the yeast cells being lower than the abundance of wild-type NaPi-2. It is more likely that the mutation influences the turnover rate of the NaPi-2 cotransporter. Consistent with this hypothesis is the finding that the first-order rate constant for phosphate uptake into cells expressing the wild-type NaPi-2 ($k = 0.06$ min^{-1}) is 3.75 times greater than the rate constant ($k = 0.016$ min^{-1}) obtained for the uptake of phosphate into cells expressing the mutant (Table 1 and Figure 6).

Phosphate uptake as a function of the phosphate concentration in the presence of saturating concentrations of sodium is considerably elevated when yeast cells express the wild-type NaPi-2 (Figure 5). In the presence of 150 mM NaCl, phosphate promotes the uptake of phosphate with a $K_{0.5}$ of 45 ± 4 μM (Table 1). This value is about 3 times lower than the K_M of 130 ± 15 μM obtained for the interaction of P_i with NaPi-2 cotransporters expressed in *Xenopus* oocytes (*7*). Possibly, variations between yeast and animal cell plasma membranes influence the activity of the transporter and account for the differences between results from this study and the previous study (*7*). On the other hand, it might also be that animal cell-specific proteins involved in regulation of NaPi-2 activity (*32*) are missing in yeast, thus allowing a shift of the relative affinity of the cotransporter toward lower P_i concentrations.

A phosphate-dependent phosphate accumulation is also observed with cells expressing the R215E mutant, but the maximum uptake of P_i is considerably lower (2.8 ± 0.14 nmol/10 min) than the uptake obtained with cells expressing the wild-type cotransporter (6.35 ± 0.2 nmol/10 min). Since wild-type or mutant NaPi-2 cotransporters are present in the plasma membrane in comparable amounts, the differences in the first-order rate constant for P_i uptake will account, at least in part, for the differences in the maximum P_i uptake. The $K_{0.5}$ of the mutants for P_i, however, is 86 ± 5 μM and therefore about 2 times higher than that obtained with the wild-type NaPi-2. Thus, the mutation, which did not have an effect on Na^+ recognition (Figure 4), clearly influences the interactions of the cotransporter with P_i. The fact that the uptake of P_i is not totally disrupted, despite the rather extreme change associated with the replacement of a positively charged amino acid by one that is negatively charged, clearly shows that ^{215}R is not essential for P_i recognition but is rather a part of a larger structure that is involved in the recognition−translocation process. This larger structure possibly includes part of the extracellular peptide sequence localized between membrane spans M3 and M4. Multiple mutation of positively charged amino acids and other amino acids of this region should help to resolve future questions concerning the mechanism Na^+/P_i cotransport and eventually of cotransport mechanisms in general.

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