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Immunodetection of a type III sodium-dependent phosphate cotransporter in tissues and OK cells

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Abstract

Polyclonal antibodies were raised in rabbits against a 14-amino acid portion of the gibbon ape leukemia virus human membrane receptor Glvr-1. This epitope also contained seven amino acids common to the receptor for the amphotropic murine retrovirus Ram-1. Antibody specificity and molecular size of Glvr-1/Ram-1-related proteins were assayed by Western blot. Using a standard Laemmli buffer system, under reducing conditions, a single band of approximately 85 kDa (designated p85) was immunodetected in membranes prepared from opossum kidney (OK) cells and in brain membranes from rat, rabbit and hamster. In mouse brain, p85 as well as a protein of 70–72 kDa were immunodetected. This protein was also present in several other mouse tissues. Limited proteolysis of p85 and the 70–72 kDa-protein from mouse yielded similar peptide fragments, suggesting that both proteins are related. Fragments of the same molecular masses were also detected in OK cell membranes following proteolysis, showing that p85 in both models (mouse brain and OK cell) share a similar sequence. p85 is not *N*-glycosylated since an assay using endoglycosidase F/*N*-glycosidase F did not alter the electrophoretic mobility of p85. We also observed that regulation of phosphate transport by incubating OK cells without any phosphate or by PTH treatment occurs without any changes in the amount of p85. In conclusion, these data demonstrate for the first time a Western blot detection of a type III phosphate transporter using polyclonal antibodies. They also suggest that, conversely to type I and type II phosphate transporters which are localized in the kidney, this third type of transporter is ubiquitous and probably absorbs the readily available phosphate from interstitial fluid for normal cellular functions in many species and tissues, serving as a housekeeping Na^+/P_i cotransport system. This is also the first report showing that p85 is not regulated in the same manner as type II phosphate transporters.

Keywords: Type III phosphate transporter; Constitutive phosphate transporter; Immunodetection; Polyclonal antibody

1. Introduction

Inorganic phosphate (P_i) is essential for cellular metabolism, signal transduction, regulation of protein enzymatic activities, nucleic acid and lipid synthesis. Regulation of phosphate homeostasis depends primarily on renal proximal tubular phosphate reabsorption [1]. About 80% of P_i in glomerular filtrate is reab-

Abbreviations: β-EtSH, β-mercaptoethanol; BBM, brush-border membrane; ECL, enhanced chemiluminescence; Glvr-1, cell-surface receptor for gibbon ape leukemia virus; OK, opossum kidney; P_i, inorganic phosphate; PTH, parathyroid hormone; PVDF, polyvinylidene difluoride; Ram-1, cell-surface receptor for amphotropic murine retrovirus

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sorbed within the proximal tubule, which is the primary site of physiological regulation of phosphate reabsorption [2]. Although the cellular mechanisms underlying phosphate reabsorption are complex and regulated by many factors, the main site of renal P_i reabsorption is the brush-border membrane (BBM) of the proximal tubule, involving predominantly a specific sodium/phosphate cotransport system [3]. A family of sodium-dependent phosphate transporters was found by expression cloning in Xenopus laevis oocytes: type I and type II Na–P_i cotransport systems (i.e. NaP_i-1 for type I and NaP_i-2,3,4,5,6 for type II) [4–8].

In addition to these two classical, well characterized types of transporters, a third type was found. It includes cell-surface virus receptors that mediate infections of gibbon ape leukemia viruses (GALV) and of mouse amphotropic retrovirus for gibbon ape leukemia virus, designated Glvr-1 and Ram-1, respectively. These membrane proteins have been cloned, sequenced and expressed in Xenopus laevis oocytes and in mammalian cells, and were found to act as phosphate carriers [9–11]. In addition to the broad tissue distribution of GVLR-1 and Ram-1 including bone marrow, thymus, brain, kidney, lung, liver and heart from rat [10], Glvr-1 mRNA is also present in a wide variety of animal species [12]. Therefore, this ubiquitous third type of phosphate cotransporter could provide a major pathway for phosphate uptake into many mammalian cells. It has been suggested that Glvr-1 and Ram-1 may act as constitutive sodium/phosphate cotransporters present in most cell types to absorb the readily available phosphate from surrounding fluids for normal cellular functions [10]. These three types of transporters share little amino acid sequence homology with each other [10] and probably have different modes of regulation. For example, type I and II are mainly found in kidney cortex while the type III phosphate transporters are present in various tissues. Furthermore, it has been clearly shown that type II is upregulated following depletion of extracellular phosphate [13]. Although a three to five-fold overexpression of Glvr-1 and Ram-1 mRNAs in rat 208F fibroblasts following P_i deprivation [10] has been reported, overexpression of the proteins following low-phosphate diet has never been presented. A recent report has shown the presence of Glvr-1 mRNA in osteoblast-like cells, and has suggested a key role for this transporter in phosphate handling by osteogenic cells [14].

Opossum kidney (OK) cells, a continuous renal tubular epithelial cell line, are useful experimental model to investigate the cellular mechanisms involved in the regulation of phosphate reabsorption (e.g. adaptation to phosphate restriction) in isolation from the complex systemic factors [3,15]. In the current report, we used biochemical and immunochemical approaches to detect and characterize type III phosphate transporters in OK cells and brain membranes from various species.

2. Materials and methods

2.1. Immunization and antibody purification

The peptide GDSGDKPLRRNNSY, corresponding to a portion of Glvr-1 ranging from amino acid 408–421, was synthesized using MAPS chemistry and was obtained from Service de Séquence de Peptides de l'Est du Québec (Centre Hospitalier de l'Université Laval, Que.) at a purity $> 85\%$. Seven of these amino acids also correspond to a portion of Ram-1, the receptor for amphotropic murine retrovirus. On day 0, three rabbits were injected subcutaneously at two sites on the back and intramuscularly at two sites on the rear hind limbs with a total of 400 μg of the Glvr-1 peptide mixed with Freund's complete adjuvant (Pierce, Rockford, IL) in a 1:1 volume ratio, i.e. 0.5 ml adjuvant + 0.5 ml of a solution containing 400 μg peptide. Booster injections with the same amount of antigen were administered on day 34 using Freund's incomplete adjuvant. The final injection (day 50) consisted of 400 μg of peptide adsorbed to aluminum hydroxide (Imject Alum, Pierce) administered intraperitoneally at two sites in the lower abdomen. The rabbits were bled 18 days after the final injection. The blood was allowed to coagulate at room temperature for 1 h and stored overnight at 4°C to allow the clot to retract. The serum was collected after removal of blood cells by centrifugation at $10\,000 \times g$ for 10 min. It was adjusted to pH 8.6 with Tris/HCl and, to collect the IgG, passed through a 22-ml protein A Sepharose 4 Fast Flow column (Pharmacia, Dorval, Que.) equilibrated with 50 mM Tris/HCl, pH 8.6 using a Fast Protein Liquid Chromatography apparatus (FPLC,

Pharmacia, Dorval, Que.). The column was washed extensively with the same buffer and IgGs were eluted with 0.2 M glycine, pH 2.2. The fractions (4 ml) were collected in 0.6 ml of 1 M Tris/HCl, pH 9. The antibodies were further purified by affinity chromatography on a peptide–Sepharose column (4 ml) prepared as described by Mumby and Gilman [16] and equilibrated with 100 mM NaCl, 20 mM Tris/HCl, pH 7.5 (buffer A). After extensive washing with buffer A, the antibodies were eluted from the column with 0.2 M glycine, pH 2.2. The fractions were collected as described for the protein A column. Neutralized fractions that contained proteins, as determined by absorbance at 280 nm, were pooled, dialyzed overnight against buffer A and adjusted to the desired concentration by ultrafiltration.

2.2. OK cell membranes preparation and treatments

Wild type OK cells, a gift from W. Pfaller (University of Innsbruck, Austria), were initially cultured in DMEM/F12 (Gibco) buffered with 15 mM Hepes/14 mM bicarbonate, containing 50 U/ml penicillin, 50 μg/ml streptomycin and 10% fetal bovine serum (FBS). Cells were used between passages 110 and 130. Cells were plated in 150 mm tissue culture dishes (Corning) and incubated in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. They were studied after 6–8 days at which time they were confluent. Membranes were prepared from cells that had been washed once with ice-cold 150 mM NaCl/5 mM Tris–HCl, pH 7.2 and once with 5 mM Hepes–KOH, pH 7.2. Cells were scraped from dishes into the latter solution and homogenized by passing six times through a number 18 needle. Nuclei and cell debris were removed by centrifugation for 5 min at $279 \times g$. The supernatant was then centrifuged for 30 min at $48\,000 \times g$ and the pellets resuspended in 5 mM Hepes–KOH, 0.5 mM EDTA, pH 7.2 [17].

The effects of high and low phosphate on OK cells were examined by incubating cells with 0 or 1 mM phosphate. In one experiment, cells were incubated for 6 h without FBS in DMEM containing 0.1% bovine serum albumin and either 0 or 1 mM phosphate before measuring uptake and isolating membranes. In another experiment, they were incubated for the first four days with DMEM/F12, 10% FBS followed by three days in DMEM 10% FBS. These cells were then incubated for 19 h in DMEM 0.1% BSA with either 0 or 1 mM phosphate. The effect of parathyroid hormone was examined in cells fed DMEM/F12 10% FBS for seven days followed by 18 h with 0.1% BSA replacing the FBS. These cells were then exposed for 2 h to 10^{-8} M PTH or its vehicle.

2.3. Phosphate uptake measurements

For measurement of ^{32}P uptake, cells were washed two times with a buffer containing 137 mM NaCl, 5.4 mM KCl, 1.2 mM $MgSO_4$, 2.8 mM $CaCl_2$, 10 mM Hepes/Tris, pH 7.4. The buffer was prewarmed to 37°C. Na-dependent uptake was measured at 37°C for 5 min by adding 0.1 mM $K_2H^{32}PO_4$ (1 μCi/ml) to the buffer. Uptake was terminated by aspirating the buffer and washing the cells four times with 1 ml of ice-cold buffered saline (145 mM NaCl, 10 mM Tris/HCl, pH 7.4). An aliquot of cells solubilized in 0.5 M NaOH was used for scintillation counting after being neutralized with 1 M CH_3COOH. Another aliquot was used for protein determination by the Lowry method. Each day measurements were carried out in duplicate or triplicate plates of cells and the results are given for the mean of at least three such experiments.

2.4. Preparation of crude membranes

Brain from human, rat, rabbit, hamster and several tissues from mouse (intestine, liver, spleen, kidney, testis, pancreas, heart, thymus, lungs, muscle and brain) were isolated and homogenized with a Polytron homogenizer (Brinkman, Switzerland). Intact cells were removed by centrifugation at $1000 \times g$ for 10 min. The supernatant was then centrifuged at $33\,000 \times g$ for 30 min to yield a pellet enriched in crude membranes. This pellet was resuspendend in buffer composed of 50 mM mannitol, 20 mM Hepes/Tris pH 7.5, and the samples were kept at −80°C until they were used.

2.5. Western blot analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was performed with a Mini-Protean II electrophoresis apparatus (Bio-Rad, Mississauga, Ont.) and the Laemmli buffer system [18]. The separating gel contained 7.5% (w/v) acryl-

amide. Unless indicated otherwise, prior to electrophoresis, the protein samples were mixed with Laemmli's sample buffer without boiling. Electrophoresis was carried out at a constant current of 10 mA/0.75 mm gel. Molecular mass determinations were based on a calibration curve prepared with standard proteins (Bio-Rad). Following electrophoresis, the proteins were transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes (Millipore, Mississauga, Ont.) with a Milliblot Graphite Electroblotter I apparatus (Millipore, Mississauga, Ont.) at a constant current of 1 mA/cm^2 for 1 h at room temperature. Hydrophobic or non-specific binding sites of PVDF membranes were blocked overnight in 150 mM NaCl, 0.2% (w/v) Tween 20 and 50 mM Tris/HCl, pH 7.5 (buffer B), at 4°C. The membranes were brought to room temperature and incubated for 1 h with the anti-peptide antibody diluted 1/10 000 in buffer B. The membranes were then washed three times by gentle agitation in buffer B for 10 min, incubated for 1 h with an anti-rabbit IgG linked to horseradish peroxidase and diluted 1/10 000 (Amersham, Oakville, Ont.) in buffer B. After a final wash, labelled antigens were revealed with enhanced chemiluminescence (ECL) detection system (Amersham, Oakville, Ont.). Fuji films which were preflashed with a Sensitize flashgun unit (Amersham, Oakville, Ont.) were exposed to the membranes, developed and analyzed using a densitometer (Molecular Dynamics).

2.6. Deglycosylation

OK cell membrane proteins were solubilized in 0.5% SDS for 10 min at room temperature and diluted 5-fold with 1% (w/v) *n*-octyl β-D-glucopyranoside, 1% (v/v) β-mercaptoethanol, 20 mM Tris/HCl, pH 8. Endoglycosidase F/*N*-glycosidase F mixture (Boehringer-Mannheim, Laval, Que.) was added at 2 U/mg protein and the reaction was allowed to proceed for 16 h at 25°C. Laemmli's sample buffer was then added and samples underwent the Western blot procedures.

2.7. Protein isolation by electroelution on a SDS–PAGE mini prep cell column

Membrane proteins (500 μg) isolated from mouse brain were resolved on a 7.5% acrylamide SDS–PAGE Mini Prep Cell column (Bio-Rad) with a running gel height of 5 cm and a stacking gel of 1 cm (4% acrylamide). The electrophoresis was carried out at a constant current of 4 mA. Fractions of 180 μl were collected at a flow rate of 60 μl/min and assayed by Western blot for immunodetection of p85 and the 70–72 kDa protein. Fractions containing these proteins were identified and pooled for further analysis.

2.8. Limited chymotryptic digestion

Membrane protein samples were solubilized with 1% (w/v) SDS for 20 min at room temperature and then centrifuged at 50 000 × *g* for 15 min. Supernatant containing solubilized proteins was then incubated with chymotrypsin for 30 min at 37°C in a buffer composed of mannitol 300 mM, Hepes/Tris 20 mM, pH 7.5. The reaction was stopped by dilution of the reaction media with the same buffer containing PEFABLOC at 1 mg/ml. Samples underwent elec-

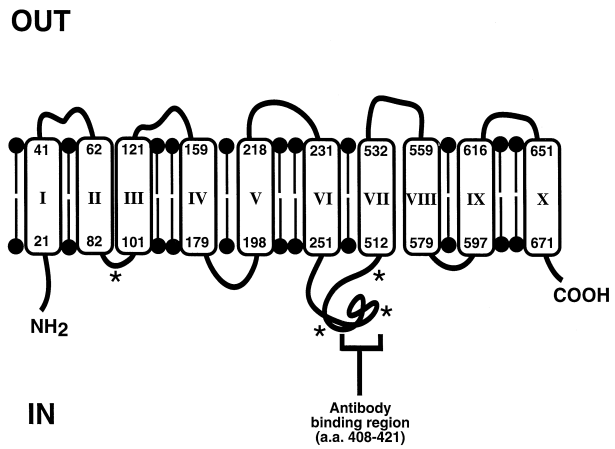

Fig. 1. Hypothetical topographic representation of Glvr-1 in the membrane based on the secondary structure of Glvr-1 proposed by Johann et al. [19] and on the analysis of the primary sequence using the MacVector software (Kodak Laboratory and Research Products). This representation is shown looking into the plane of the bilayer. According to this model, Glvr-1 could possess up to ten transmembrane domains with both N- and C-terminal segments facing the cytoplasmic side of the membrane. Numbers at each end of transmembrane segments represent residues. The peptide region against which the antibodies have been raised is located within the large hydrophilic loop (residues 251–512). Potential *N*-glycosylation sites are indicated by an asterisk (*).

trophoresis (15% acrylamide gels) and Western blot analysis was carried out as described previously.

3. Results

The predicted Kyte and Doolittle hydropathy profile suggests that Glvr-1 possesses ten transmembrane domains (Fig. 1). According to this model, both amino and carboxy ends would be within the cytoplasmic surface of the membrane. The region against which the antibodies are directed is located in the large intracytoplasmic loop of Glvr-1 (residues 251–512).

Polyclonal antibodies directed against a 14-amino acid portion of the cloned sodium/phosphate cotransporter Glvr-1 were raised in rabbits, affinity-purified and used to determine the presence of related proteins in brain crude membranes from human, rat, rabbit, hamster, mouse, and in plasma membranes from OK cells and umbilical cord endothelial cells (Fig. 2(A)). The last seven C-terminal amino acids of this peptide also corresponds to a portion of the rat Ram-1 transporter. Analysis revealed a protein of about 85 kDa (designated p85) in brain of rat, rabbit, hamster, mouse and in OK cells. No proteins were detected in crude membranes from human brain or in umbilical cord endothelial cells. In mouse brain, another strong band was also detected, which corresponds to a protein of about 70–72 kDa. This mouse protein was peptide displaceable, showing the specificity of the antibodies for this protein (not shown). p85 was also detected in several mouse tissues such as kidney, heart, thymus, muscle and brain, although in liver and testis, there was slight variations in electrophoretic mobility of the protein. However, p85 was not detected in intestine, spleen, pancreas or lungs, possibly because of its absence or its weak expression in those tissues (Fig. 2(B)). In a previous study, Glvr-1 and Ram-1 mRNA was detected in all rat tissues except in spleen, although intestine, testis and pancreas were not assayed [10]. In another report, Glvr-1 mRNA was detected in all mouse tissues except for stomach (intestine, testis and pancreas were not tested) [19].

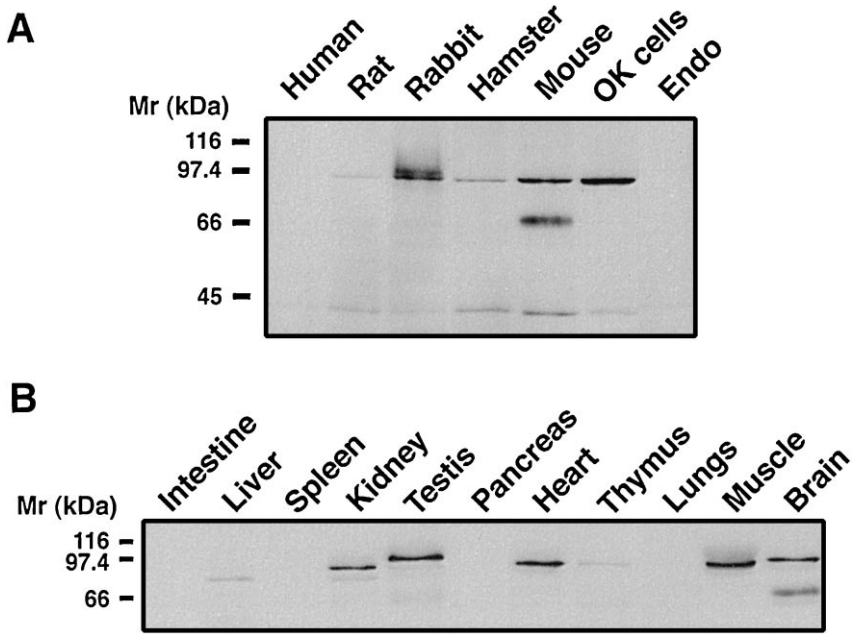

Fig. 2. Immunodetection of proteins in crude membrane preparations from OK cells and umbilical cord endothelial cells (Endo) and from human, rat, rabbit, hamster and mouse brain (A) and mouse tissues (B) using polyclonal antibodies directed against type III sodium/phosphate cotransporter Glvr-1/Ram-1. Protein samples (12 μg) were resolved by SDS–PAGE using 7.5% polyacrylamide gels. Immunodetection was performed as described under Section 2 and antibody binding was detected using donkey horseradish peroxidase-linked anti-rabbit IgG antibodies. Labelled antigens were revealed by ECL detection system. Comparable data were obtained in three independent experiments performed in duplicate.

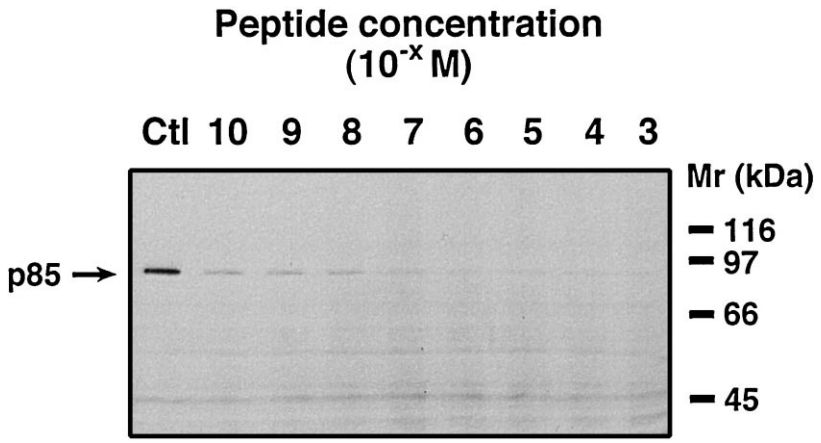

Fig. 3. Antibody specificity and displacement studies. Western blot analysis of OK cell membrane proteins was performed as described under Section 2 with addition of antigenic peptide during the incubation with the primary antibody. The control sample (i.e. without any peptide) is represented by ''Ctl'' and the peptide concentrations utilized ranged from 10^{-10} to 10^{-3} M.

The specificity of the antibody was examined by comparing the detection profiles obtained after incubation of the PVDF membranes with affinity-purified antibodies in the presence of increasing concentrations of antigenic peptide (Fig. 3). The detection of p85 from OK cell membranes was reduced approximately 84% that of the control by the addition of 10^{-10} M peptide for a 1/10 000 dilution of the antibody preparation and 10^{-6} M peptide was sufficient to prevent any binding of the antibodies to p85.

Since previous reports have demonstrated a critical role of sulfhydryl groups on the function and structure of sodium-phosphate uptake [20–23], the influence of β-mercaptoethanol (β-EtSH) and heating on the immunodetection of p85 was investigated in OK cells (Fig. 4). Denaturation by heating of the samples prior to electrophoresis had no apparent influence on the detection of p85. When electrophoresis was carried out in the presence of β-EtSH, the immunodetection of the 85 kDa protein was enhanced compared to Western blots where β-EtSH was excluded from samples. However, under all conditions only one major band corresponding to p85 was observed. To further characterize this protein, the addition of β-EtSH to protein samples (without boiling) before electrophoresis was used in the following experiments.

Type II phosphate transporters are up- and down-regulated by a low-phosphate diet and by PTH, respectively [13,24]. To study the effects of those strong phosphate uptake modulators on p85, OK cells were cultured in absence of phosphate or in presence of PTH. Following culture in absence of phosphate, phosphate uptake was 2–3 times higher than the uptake of cells cultured in presence of 1 mM phosphate (Table 1). Also, uptake in PTH-treated cells showed a 75% decrease compared to control cells (Table 2). However, these treatments did not alter the net level of p85 present in the membrane (Fig. 5). This clearly shows that p85 expression is not affected by extracellular phosphate levels in OK cells or in response to treatment with PTH.

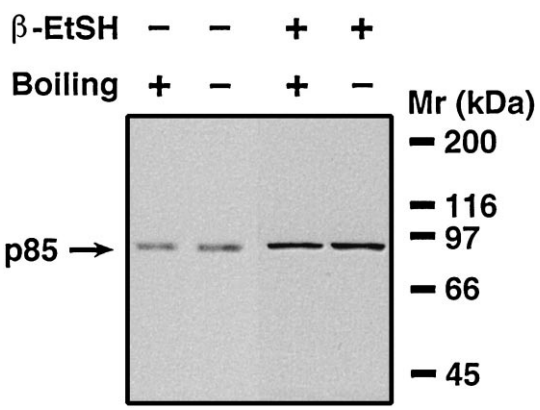

Fig. 4. Immunodetection of p85 from OK cell membranes under various conditions. Prior to electrophoresis, protein samples were either boiled (+) or not (–) in the presence (+) or absence (–) of β-EtSH. Proteins (12 μg/well) were resolved by SDS–PAGE using 7.5% polyacrylamide gels and p85 was immunodetected as described under Section 2.

Table 1
Effect of phosphate treatments on phosphate uptake in OK cells

Treatment	Phosphate uptake in cells cultured without any phosphate (nmol/mg protein/5 min)	Phosphate uptake in cells cultured in presence of 1 nM phosphate (nmol/mg protein/5 min)
Treatment A	17.94	9.68
	17.80	8.72
	16.75	8.10
Treatment B	12.43	3.85
	12.66	4.05
	12.29	4.99

In treatment A, cells were incubated for 6 h without FBS in DMEM containing 0.1% bovine serum albumin and either 0 or 1 mM phosphate before measuring uptake and isolating membranes as described under Section 2. In treatment B, they were incubated for the first four days with DMEM/F12, 10% FBS followed by three days in DMEM 10% FBS. These cells were then incubated for 19 h in DMEM 0.1% BSA with either 0 or 1 mM phosphate. The results are given for three experiments for each treatment.

Since the amino acid sequence of Glvr-1 possesses four potential *N*-glycosylation sites, glycosidase treatments were performed on OK cell membrane proteins to investigate the glycosylation state of p85 (Fig. 6). Protein samples were treated for 16 h with a mixture of endoglycosidase F/*N*-glycosidase F, which hydrolyzes *N*-glycans of the ''high mannose'' and the ''complex'' type [25], and then processed for Western blotting. The Glvr-1/Ram-related protein p85 showed no alteration in its electrophoretic mobility following glycosidase treatment.

Table 2
Effect of PTH treatment on phosphate uptake in OK cells

Phosphate uptake in cells cultured without any PTH (nmol/mg protein/5 min)	Phosphate uptake in cells cultured in presence of 10^{-8} M PTH (nmol/mg protein/5 min)
13.5	3.60
13.8	3.20

The effect of parathyroid hormone was examined in cells fed DMEM/F12 10% FBS for 7 days followed by 18 h with 0.1% BSA replacing the FBS. These cells were then exposed for 2 h to 10^{-8} M PTH or its vehicle before measuring uptake and isolating membranes as described under Section 2. The results are given for two experiments.

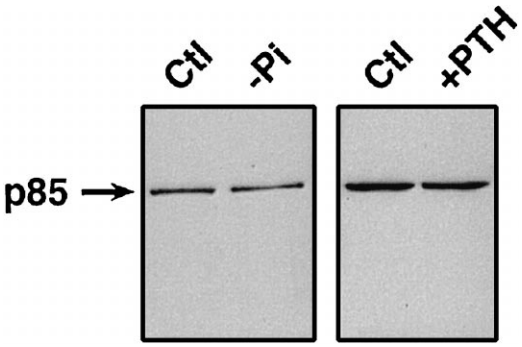

Fig. 5. Effect of phosphate and PTH on the immunodetection of p85. Membranes were isolated from OK cells grown under control conditions (Ctl), in absence of inorganic phosphate ($-P_i$), or in presence of PTH (+PTH) in culture medium, as described in Section 2. Protein samples were analyzed by Western blot as described under Section 2.

We investigated the relationship between the two proteins detected in mouse brain, i.e. p85 and the 70–72 kDa protein. First, both proteins were isolated using an SDS–PAGE column to study them separately (Fig. 7). Western blots were done with every second fraction collected to determine in which fractions we could find proteins around 70–72 and 85 kDa. Fractions containing each of these proteins were pooled separately, i.e. fractions 22–24 for the 70–72 kDa protein and fractions 44–48 for p85. Af-

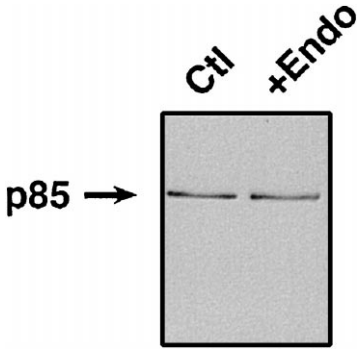

Fig. 6. Effect of endoglycosidase F/*N*-glycosidase F treatment on the electrophoretic mobility of p85 from OK cells. Protein samples were first solubilized in 0.5% SDS and then diluted 5-fold with 1% (w/v) *n*-octyl β-D-glucopyranoside, 1% (v/v) β-mercaptoethanol, 20 mM Tris/HCl, pH 8. The samples were incubated at 25°C for 16 h with (+Endo) or without (Ctl) 2 U/mg of the enzymatic mixture. Protein samples were analyzed by Western blot as described under Section 2.

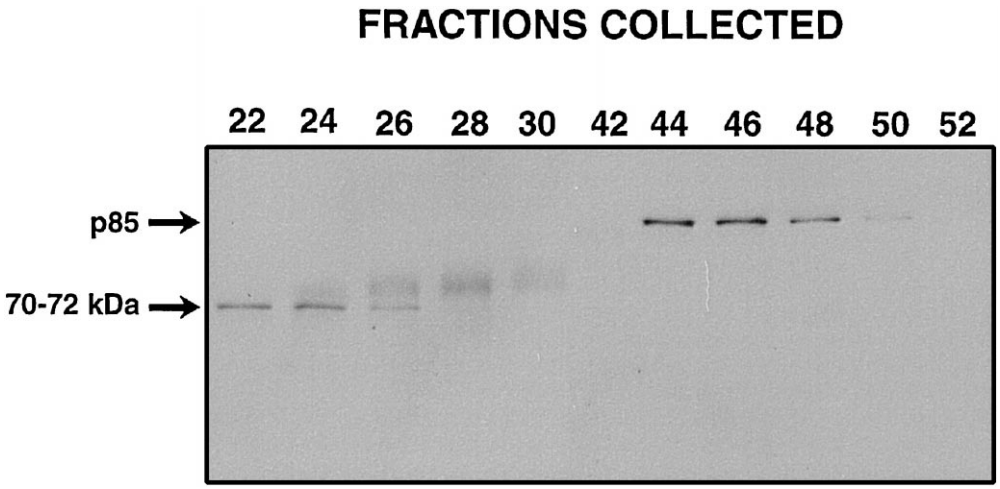

Fig. 7. Isolation of mouse brain proteins using an SDS–PAGE column. Membrane proteins (500 μg) isolated from mouse brain were resolved on a SDS–PAGE column (Bio-Rad) as described in Section 2. Fractions of 180 μl were collected at a rate of 60 μl/min and assayed for immunodetection of p85 and proteins of 70–72 kDa. Fractions containing these proteins were identified and pooled for further analysis.

ter limited chymotrypsin proteolysis, two fragments were obtained from p85 (Fig. 8(A)) which were identical to those found in the 70–72 kDa protein fragment pattern (Fig. 8(B)). These fragments are identified as F26 and F17. This finding demonstrates that p85 and the 70–72 kDa protein from mouse brain are very similar in their amino acid sequence and possess common chymotrypsin digestion sites.

Third, to investigate the relationship between p85 from OK cells with p85 from mouse brain, we also

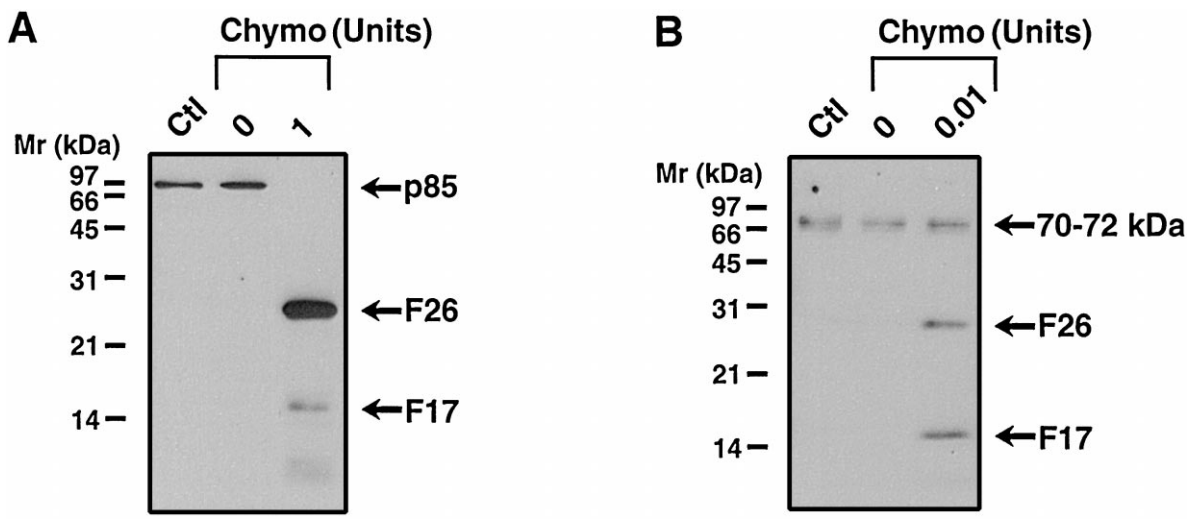

Fig. 8. Chymotrypsin proteolysis of isolated fractions containing p85 and 70–72 kDa-protein from mouse brain. Isolated protein fractions containing either p85 (A) or 70–72 kDa (B) were incubated with 0 and 1 (A) or 0.01 U (B) chymotrypsin for 30 min at 37°C and were used for electrophoresis (on 15% acrylamide gels) and Western blot analysis as described under Section 2. As controls (Ctl), protein samples were simply loaded on the gel for electrophoresis without any treatment.

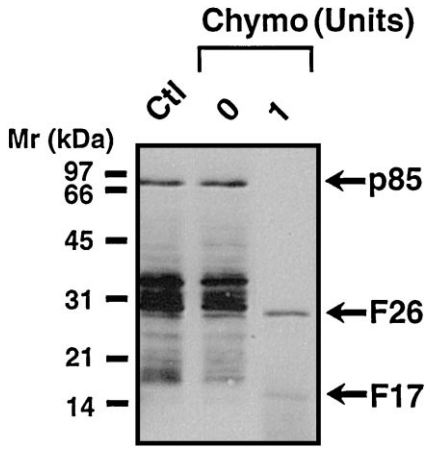

Fig. 9. Chymotrypsin proteolysis of OK cell membrane proteins. Membrane protein samples were solubilized with 1% (w/v) SDS for 20 min at room temperature and then centrifuged at 50 000× *g* for 15 min. Supernatant containing solubilized proteins was then incubated with 0 and 1 U chymotrypsin, as described under Section 2. Samples underwent electrophoresis on 15% acrylamide gels and Western blot analysis as described under Section 2. As a control (Ctl), protein sample was simply loaded on the gel for electrophoresis without any treatment.

performed limited chymotrypsin proteolysis on OK cell membranes (Fig. 9). After enzymatic cleavage, both F26 and F17 fragments were also detected in membranes from OK cells, demonstrating that p85 detected in these two species possesses a similar amino acid sequence. In the control, the many bands detected below 45 kDa are due to non-specific binding of the antibodies since they were not displaced by the antigenic peptide (not shown).

4. Discussion

To study the human receptor for the gibbon ape leukemia virus Glvr-1, polyclonal antibodies were raised in rabbits against a synthetic peptide corresponding to a 14-amino acid segment of the cloned receptor's deduced sequence [9]. The Gly-408 to Tyr-421 segment was chosen because hydropathy analysis [26] of the amino acid sequence of Glvr-1 suggests that it is fairly hydrophilic and because its antigenic index seemed adequate. This segment had to be very carefully selected since Glvr-1 has a relatively hydrophobic amino acid composition. The epitope we used to raise these antibodies is present in homologs to human receptor Glvr-1, two different murine homologs [12,19] and a Chinese hamster homolog [27]. The last seven C-terminal amino acids of this epitope are also present in murine Ram-1, thus cross-reactivity of the antibodies with this other member of the type III phosphate transporter gene family is possible. The affinity-purified antibodies were used in Western blot experiments to identify immunoreactive proteins in OK cell membranes as well as in brain membranes from various species. Using this technique, a single band representing a protein of about 85 kDa (p85) was immunodetected in rat, rabbit, hamster, mouse and OK cells. In mouse brain, a second protein of about 70–72 kDa was also detected. These apparent molecular masses are somewhat in good agreement with that calculated from the amino acid sequences of Glvr-1 or Ram-1 which are 74 and 70 kDa, respectively, without any post-translational modifications [9]. However, distinction between Glvr-1 and Ram-1 cannot be done since a portion (seven amino acids) of the epitope chosen to raise our antibodies is identical in both transporters. Although detection of Glvr-1 and Ram-1 mRNAs in several rat tissues were performed by Northern blot analysis [10], this is the first report showing the Western blot detection of Glvr-1/Ram-1 by polyclonal antibodies. The cross-reactivity of our antibodies with proteins from rat, rabbit, hamster, mouse and OK cell indicates that this receptor, which is a sodium/phosphate cotransporter, is a well conserved protein since all these species possess a similar epitope. The detection of Glvr-1/Ram-1 in several mouse tissues is in agreement with a constitutive (''housekeeping'') role of type III phosphate transporters in most cells to absorb the readily available phosphate from interstitial fluid for normal cellular functions. A previous study has also shown the presence of Glvr-1 and Ram-1 mRNA in many rat tissues [10]. The role of type III phosphate transporters greatly differs from those of the adaptive type II system which is strictly localized in the kidney [28]. The absence of p85 in human brain can be explained by degradation of the sample since many hours elapsed between removal of the sample from the patient and the resulting membrane preparation.

β-EtSH reacts with the existing disulfide bonds in a protein to reduce them. In this study, the presence of β-EtSH increased the amount of the immunode-

tected p85, but there was no change in its apparent molecular mass. This suggests that β-EtSH enhances the accessibility of the epitope to the antibodies by reducing disulfide bonds.

Dietary phosphate restriction produces an adaptive increase in sodium/phosphate cotransport by OK cells [29] which is characterized by an increase in the mRNA and protein levels of NaP_i-4, a type II sodium/phosphate cotransporter [30]. Accordingly, exposure of rat fibroblasts to phosphate-free medium has been shown to cause a 3-fold increase in phosphate transport, which is accompanied by a 3- to 5-fold increase in Glvr-1 and Ram-1 mRNA levels (Northern blot experiments) [10]. In our study, Western blots experiments showed that the expression of p85 was not affected by phosphate deprivation. These contrasting results could be explained by different modes of regulation in the species studied (i.e. rat fibroblasts vs. OK cells), by the conditions under which the experiments were carried out (duration of the diet), or by the delay of mRNA to be translated into detectable membrane proteins. It has already been established that OK cells possess at least one other type of sodium/phosphate cotransporter (type II), NaP_i-4, which is up-regulated by phosphate deprivation [31]. However, such a discrepancy between overexpression of mRNA in rat fibroblasts and steady amount of p85 in OK cell membranes following phosphate deprivation remains to be elucidated.

Since regulation of phosphate transporter function may occur through PTH activation of kinase, and since multiple consensus phosphorylation sites are present in Glvr-1, particularly in the large cytoplasmic loop [32], we examined the effect of PTH on p85 in OK cells. PTH did not alter the expression of p85 in OK cells, contrarily to NaPi-II-type transporters which are tightly regulated following PTH treatments [24].

According to its amino acid sequence, Glvr-1 possesses four potential sites of *N*-glycosylation: Asn-96, Asn-371, Asn-415 and Asn-497 [9]. Since the apparent molecular mass obtained for Glvr-1/Ram-1-related protein was 85 kDa, and the predicted molecular mass from the cDNA is 74 kDa for Glvr-1 and 70 kDa for Ram-1 [9], the glycosylation state of p85 was investigated. According to our results, the discrepancies between both observed and predicted molecular masses cannot be explained by *N*-glycosylation since no variation of the immunodetection of p85 was observed following glycosidase treatment. This result is not surprising since the hypothetical topographic representation of Glvr-1 in its lipid bilayer shows all three potential *N*-glycosylation sites facing the cytoplasm [11,19]. Possibly, Glvr-1/Ram-1 possesses other post-translational modifications that could alter its electrophoretic mobility, or is also present as a larger precursor.

The two identical fragments obtained for p85 from mouse and OK cells and for the 70–72 kDa protein from mouse after chymotryptic cleavage demonstrate the very close structural relationship between these proteins. These fingerprinting experiments show that p85 from both species possesses a common portion of their amino acid chains that contains the same chymotryptic cleavage sites. Also, these findings suggest that the mouse 70–72 kDa protein could be a peptide fragment from p85, or a type III transporter (Glvr-1 or Ram-1) without any post-translational modification since its mass is closer to the predicted mass of these transporters.

In conclusion, these data demonstrate for the first time a Western blot detection of a type III phosphate transporter using polyclonal antibodies. This report provides evidence that, conversely to type I and adaptive type II phosphate transporters which are localized in the kidney, this third type of transporter is ubiquitous and probably absorbs the readily available phosphate from interstitial fluid for normal cellular functions in many species, serving as a ''housekeeping'' transporter. This is also the first report showing that p85 is not regulated by low extracellular phosphate nor by PTH, contrarily to type II phosphate transporters.

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